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**Understanding potentials and restrictions of solvent-free enzymatic polycondensation of itaconic acid: an experimental and computational analysis**Livia Corici<sup>1,ψ</sup>, Alessandro Pellis<sup>2,§</sup>, Valerio Ferrario<sup>2</sup>, Cynthia Ebert<sup>2</sup>, Sara Cantone<sup>1</sup>, Lucia Gardossi<sup>2,\*</sup><sup>1</sup> SPRIN S.p.a. c/o BIC Incubatori FVG, Via Flavia 23/1, 34148, Trieste (TS), Italy<sup>2</sup> Laboratory of Applied and Computational Biocatalysis, Dipartimento di Scienze Chimiche e Farmaceutiche, Università degli Studi di Trieste, Piazzale Europa 1, 34127, Trieste (TS), Italy

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Itaconic acid is a chemically versatile unsaturated diacid that can be produced by fermentation and potentially it can replace petrol based monomers such as maleic and fumaric acids in the production of curable polyesters or new biocompatible functionalized materials. Unfortunately, due to the presence of the unsaturated C=C bond, polycondensation of itaconic acid is hampered by cross reactivity and isomerization. Therefore, enzymatic polycondensations would respond to the need of mild and selective synthetic routes for the production of novel bio-based polymers. The present work analyses the feasibility of enzymatic polycondensation of diethyl itaconate and, for the first time, provides comprehensive solutions embracing both the formulation of the biocatalyst, the reaction conditions and the choice of the co-monomers. Computational docking was used to disclose the structural factors responsible for the low reactivity of dimethyl itaconate and to identify possible solutions. Surprisingly, experimental and computational analysis revealed that 1,4-butanediol is an unsuitable co-monomer for the polycondensation of dimethyl itaconate whereas the cyclic and rigid 1,4-cyclohexanedimethanol promotes the elongation of the oligomers.

**Keywords:** enzymatic polyester synthesis, itaconic acid, lipase B from *Candida antarctica*, curable polyesters, thin film reaction

**Introduction**

The demand for novel bio-based functionalized polyesters is incredibly rising<sup>[1]</sup> and new synthetic

biocatalyzed routes can concretely respond to this challenge by combining benign conditions with efficiency and selectivity of enzymes.<sup>[2-5]</sup>

Aliphatic polyesters are of particular interest due to their biodegradable and non-toxic properties and the need for novel biodegradable polymers in biomedical fields increased the interest of researchers to develop new polymers.<sup>[6-8]</sup> Moreover, bio-based chemistry has recently demonstrated the feasibility of the synthesis of value-added polyesters derived from renewable monomers, both polyols and dicarboxylic acids, obtainable via fermentation.<sup>[9-13]</sup> These challenges have been also addressed by using enzymes *in vitro*, and hydrolases in particular, because they are attractive and sustainable alternatives to toxic catalysts used in polycondensation,<sup>[14]</sup> such as tin and other metals.<sup>[15]</sup> The synthesis of an array of polyesters with medium or modest MW can be catalyzed by lipase B from *Candida antarctica* (CaLB), a robust enzyme that works efficiently in solvent-free systems and at temperatures below 90 °C.<sup>[16-18]</sup> Notably, such conditions are compatible with the polycondensation of unsaturated di-acids, as itaconic acid for instance, which suffer from isomerization or cross linking under the harsh conditions requested by conventional chemical processes (i.e. temperatures >150 °C).<sup>[19]</sup> Therefore, the use of the highly active and stable CaLB at mild temperature represents a route for the production of polyesters bearing reactive functional groups (e.g. C=C bonds), prone to further chemical modifications or molecular weight enhancement by combining chemical and thermal polymerization.<sup>[18,19]</sup> Itaconic acid (IA) is a fully sustainable industrial building block that can be produced industrially by employing strains of *Aspergillus terreus* that ferment sugars extracted from hardwood and agricultural residues.<sup>[20]</sup> For that reason IA is the main “renewable” candidate for the substitution of maleic and fumaric acids, two largely used petrol-based chemicals, in the production of reticulate polymers.<sup>[21]</sup> The vinyl moiety and the 1,4 carboxylic functionalities provide routes towards molecular complexity<sup>[15,16,21,22]</sup> and, conversely, new materials applicable in drug-release systems, tissue engineering and other biomedical and biotechnological applications.<sup>[23]</sup> Itaconic acid has been also combined with adipic acid and trimethylolpropane in the thermal synthesis of branched photocurable polyesters.<sup>[19]</sup> Additionally, water-soluble polymers, such as poly(itaconate sorbitol *co*-succinate sorbitol),

have attracted huge interest as potential important materials for hydrogels design.<sup>[10,19]</sup> Despite the interest and the considerable number of studies addressing *in vitro* enzymatic polycondensation, insufficient progresses have been documented in the last two decades towards the preparative and industrial application of this methodology and major bottlenecks have been reviewed and discussed.<sup>[24,25]</sup> Recently, we have investigated the feasibility of solvent-free polycondensation of different monomers, including esters of itaconic acid. More specifically, it has been shown that CaLB catalyzed polycondensation of dimethyl itaconate (DMI) suffers from slow reaction kinetics<sup>[19]</sup> caused by the poor reactivity of the acyl group conjugated with the C=C bond.<sup>[24,26]</sup> Moreover, we have observed that CaLB immobilized *via* adsorption on methacrylic resins (Novozym 435<sup>®</sup>) releases a considerable amount of free enzyme and it was concluded that covalent immobilization of CaLB is necessary.<sup>[24]</sup> Starting from those preliminary observations, the present work intends to analyse more in detail the feasibility of enzymatic polycondensation of DMI, by addressing the different chemical and enzymatic problems according to an integrated approach. The different reactivity of the two acyl groups of DMI was investigated both experimentally and computationally to understand the structural basis of the unsatisfactory elongation of the oligomeric chain. Docking studies were carried out to shed light on the influence of the structure of polyols and the effect of competing nucleophilic species, thus setting the basis for a rational planning of reaction conditions. All enzymatic reactions were carried out in neat substrate mixtures, to meet sustainability criteria. The combined use of covalently immobilised CaLB and thin film systems allowed to prevent the detachment of the enzyme from the carrier and the mechanical crushing of immobilization support.<sup>[24,27]</sup> The data here presented, although confirming the low reactivity of itaconic acid, indicates new solutions for improving reaction kinetics of polycondensation of DMI, both by selecting appropriate diols and by a rational planning of reaction conditions. Conclusions demonstrate how the design of biocatalysts, the choice of comonomers and the tuning of the chemical process must proceed synchronized. Therefore, these

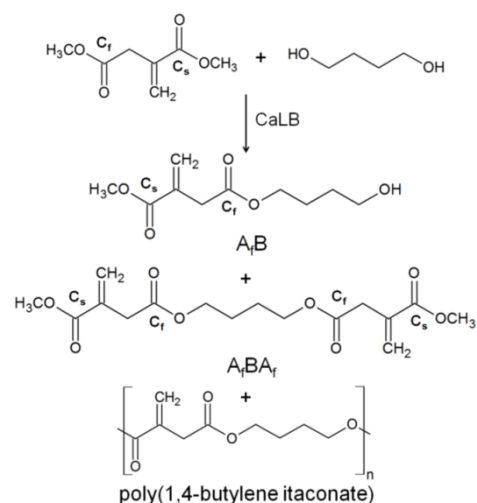
findings have a wider validity and the approach is applicable to the optimization of further biocatalyzed synthesis of polyesters.

## Results and Discussion

### *Effect of the distribution of the biocatalyst*

As discussed above, the enzymatic polycondensation of itaconic acid and its esters is severely hampered by the lower electrophilicity of the acyl carbon adjacent to the vinyl group ( $C_s$  in Scheme 1).<sup>[2,24]</sup> Previous investigations of polycondensation of dimethyl itaconate (DMI) were mainly focused on the possibility of achieving oligomers of acceptable molecular weight, either in solvent free systems<sup>[19]</sup> or in the presence of solvents.<sup>[28]</sup> Limited attention has been devoted so far to the understanding of the process at molecular level or to the identification of chemical species formed in the course of the reaction.

In the present study we investigated the solvent-free synthesis of poly(1,4-butylene itaconate) (PBI) as a model reaction (Scheme 1) with the aim of understanding the effect of a number of variables on transesterification kinetics. The initial focus was on the accessibility and distribution of the biocatalyst in the reaction mixture. All polycondensations were performed without any pre-treatment of the reagents, in order to be as close as possible to conditions applicable at industrial scale, where purification/dehydration steps would cause an unacceptable increase of production costs. In order to evaluate the amount of water available in the different phases, the water activity ( $a_w$ ) was measured after 24 h of equilibration of the reaction mixture in the closed vessel and it resulted to be in the range of 0.28-0.30 in all experimental systems.<sup>[29]</sup> The  $a_w$  data indicate that the results observed during the reactions reported herein can be compared and analyzed with sufficient confidence and there is a low amount of water available for promoting competing hydrolytic reactions.



Scheme 1. Enzymatic synthesis of poly(1,4-butylene itaconate) and structures of AB and ABA, which are the main products observed.  $C_f$  corresponds to the fast reacting acyl carbon whereas  $C_s$  indicates the slow reacting acyl carbon.

The integrity of the carrier was preserved by avoiding any mechanical or magnetic mixing<sup>[30]</sup> but rather mass transfer was assured by working with a thin film of reaction mixture.<sup>[24,27]</sup> That was achieved by carrying out the reaction in a round bottom flask connected to a rotary evaporator (80 rpm) allowing, when required, to work under reduced pressure (70 mbar) and facilitate the removal of volatile by-products.

A typical polycondensation reaction was carried out by suspending dimethyl itaconate (DMI) in the liquid 1,4-butanediol (BDO) (1.0:1.1, molar ratio) and the suspension was warmed to achieve a fluid slurry. The reaction was started by the addition of the biocatalyst and the slurry progressively became a homogeneous transparent solution as the transesterification proceeded. Reactions were performed at a maximum temperature of 50 °C because, when operating under reduced pressure (70 mbar) DMI evaporates due to its relatively high vapor tension (vapor pressure: 219 mmHg at 25 °C). These experimental conditions are milder as compared to the previous study of Barrett,<sup>[19]</sup> which described the polycondensation of DMI and different polyols catalyzed by Novozym 435<sup>®</sup> at 90 °C for 48 hours with the application of reduced pressure only during the last 46 hours of the reaction. However, all attempts of carrying out the reaction at temperature equal or above 80 °C led to the formation of solid products insoluble in a number of solvents tested (see Experimental Session for details) and these experimental

observation suggests the formation of cross-links among the vinyl groups of DMI.

Figure 1 illustrates the ESI-MS spectra of the polycondensation catalyzed by 10% w/w (referred to the global amount of monomers) of a commercial adsorbed preparation of CaLB (Novozym 435<sup>®</sup>, hydrolytic activity 2200 U g<sub>dry</sub><sup>-1</sup>). After 72 h of reaction there is the formation of oligomers made by 8-9 units and more than 90% of C<sub>f</sub> and about 60% of C<sub>s</sub> of DMI reacted. This information is attainable by comparing the <sup>1</sup>H-NMR signals of the two methoxy groups of DMI (SI, Figure S5).

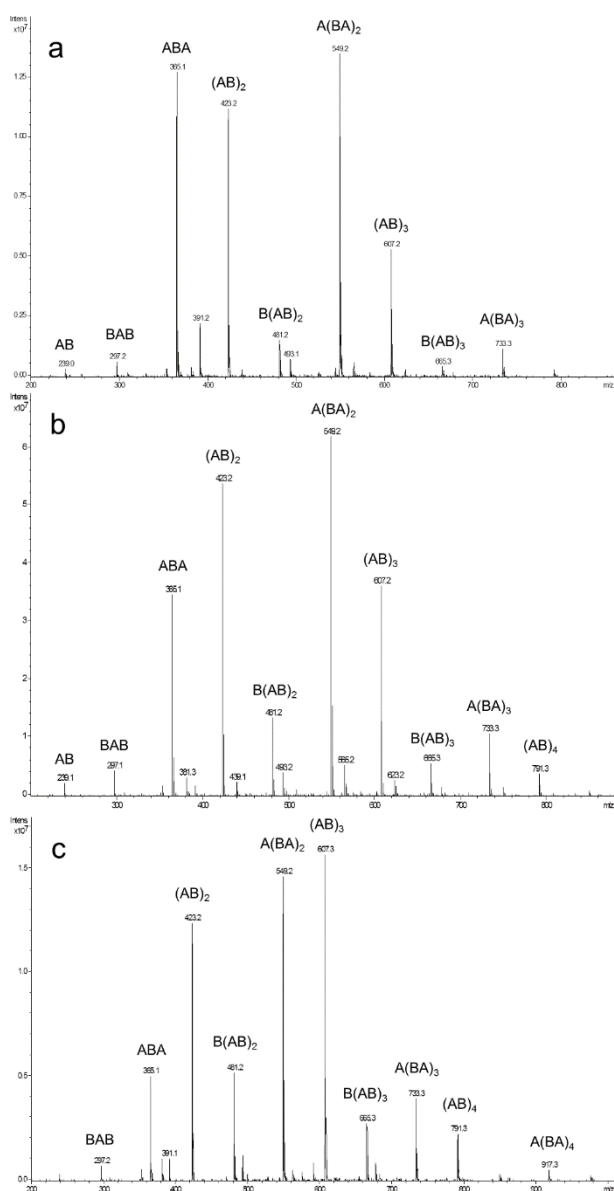


Figure 1: ESI-MS positive ion mass spectrum of polycondensation products of DMI (A) with BDO (B) (molar ratio 1:1.1). The reaction was catalyzed by 10% w/w of adsorbed CaLB (Novozym 435<sup>®</sup>, activity 2200 U g<sub>dry</sub><sup>-1</sup>) corresponding to 220 U per g of substrate monomers. a) 24, b) 48, c) 72 h.

Being aware of the fact that Novozym 435<sup>®</sup> releases a considerable amount of active enzyme in the reaction mixture,<sup>[24]</sup> the effect of the distribution and accessibility of the biocatalyst was investigated also by using formulations of CaLB covalently immobilised on similar methacrylic resins.

Figure 2a illustrates the poor results obtained by using 10% w/w of a formulation of CaLB covalently immobilized on epoxy methacrylic resins and endowed with an experimentally determined activity of 2400 U g<sub>dry</sub><sup>-1</sup> (SPRIN Epobond CaLB, referred herein as CaLB<sub>2400</sub>).<sup>[31]</sup> There is an accumulation of AB dimer and ABA trimer within the first 24 h but the elongation apparently stops as demonstrated by HPLC-DAD and ESI-MS recorded during the 72 h of reaction. The explanation of the limited elongation comes from <sup>1</sup>H-NMR spectra that indicate how, after 24 h, 95% of the reacted acyl groups correspond to C<sub>f</sub> and less than 5% to C<sub>s</sub> (Figure S9 and S10 in SI). It must be noted that the inactivation of the enzyme was excluded (see Figure S12 in SI).

By doubling the amount of biocatalyst (20% w/w, Figure 2b) the polycondensation proceeds with the formation of oligomers slightly longer, indicating that the polycondensation of DMI would require an exceeding amount of covalently immobilized CaLB for achieving acceptable kinetics. Consequently, the process would be economically unfeasible.

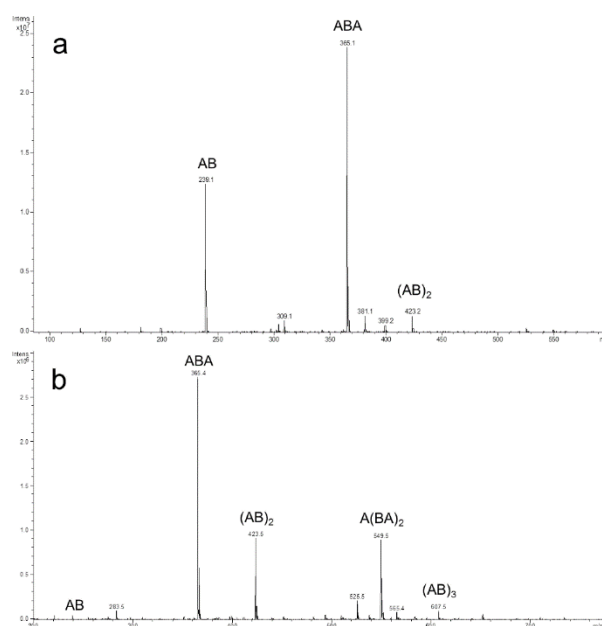


Figure 2. ESI-MS positive ion mass spectrum of polycondensation products of DMI (A) with BDO (B) catalyzed by 10 (a) and 20% w/w (b) of CaLB<sub>2400</sub> at 24 h.

All experimental evidences as well as data reported in a previous study [24] indicate that, in the case of Novozym 435<sup>®</sup>, the presence of the native enzyme homogeneously dispersed in the reaction mixture plays a major role in promoting polymer elongation. Moreover, it has been previously verified that the active site of the covalently immobilized CaLB is accessible [24] and the method of covalent immobilization is not responsible for a reduced efficiency of the biocatalyst.

As an alternative solution to the use of large amounts of highly active covalently immobilized biocatalysts, we thought to distribute the enzyme on a wider carrier surface. Therefore, new enzymatic preparations were conceived, having a much lower loading of protein still covalently anchored on the same epoxy-functionalized carrier. For this purpose, covalently immobilized CaLB<sub>350</sub> (displaying a hydrolytic activity of 350 U g<sub>dry</sub><sup>-1</sup>) and CaLB<sub>230</sub> (230 U g<sub>dry</sub><sup>-1</sup>) were prepared by applying the same protocol but changing the protein loadings. [32,33]

A further confirmation of what reported above comes from the polycondensations catalyzed by using 10% w/w of either CaLB<sub>350</sub> or CaLB<sub>230</sub> (Figure S7 and S9 in SI). The products were comparable to what observed employing 10% w/w of CaLB<sub>2400</sub>, which has 7-8 fold higher activity. Overall, the data indicate that the specific activity of the biocatalyst has a much lower effect on the polycondensation as compared to mass transfer.

On the other hand, a considerable improvement was achieved when using 30% w/w of covalently immobilized CaLB<sub>230</sub>. Notably, 69 U per gram of substrate monomers were sufficient to synthesize oligomers made by 5-9 units (Figure 3).

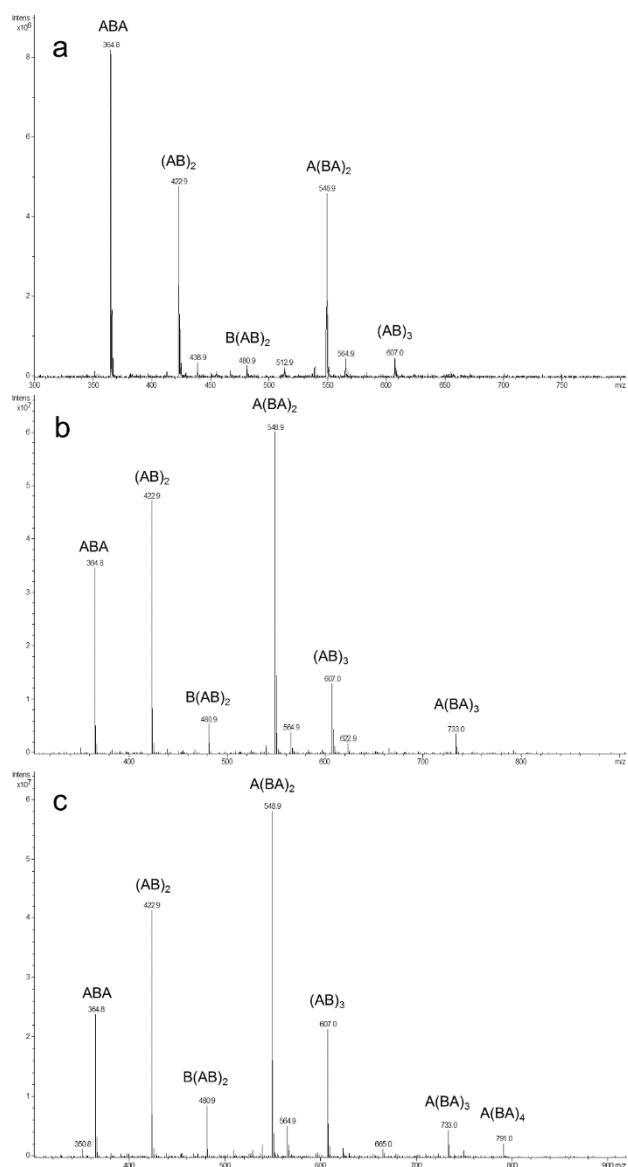


Figure 3. ESI-MS positive ion mass spectrum of polycondensation products of DMI (A) with BDO (B) catalyzed by 30% w/w of CaLB<sub>230</sub> (a), 48 (b) and 72 h (c).

As shown by <sup>1</sup>H-NMR data in Table 1, after 24 h most of C<sub>f</sub> reacted whereas the percentage of reacted C<sub>s</sub> increases slowly during the whole course of the reaction (22% of C<sub>s</sub> reacted within 72 h). It must be noted, that the polycondensation catalyzed by 10% w/w of CaLB<sub>2400</sub> led the transesterification of less than 5% of C<sub>s</sub> after 72 h, despite the use of a 3 folds larger amount of enzymatic units (see SI, Figure S10a).

The acylation of BDO was also estimated by calculating the ratio between the average of the intensity of vinyl proton of DMI (assumed as constant) and the signals of methylenic protons of BDO after esterification.

Table 1. Different reactivity of the two acyl groups of DMI in the reaction with BDO catalyzed by 30% w/w of CaLB<sub>230</sub>

Time (h)	Esterified -CH <sub>2</sub> BDO*	Reacted DMI C <sub>r</sub> *	Reacted DMI C <sub>s</sub> *
24	56%	89%	7%
48	60%	>95%	16%
72	65%	>95%	22%

\* Calculated via <sup>1</sup>H-NMR. The reactivity of the two acyl groups is attainable by comparing the <sup>1</sup>H-NMR signals of the methoxy groups of DMI. The conversion of BDO was calculated by comparing the ratio between the area of methylene groups adjacent to -OH groups and the average area of vinyl protons of DMI (assumed as constant). See Figure S6 in SI.

Although the result of Table 1 is below the percentage achieved using 220 U of adsorbed Novozym 435<sup>®</sup> (about 60% C<sub>s</sub> reacted) there is a clear evidence that by immobilizing covalently a lower amount of CaLB on a wider surface, the polycondensation proceeds and this route is promising in the perspective of reaching a compromise between polymer elongation and prevention of the contamination of the product. More specifically, since the enzyme is not solubilized but it works suspended in a viscous reaction mixture, kinetics are mainly hampered by diffusion limitations and by the difficulty encountered by substrates to access the enzyme active site. Therefore, having a high amount of enzymatic units concentrated within a small volume is not of great aid for increasing the reaction rate. Rather, distributing the catalyst on the widest surface will facilitate the enzyme-substrate approach and promote the polycondensation.

The effect of the amount of biocatalyst was also evidenced by monitoring the acylation of BDO in two reactions carried out under similar conditions but catalyzed by either 20 or 30% w/w of CaLB<sub>350</sub> (Table 2).

Table 2. Acylation progression (24, 72 and 96 h) of the hydroxyl groups of BDO in different reaction conditions evaluated via <sup>1</sup>H-NMR by comparing the ratio between the area of methylene groups adjacent to hydroxyl group of alcohols and the average area of vinyl protons of DMI (assumed as constant).

CaLB <sub>350</sub>	Time (h)	Area -CH <sub>2</sub> -OCO- (A <sub>1</sub> )*	Area -CO-C=CH <sub>2</sub> (A <sub>2</sub> )*	Ratio A <sub>1</sub> /A <sub>2</sub> *
20% w/w	24	7.79	7.68	1.01
	72	8.99	5.68	1.58
	96	9.63	5.83	1.65
30% w/w	24	6.91	5.53	1.25
	72	9.32	5.73	1.63
	96	11.35	6.03	1.88

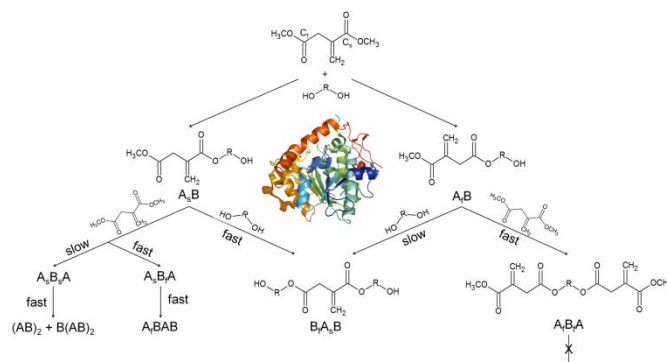
\* Calculated via <sup>1</sup>H-NMR as described in Table1.

The biocatalyst shows a remarkable stability, since it continues to catalyze the reaction even after 96 h (see also HPLC-DAD in SI, Table S1). Of course, further optimization studies would be necessary to identify the most convenient enzyme

loading for the immobilized biocatalyst referred to a desired reaction configuration.

### Effect of diol concentration

Generally speaking, the esterification catalyzed by CaLB follows a ping-pong bi-bi mechanism.<sup>[34]</sup> The enzyme stabilizes the negatively charged oxyanion thanks to electrostatic interactions between Thr40 and Gln106 inside the so-called oxyanion hole. Along the course of the catalytic reaction and after the acylation of Ser105, the His224 residue receives a proton that is transferred from the alcohol that is entering the active site. The latter acts as nucleophile by attacking the acylated-seryl ester, which leads to the ester formation. The rate determining step of the reaction can be either the formation of the acyl-enzyme (acylation) or the deacylation steps. All data reported so far demonstrate that in the transesterification of DMI with BDO the formation of the acyl-CaLB on the slow reacting carbon C<sub>s</sub> is rate limiting. Of course, the overall course of the polycondensation process is far too complex for drawing any exhaustive model. Nevertheless, some preliminary elements describing the first steps of the polycondensation can be schematized as reported in Scheme 2.



Scheme 2. Initial steps of the polycondensation between DMI and a general diol catalyzed by *Candida antarctica* lipase B (CaLB).

It appears evident that elongation is hampered by the formation of A<sub>2</sub>B<sub>3</sub> trimer, which causes the stop of the reaction due to the presence of two slow reacting ends. On the other side, the A<sub>2</sub>B dimer is a good acylating agent that might react also as nucleophile.

Therefore, one of the major challenges is the increase of the kinetic of the formation of the acyl-enzyme A<sub>s</sub>-CaLB, which is rate determining. Consequently, the apparent concentration of DMI should be as high as possible and this is confirmed

by Figure 4b that shows the formation of tetramers and pentamers in a reaction catalyzed by 10% w/w of CaLB<sub>2400</sub> but where the diol (BDO) was added stepwise (see Figure S8 in SI). It must be recalled that all reactions were conducted using the neat substrates.

The advantage of maintaining the BDO concentration as low as possible was also demonstrated by comparing the products obtained in two reactions catalyzed by 20% w/w CaLB<sub>350</sub> and carried out initially with a ratio of 1:0.5 between DMI and BDO. After the first 24 h of reaction, 0.5 equivalent of BDO were added in one of the two reactions.

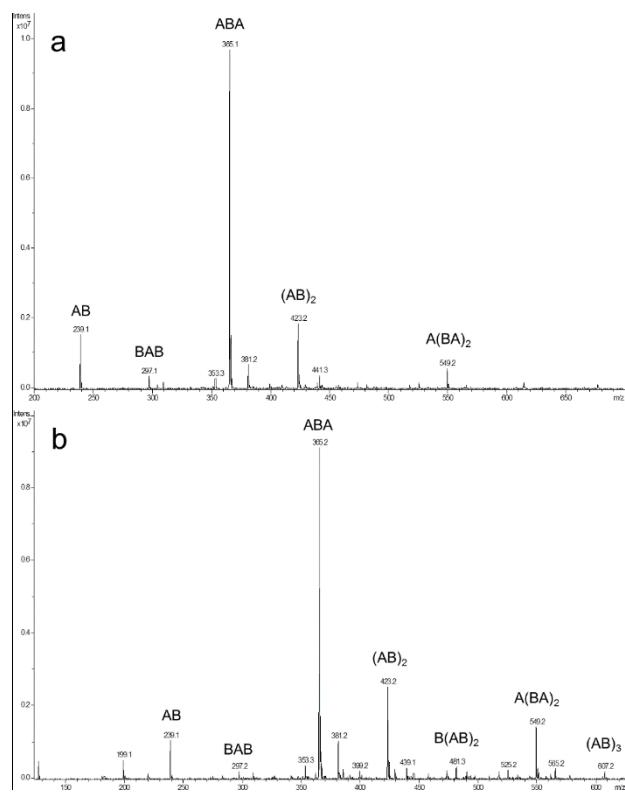


Figure 4. ESI-MS positive ion mass spectrum of polycondensation products of DMI (A) with BDO (B) catalyzed by 20% w/w of CaLB<sub>350</sub> at 72 h. a) A:B=1.0:1.0; b) A:B=1.0:0.5

Notably, the elongation is favored when an excess of DMI is present and at 72 h species such as B(AB)<sub>2</sub> and (AB)<sub>3</sub> are formed, although as minor components. Despite the defect of BDO, the progress of the reaction appears evident even after 72 h (Table 3).

Table 3. HPLC-DAD analysis of products for the reaction DMI (A) + BDO (B) catalyzed by 20% w/w of CaLB<sub>350</sub> in the presence of an excess of DMI.

Time (h)	AB/DMI	(AB) <sub>2</sub> /DMI	ABA/DMI	A(BA) <sub>2</sub> /DMI
24	0.548	0.027	0.384	-
48	0.643	0.074	0.754	0.025

72	0.660	0.126	0.952	0.071
*Calculated as the ratio between the areas of peaks of different oligomers formed and the peak of DMI still present in the reaction mixture.				

Therefore, the concentration of BDO emerges as a further relevant factor to be considered in future optimization studies.

#### *Effect of different polyol structures: synthesis of poly(1,4-cyclohexanedimethanol itaconate) and poly(glycerol itaconate)*

In the attempt of better understanding the molecular basis of polycondensation of DMI and exploring possible solutions for overcoming the bottlenecks evidenced above, different polyols were considered. While we assumed that the formation of the CaLB-A<sub>s</sub> acyl enzyme is rate determining, it is more difficult to understand how a bulky polyol might affect the rate of the other steps of the polycondensation. For instance, it is known that in the case of transesterifications of secondary alcohols, the deacylation step is often rate limiting, especially in the case of bulky secondary alcohols.<sup>[12]</sup>

Starting from these considerations, glycerol (GLO) and the cyclic and rigid 1,4-cyclohexanedimethanol (CHDM) were included in our investigation. CHDM was employed as a commercial 30:70 mixture of the *cis*- and *trans*-stereoisomers, as confirmed also by <sup>1</sup>H-NMR (see Figure S11 in SI). The interest towards CHDM was also induced by the possibility of incorporating structural elements that confer rigidity to the oligomeric chain. That represents a route for modulating the final properties of the final polymer, either by using combinations of different diols or by changing their relative molar ratio.

The synthesis of poly(1,4-cyclohexanedimethanol itaconate) (PCI) and poly(glycerol itaconate) (PGI) was performed in the presence of 10% w/w of CaLB<sub>2400</sub> at 50 °C, reduced pressure (70 mbar) and it was monitored for 72 hours. The unsaturated C=C bonds were well preserved under such conditions, as confirmed by <sup>1</sup>H-NMR spectra, which also attest the formation of the new ester bonds (SI, Figure S10).

The signals of the products obtained in the polycondensation of GLO evidence the regioselective acylation of primary alcohols in 1,3 positions (SI, Figure S10).

The progress in the acylation of the three polyols within 72 h was monitored by calculating the ratio between the area of the signals corresponding to the protons of methylene adjacent to the newly formed ester group and the average of vinyl protons of DMI (assumed as constant). As can be seen from Table 4, the ester bonds are synthesized gradually for all polyols but in the case of BDO the reaction appears by far the slowest.

Table 4. Acylation progression (24, 48 and 72 h) of the primary -OH groups of BDO, CHDM and GLO evaluated via  $^1\text{H-NMR}$  by comparing the ratio between the area of methylene groups adjacent to -OH and the average area of vinyl protons of DMI (assumed as constant).

Used polyol	Time (h)	Area -CH <sub>2</sub> -OCO- (A <sub>1</sub> )	Area -CO-C=CH <sub>2</sub> (A <sub>2</sub> )	Ratio A <sub>1</sub> /A <sub>2</sub>
BDO	24	9.6	7.4	1.30
	48	12.5	7.6	1.64
	72	13.5	6.9	1.96
CHDM	24	8.6	4.2	2.05
	48	9.7	4.5	2.15
	72	11.7	4.2	2.79
GLO	24	13.6	6.0	2.27
	48	14.5	6.2	2.34
	72	14.3	5.8	2.46

\* Calculated via  $^1\text{H-NMR}$  as described in Table 1.

The reaction carried out with GLO leads to the full conversion of DMI after 72 hours and ESI-MS spectrum of PGI confirms the presence of oligomers with a molecular weight in the range of 344 and 716  $\text{m z}^{-1}$  (Figure 5) as a result of the acylation of the primary hydroxyl groups.

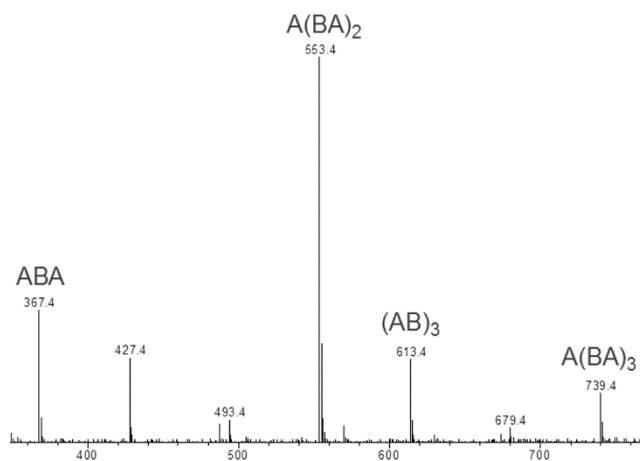


Figure 5. ESI-MS positive ion mass spectrum of polycondensation product of DMI (A) with GLO (B) recorded after 72 h of reaction carried out with 1:1.1 molar ratio of monomers and catalysed by 10% w/w of SPRIN Epobond CaLB (activity 2400  $\text{U g}_{\text{dry}}^{-1}$ ).

Surprisingly, the polycondensation of DMI with the cyclic CHDM gives oligomers ranging between 3 and 13 units and characterized by  $\text{m z}^{-1}$  between 382 and 1586 (Figure 6). The improved reactivity of DMI is documented by  $^1\text{H-NMR}$

spectra, indicating that 35% of C<sub>s</sub> has reacted after 72 h, as compared to <5% in the case of BDO (see SI, Figure S10).

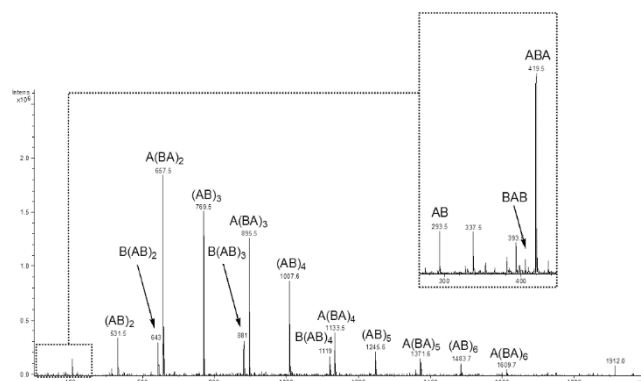


Figure 6. ESI-MS positive ion mass spectrum of polycondensation products of DMI (A) with CHDM (B) after 72 h. The reaction was carried out with 1:1.1 molar ratio of monomers and it was catalysed by 10% w/w of CaLB<sub>2400</sub>. The amount of biocatalyst corresponds to 240 U per gram of substrate monomers. ESI-MS spectra recorded after 24 and 48 hours of reactions are available in the supplementary materials, Figure S13.

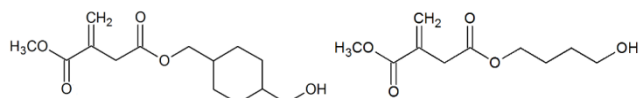
It must be noted that one significant and positive difference, as compared to the polycondensation with BDO, stays in the formation of the tetramer (AB)<sub>2</sub> already during the first 24 h of the reaction. This is clearly documented by HPLC-DAD profiles of the reaction that was monitored for 72 h, until DMI was quantitatively converted (SI, Figure S12).

#### Computational analysis of the different reactivity of A<sub>f</sub>B dimers

The unexpected behavior of the bulkier and rigid CHDM cannot be explained on the basis of its reactivity but must be investigated within the context of the active site of the enzyme, especially by analyzing differences as compared to BDO. The formation of the (AB)<sub>2</sub> will be favored either by the formation of A<sub>s</sub>B, which is a good acylating agent, or to the transesterification of A<sub>f</sub>B on the slow reacting group. Although A<sub>f</sub>B is a bad acylating agent, it can act also as nucleophile and be acylated to the free -OH group leading to the “dead-end” intermediate A<sub>s</sub>BA<sub>s</sub> (Scheme 2). Although the description of any kinetic model is beyond the scope of the present investigation, we tried to shed light on the differences of the reactivity of the A<sub>f</sub>B intermediates for the two diols inside the active site of CaLB, assuming that the electrophilicity of the acyl groups of A<sub>f</sub>-BDO and A<sub>f</sub>-CHDM is comparable (Scheme 3). The



two dimers were docked in the CaLB structure taken from the Protein Data Bank (PDB), with the code 1TCA.<sup>[35,36]</sup>



Scheme 3. The two A<sub>f</sub>B dimers (mono-acylated diols) that were docked in the active site of CaLB.

The software AutoDock (version 4.2)<sup>[37]</sup> was used for the docking procedure<sup>[38]</sup> and 100 docking generations, each one comprising 250 docking poses, were calculated using a Lamarckian genetic algorithm. Finally, the best 100 poses were selected according to AutoDock scoring functions mainly based on binding energies. Each selected pose was manually inspected in order to select only productive conformations where the substrate assumes a Near Attack Conformation (NAC) compatible with the attack of the catalytic serin to one of the two acyl groups of the dimer.<sup>[39]</sup>

When A<sub>f</sub>-BDO was docked into the CaLB active site, 27 out of 100 generated poses resulted to assume a conformation that enables the generation of an acyl-Enzyme complex. The results concerning the A<sub>f</sub>-CHDM intermediate showed only 11 productive poses, out of 100, which suggest an even slower formation of any CaLB-CHDM acyl-enzyme.

Concerning the nature of the productive poses, it is interesting to note that in the case of A<sub>f</sub>-BDO, only 2 conformations are compatible with the geometry required by the elongation reaction (attack of Ser 105 to the slow reacting acyl group) whereas the other 25 poses are in agreement with the attack of the fast reacting acyl group. The latter, however, is already esterified by the BDO but it will be prone to be attacked by any available nucleophile as, for instance, the methanol released during the acyl-enzyme formation or residual molecules of water.

On the contrary, in the case of A<sub>f</sub>-CHDM intermediate, 9 conformations (out of the 11 selected productive poses) are compatible with the attack of Ser 105 to the slow reacting acyl group (C<sub>s</sub>) and potentially leading to the elongation reaction. Only 2 poses are consistent with the attack of Ser 105 to the esterified fast reacting acyl group and the reversal of the reactions. Examples of productive docking poses are shown in Figure 7.

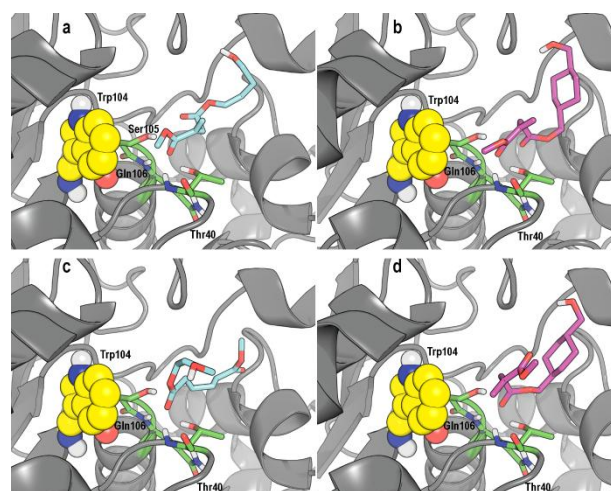


Figure 7. Docking of A<sub>f</sub>-BDO. a) Example of docking pose in agreement with the elongation reaction (attack of Ser105 to the slow reacting ester). c) Example of docking pose in agreement with the reversing or hydrolysis of the reaction (attack of Ser105 to the fast reacting ester).

Docking of A<sub>f</sub>-CHDM intermediate. b) Example of docking pose in agreement with the attack of Ser105 to the slow-reacting acyl group, thus leading to the elongation reaction. d) Example of docking pose in agreement with the attack of Ser105 to the fast reacting ester.

Substrates are represented in cyan sticks mode for A<sub>f</sub>-BDO and in pink sticks mode for A<sub>f</sub>-CHDM. The catalytic serine and the residues of the oxyanion hole are highlighted in green sticks mode; bulky Trp104 highlighted in yellow sphere mode.

One major structural factor responsible for the different conformational behaviour of the two dimers is represented by the steric hindrance of the bulky Trp104<sup>[40]</sup> that characterizes the narrow alcoholic subsite of CaLB, which is also responsible for enantiodiscrimination (Figure 7). As a consequence, no pose of the bulkier and rigid A<sub>f</sub>-CHDM, among the 100 characterized by the highest binding energy, locates the diol moiety inside the alcoholic pocket but rather it finds an unconventional location within the wider acylic subsite.

On the contrary, the flexible 1,4-butanediol moiety fits inside the alcoholic subsite of CaLB by assuming a globular shape (Figure 7c) and that increases the conformational chances for the nucleophilic attack to C<sub>f</sub> and the reversal of the reaction (or hydrolysis). That can occur when A<sub>f</sub>-BDO locates the alcohol functionality in any of the two subsites of CaLB.

These computational results provide at least some preliminary rationale to explain why the polycondensation of DMI with 1,4-*trans*-dimethanocyclohexane leads to oligomers with higher molecular weight. On the other hand,

docking studies confirms that the flexible BDO is not an optimal diol for the polycondensation of DMI since the dimer A<sub>f</sub>-BDO is more prone to the attack of the C<sub>f</sub> acylated group that causes the reversing of the reaction. In that case, the continuous removal of the released alcohol or residual water from the reaction system will be crucial. It must be noted that thin-film processes provide an easy route for fast removal of volatile components under reduced pressure, as previously demonstrated both at lab scale<sup>[24]</sup> and in pilot plant.<sup>[27]</sup> Therefore, future investigations will be aimed at integrating robust covalently immobilized CaLB with thin film processes for the synthesis of functionalized copolymers endowed with desired chemical properties.

## Conclusion

The present study for the first time sheds light on a number of factors affecting the polycondensation of DMI and it sets the basis for further optimization studies aiming at overcoming the low reactivity of DMI. The evidences here reported open new perspectives for improving the kinetics of polycondensation of DMI, either by optimizing the reaction configuration and by selecting the proper co-monomers.

Optimal mass transfer and a homogeneous dispersion of the enzyme in the reaction mixture is the major condition for achieving a reasonable elongation of the oligomers. Of course, a mono-molecular dispersion of the native enzyme would lead to the highest reaction rate but the contamination of product with the free enzyme must be avoided. Moreover, the recycling of the biocatalyst is mandatory for the economics of the process, which is strongly affected by the cost of the enzyme. On that respect, the data here reported demonstrate that biocatalysts consisting of CaLB covalently immobilized on carriers with low protein loading would be a practical and economical solution to the problem, although a further improvement might come from the development of novel cheaper, renewable and efficient carriers. Of course, this conclusion is applicable more generally to solvent-free enzymatic polycondensations but also to any biocatalyzed process hampered by mass transfer limitations and viscosity.

The second major factor affecting the course of the polycondensation consist in the concentration

and structure of the diols. Interestingly, computational and experimental data showed that the bulky and rigid CHDM is a promising candidate for achieving a better elongation of DMI polyesters. We have also demonstrated that in the case of BDO not only its concentration must be maintained low throughout the process but also that its structure is not favorable for the elongation. These findings encourage further investigations towards the optimization of the synthesis of polyesters of DMI and, more generally, the rational planning of *in vitro* enzymatic polycondensation.

## Experimental Section

### Chemicals and reagents.

Dimethyl itaconate (99%), 1,4-butanediol (99%), dichloromethane ( $\geq 99.9\%$ , GC grade), deuterated chloroform (CDCl<sub>3</sub>) (99.8 D-atoms, 0.03% v/v of TMS), tributyrin (98%) and ethyl acetate ( $\geq 99.5\%$ ) were purchased by Sigma-Aldrich. Acetonitrile ( $\geq 99.5\%$ ) was purchased from Riedel-de-Haën. n-Heptane (98.9%) and all the other solvents and chemicals were purchased from AnalR Normapur. All reagents were of analytical grade and were used as received without further purification if not otherwise specified.

### Enzymatic preparations.

Novozym 435<sup>®</sup> is a commercial formulation of lipase B from *Candida antarctica* (CaLB), adsorbed on a macroporous methacrylic resin. The biocatalyst was kindly donated by Novozymes (DK). The activity, assayed in the hydrolysis of tributyrin, resulted to be 2200 U g<sub>dry</sub><sup>-1</sup>. It has been demonstrated that most of the enzyme molecules of Novozym 435<sup>®</sup> are localized in a shell of the bead with a thickness of  $\sim 100$   $\mu\text{m}$ .<sup>[5]</sup>

CaLB<sub>2400</sub> correspond to the commercial biocatalyst SPRIN Epobond CaLB consisting of CaLB covalently immobilized on epoxy acrylic resin and experimentally determined activity of 2400 U g<sub>dry</sub><sup>-1</sup>. The covalent immobilization of CaLB<sub>350</sub> and CaLB<sub>230</sub> were carried out using as carrier an epoxy acrylic resin (Relizyme<sup>®</sup> EC-EP; average pore diameter 40-60 nm) according to protocols previously reported<sup>[33]</sup> and using a loading of the enzyme of 12000 and 10000 U per gram of methacrylic resin. All biocatalyst employed in the study have particle diameter in the range of 200-500  $\mu\text{m}$ .

It must be underlined that the robustness and recyclability of covalently immobilized CaLB have been already described in a previous study.<sup>[24]</sup>

### Assay of hydrolytic activity of lipases.

The activity of enzymatic preparations was assayed by following the tributyrin hydrolysis and by titrating, with 0.1 M sodium hydroxide, the butyric acid that is released during the hydrolysis. An emulsion composed by 1.5 mL tributyrin, 5.1 mL arabic gum emulsifier (0.6% w/v) and 23.4 mL water was prepared in order to obtain a final molarity of tributyrin of 0.17 M. Successively, 2 mL of K-

phosphate buffer (0.1 M, pH 7.0) were added to 30 mL of tributyrin emulsion and the mixture was incubated in a thermostated vessel at 30 °C, equipped with a mechanical stirrer. After pH stabilization, 50 mg of biocatalyst were added. The consumption of 0.1 M sodium hydroxide was monitored for 15-20 min. One unit of activity was defined as the amount of immobilized enzyme required to produce 1  $\mu$ mol of butyric acid per min at 30 °C.

#### HPLC analysis.

The polymerization products were analyzed by HPLC-DAD using a Phenomenex Gemini-NX C18 5  $\mu$ m (4.6 mm ID x 250 mm L) column and a Phenomenex Menex IB-Sil C8 5  $\mu$ m (4.6 mm ID x 30 mm L) pre-column connected to a Gilson HPLC system equipped with diode array detector Agilent 1100 Series and auto sampler. The elution of the compounds has been done isocratic using a mixture of ultrapure water (0.05 % trifluoroacetic acid) and AcN (0.05 % trifluoroacetic acid) with a flow rate of 1 mL min<sup>-1</sup> and the sample injection volume of 10  $\mu$ L. The eluting components were detected at 210 and 230 nm. Different gradient concentrations of acetonitrile and ultrapure water were used and the details are reported in Supplementary Information.

#### <sup>1</sup>H-NMR spectroscopy.

<sup>1</sup>H-NMR spectra were recorded on a Varian® Gemini 200 MHz spectrometer operating at 200 MHz or using a Jeol 270 MHz spectrometer operating at 270 MHz.

#### Water Activity.

Water activity of the reaction system was determined at 30 °C by using a hygrometer (DARAI-Trieste, Italy) operated and calibrated as previously reported.<sup>[29]</sup> The sensor was sealed, until constant reading, into the reaction vessel that had been previously equilibrated for 24 h at 30 °C.

#### Electrospray Ionization Mass Spectrometry (ESI-MS).

The crude reaction mixtures were analyzed on Esquire 4000 (Bruker) electrospray positive ionization by generating the ions in an acidic environment. Around 10 mg of sample was dissolved in 1 mL methanol containing 0.1% v v<sup>-1</sup> formic acid. The generated ions were positively charged with m z<sup>-1</sup> ratio falls in the range of 200-1000. The subsequent process of deconvolution allows the reconstruction of the mass peaks of the chemical species derived from the analysis of the peaks generated.

#### Enzymatic synthesis of PBI: polycondensation of dimethyl itaconate and 1,4-butanediol.

Dimethyl itaconate, (35 mmol), BDO (38,5 mmol) and the biocatalyst CaLB-cov (10% w/w with respect to the total amount of monomers) were mixed in a 250-mL reaction flask and the reaction proceeded connected with a rotary evaporator under reduced pressure (70 mbar) at 50 °C. The molar ratio of diester and polyol used was 1.0:1.1 unless differently stated. During the polymerization process the biphasic system became a monophasic homogeneous transparent solution. The final product was a viscous sticky colorless liquid, which was solubilized in DCM. After solvent evaporation, the crude product was analyzed by HPLC-DAD, ESI-MS and <sup>1</sup>H-NMR and TLC without any further purification. It was also verified that no reaction occurred in the absence of enzyme.

#### Attempts of performing polycondensations at 80°C

35 mmol DMI and 38,5 mmol of diols (either BDO or CHDM or a mixture consisting in 82.5% of BDO and 27.5% of CHDM) were mixed in a 250-mL reaction flask with the biocatalyst CaLB<sub>350</sub> (20% w/w with respect to the total amount of monomers). The reaction was carried out at 80 °C, 80 rpm, by connecting the flask to the rotary evaporator and under reduced pressure (800 mbar). During the polymerization process the biphasic system became a monophasic homogeneous transparent solution. After 42-48 h the products appeared as crystalline solids, that were insoluble in all solvents tested (DCM, methanol, THF, n-hexane, ethyl acetate). The insolubility of the reaction products suggest that the vinyl groups of DMI underwent a radical cross-linking.

#### Computational investigations

Crystal structure of CaLB was retrieved from Protein Data Bank (PDB); PDB ID: 1TCA.<sup>[35,36]</sup> CaLB structure was preprocessed by using the software PyMOL: all molecules but proteins and crystal water were deleted (i.e. inhibitors, glycosylation residues, etc.). Substrate molecules (A<sub>F</sub>-BDO and A<sub>F</sub>-CHDM) were in-silico generated by using PyMOL build tool. Only the *trans* CHDM was considered for the docking study since it is the predominant stereoisomer used in the polycondensation mixture. AutoDock version 4.2<sup>[34]</sup> was used for performing the docking calculations with Lamarckian genetic algorithm. 100 docking generation, each one composed of 250 docking poses were generated. Finally the 100 best generated poses were selected according to AutoDock scoring function. A further selection was performed by manual inspection for selecting productive Near Attack Conformation (NAC) docking poses.<sup>[39]</sup> More in detail, NACs correspond to conformers where the atoms involved in the bond formation are at van der Waals distance. At the same time, the angle of approach for the atoms must be  $\pm 15^\circ$  with respect to the angle of the bond formed in the transition state (TS). A numerous population of NACs indicates that low free energy changes are required to reach such NACs. Therefore, the population of NACs provides an indirect idea of the reaction rate.

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