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Poly(3-hydroxyoctanoate) depolymerase from *Pseudomonas fluorescens* GK13: Catalysis of ester-forming reactions in non-aqueous media

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ABSTRACT

Several industrial processes based on lipase catalysis have been established. However, since there are still a vast number of catalytic processes that lack a suitable enzyme, the discovery of new biocatalysts is required to fulfil this purpose. The potential of using the medium-chain-length (mcl)-PHA depolymerase from *Pseudomonas fluorescens* GK13 in anhydrous media to catalyze ester-forming reactions has been investigated and compared with that of Novozyme 435. The mcl-PHA depolymerase catalyzes the ring-opening polymerization of racemic β-butyrolactone (β-BL), L- and D-lactide (LLA, DLA) with high yield resulting in low molecular weight polymers. On the other hand, ε-caprolactone and pentadecalactone, which show high polymerizability using Novozyme 435 as catalyst, were not polymerized by mcl-PHA depolymerase. Besides, the activity of mcl-PHA depolymerase toward transesterification and esterification of ethyl-3-hydroxyoctanoate, lauric acid, (*R,S*)-β-BL, LLA and DLA has been studied.

1. Introduction

Polyhydroxyalkanoate (PHA) depolymerases are microbial enzymes that degrade polyhydroxyalkanoates, biopolyesters that are synthesized and accumulated intracellularly by a wide range of bacteria [1]. PHAs are biopolyoxoesters composed of (*R*)-3-hydroxy fatty acids which represent a complex class of storage polyesters [2]. The study of the PHAs hydrolysis by PHA depolymerases has attracted much attention not only due to their potential use as bioplastics or biomaterials [3], but also because their potential use in the production of chiral (*R*)-3-hydroxyalkanoic acids [4]. PHA depolymerases can be divided into intracellular and extracellular depolymerases [5]. These enzymes are specific for either short-chain-length (scl)-PHAs (3–5 carbon atoms, EC 3.1.1.75) or for medium-chain-length (mcl)-PHAs (6–14 carbon atoms, EC 3.1.1.76) [5]. Until now, more than 80 extracellular PHA depolymerases have been purified and characterized from various microorganisms but most of the isolated enzymes are specific to scl-PHA [1]. In general, mcl-PHA depolymerases differ considerably from scl-PHA depolymerases in terms of primary sequence and polymer-binding [5].

Among the mcl-PHA depolymerases characterized, the prototype of extracellular mcl-PHA depolymerase is that of *Pseudomonas fluorescens* GK13 ($PhaZ_{GK13}$) [6,7]. This enzyme consists of a signal peptide, an N-terminal substrate binding domain and a C-terminal catalytic domain [6]. Although mcl-PHA depolymerases do not display significant overall sequence homology to lipases or to other esterases, these enzymes possess a catalytic triad consisting of serine, histidine, and aspartate residues which have been demonstrated to form the active site in all known serine hydrolases. Moreover, the conserved Ser is a part of the lipase consensus sequence motif Gly-X_1-Ser-X_2-Gly [1,6]. Apart from this, $PhaZ_{GK13}$ is active for p-nitrophenylacyl esters, which are typical substrates for the determination of esterase activity. However, $PhaZ_{GK13}$ does not show significant lipase activity being unable to catalyze the hydrolysis of triolein and Tween 80 [8]. On the contrary, some lipases are able to hydrolyze polyesters consisting of an ω-hydroxyalkanoic acid, such as poly(4-hydroxybutyrate) and poly(6-hydroxyhexanoate) but not to hydrolyze poly-β-hydroxyalkanoates [9]. Apparently, lipases and PHA depolymerases may follow the same reaction mechanism, but differing in the spatial arrangement of their active sites.

The syntheses of aliphatic polyesters have been extensively studied by both fermentation and chemical processes in the field of biodegradable materials science [10]. Nevertheless, microbial syntheses generally give random copolyesters with relatively high production costs; and chemical modifications involve the use of toxic chemicals or catalysts. Alternatively, extensive research proved that a number of hydrolases can catalyze very efficiently ester and amide bond formation in non-aqueous media allowing

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the synthesis of a number of functional aliphatic polyesters [11]. Enzymatic polymerization has provided novel synthetic strategies for the production of different types of polymers affording cleaner products under milder conditions than conventional chemical catalysts [12]. Reactions leading to polymers include step growth reaction like polycondensation, and chain growth reaction like ring-opening polymerization [12]. Surprisingly, the majority of enzymes studied for polymerization reactions are members of the lipase family with Lipase B from *Candida antarctica* (CALB) immobilized on an acrylic resin (Novozyme 435) as the dominant enzyme [11]. This commercially available biocatalyst has proven to be highly useful and versatile. Although numerous reactions have been performed using lipases and related enzymes, it is still a challenge to identify the optimal biocatalyst and reaction conditions for a specific reaction [13].

Previous work describing scl-PHA depolymerase activity in non-aqueous media have been reported [14–17]. Kumar et al. [14] reported that scl-PHA depolymerase from *Pseudomonas lemoignei* has the potential to catalyze ester-forming reactions starting with cyclic esters and carbonates. Specifically, they reported the ring-opening of the lactones and propylation of their carboxyl group with 1-propanol in organic solvents. On the other hand, Suzuki et al. [15] provided the first report on polymerization of β-butyrolactone (β-BL) into poly(3-hydroxybutyrate), P(3HB), by scl-PHA depolymerase from *Pseudomonas stutzeri*. Lately, they subjected unsubstituted lactones of different ring size to ring-opening polymerization using an extracellular P(3HB) depolymerase from *Alcaligenes faecalis* T1 and obtained best results when using small ring-sized lactones as monomers [17]. However, the use of mcl-PHA depolymerases to catalyze bond formation in non-aqueous media has not yet been investigated. Hence, in this work, we prepared and purified the mcl-PHA depolymerase from *P. fluorescens* GK13 and studied its activity and specificity to catalyze ester-bond forming reactions in non-aqueous media in comparison to those of the commercially available immobilized lipase Novozyme 435.

2. Materials and methods

2.1. Materials and reagents

β-Propiolactone (β-PL, 98% purity, Aldrich), β-butyrolactone (β-BL, 98% purity, Aldrich), pentadecanolide (PDL, 99.0% purity, Fluka), ethyl-3-hydroxyoctanoate (Penta) and lauric acid (99.5% purity, Acros Organics) were used without further purification. ε-Caprolactone (ε-CL, 99% purity, Acros Organics) was distilled over calcium hydride *in vacuo*. D-Lactide (DLA, >99.5% purity, a gift from PURAC) was purified by recrystallization in ethyl acetate and sublimation in vacuum at 80 °C. L-Lactide (LLA, Boehringer) was purified by sublimation *in vacuum* at 80 °C.

All esterification reactions were carried out under a N_2 atmosphere. Nitrogen (Linde, 5.0) was passed over molecular sieves (4 Å) and finely distributed potassium on aluminium oxide. Toluene was distilled over sodium. Novozyme 435 (10,000 U/g, Novo Nordisk) was dried at room temperature for 24 h *in vacuum* and stored under N_2. $PhaZ_{GK13}$ was purified from the culture broth of cells grown in mineral medium supplied with poly(3-hydroxyoctanoate) [P(3HO)] as described by Gangoiti et al. [18], and used after lyophilisation.

2.2. Measurements

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 FT-NMR spectrometer at 400 MHz and 101 MHz, using deuterated chloroform ($CDCl_3$) as the solvent. Chemical shifts were reported relative to tetramethylsilane signals.

Size exclusion chromatography (SEC) was carried out using chloroform as eluting solvent. A high pressure liquid chromatography pump (Jasco PU-2080-Plus), a Jasco 2031-Plus RI detector and four MZ-SD Plus gel columns (50 Å, 100 Å, 1000 Å and 10,000 Å) were used in series at 30 °C with a flow rate of 1.0 mL/min. The molecular weights and polydispersities were determined using narrow distributed poly(methyl methacrylate) (PMMA) standards.

The catalytic activity of $PhaZ_{GK13}$ was determined at 30 °C by measuring for 5 min the turbidity decrease at 650 nm of P(3HO) suspensions [18]. The standard assay mixture consisted of 500 μg of P(3HO) in 200 mM Tris–HCl (pH 8.0) in a total volume of 1 mL. After incubation at 30 °C for 10 min, the reaction was started by the addition of the purified enzyme.

2.3. General procedure for transacylation reactions using mcl-PHA depolymerase as the catalyst

The enzyme-catalyzed transacylation of lactides, β-BL, ethyl-3-hydroxy-octanoate and lauric acid were performed as follows. The monomer (100 mg) and the powdered enzyme (1 wt%) were transferred to a dry flask. An equimolar amount of the alcohol with respect to the acyl-compound was added. The glass flask was then placed in an oil bath at 80 °C with agitation. Depending on the monomer, the reactions were performed in bulk or in toluene. In the absence of enzyme no significant transacylation was observed.

n-Propyl-3-hydroxy-octanoate (1): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.90 (t, J = 8.0 Hz, CH_3CH_2), 1.6 (m, J = 7 Hz, $CH_2CH_2CH_3$), 4.02 (t, J = 6.8 Hz, OCH_2CH_2), 2.46; 2.35 (ddd, J = 7 Hz, $HOCHCH_2CO_2$), 3.94 (m, J = 7 Hz, $CH_2CH(OH)CH_2$), 1.44 (q, J = 7.1 Hz, $CH_2CH_2CH_2OH$), 1.28 (m, J = 7.1 Hz, $CH_2CH_2CH_2CH_2$), 1.31 (m, J = 7.1 Hz, CH_3CH_2), 0,88 (t, J = 7.6 Hz, CH_3CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 173.1, 67.9, 66.14, 41.6, 36.46, 31.71, 25.12, 21.87, 21.8, 13.95, 10.26.

2-Propyl-3-hydroxy-octanoate (2): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.30 (d, J = 6.8 Hz, $OCH(CH_3)_2$), 5.06 (m, J = 6.8 Hz, $OCH(CH_3)_2$, 2.46; 2.35 (ddd, J = 7 Hz, $HOCHCH_2CO_2$), 3.94 (m, J = 7 Hz, $CH_2CH(OH)CH_2$), 1.44 (q, J = 7.1 Hz, $CH_2CH_2CH_2OH$), 1.28 (m, J = 7.1 Hz, $CH_2CH_2CH_2CH_2$), 1.31 (m, J = 7.1 Hz, CH_3CH_2), 0,88 (t, J = 7.6 Hz, CH_3CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 171.4, 68.9, 68.04, 41.6, 36.46, 31.76, 25.20, 22.58, 21.46, 14.18.

n-Propyl laurate (3): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.91 (t, J = 8.0 Hz, CH_3CH_2), 1.26–1.29 (m, J = 7.1 Hz, $CH_2(CH_2)_8CH_2$), 1.31 (m, J = 7.5 Hz, $CH_3CH_2CH_2$), 1.61 (m, J = 7.1 Hz, $CH_2CH_2CH_2COOH$), 1.73 (m, J = 7.5 Hz, $CH_3CH_2CH_2$), 2.3 (t, J = 7.1 Hz, CH_2CH_2COOH), 4.02 (t, J = 7.0 Hz, $OCOCH_2CH_2$). ^{13}C NMR ($CDCl_3$): δ = 173.9, 65.7, 34.4, 31.9, 29.6, 25.1, 22.8, 21.4, 14.1, 10.3.

Propyl-(3R/3S)-3-hydroxybutanoate (4): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.19 (q, J = 6.3 Hz, $CH_2CHOHCH_3$), 3.97 (t, J = 6.7 Hz, CH_2CH_2OCO), 2.31 (ddd, J = 8.2 and 16.0 Hz, 4.1 and 16.0 Hz, $OCOCH_2CHOHCH_3$), 1.63 (m, J = 6.7 Hz, $CH_3CH_2CH_2$), 1.19 (d, J = 6.3 Hz, $CH_2CHOHCH_3$), 0.91 (t, J = 6.7 Hz, CH_3CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 172.89, 66.14, 64.50, 44.16, 22.30, 21.87, 10.26.

n-Propyl lactide (5): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.18 (q, J = 7.1 Hz, OCH_3CHCO), 4.33 (q, J = 6.9 Hz, $OHCHCH_3CO$), 4.08 (t, J = 6.8 Hz, OCH_2CH_2), 1.63 (m, $CH_2CH_2CH_3$), 1.56 (d, J = 7.1 Hz, OCH_3CHO), 1.48 (d, J = 6.9 Hz, CH_3CHOH), 0.92 (t, J = 6.8 Hz, CH_2CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.06, 170.09, 69.35, 66.94, 66.6, 21.82, 21.35, 16.8, 10.15.

n-Propyl lactate (6): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.18 (q, J = 7.1 Hz, $O(CH_3)CHCO$), 4.23 (q, J = 6.9 Hz, $HOCHCH_3CO$), 4.08 (t, J = 6.8 Hz, OCH_2CH_2), 1.63 (m, $CH_2CH_2CH_3$), 1.56 (d, J = 7.1 Hz, OCH_3CHO), 1.39 (d, J = 6.9 Hz, CH_3CHOH), 0.92 (t, J = 6.8 Hz, CH_2CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.69, 67.0, 66.6, 21.82, 21.35, 10.15.

2.4. Typical procedure for mcl-PHA depolymerase catalyzed ring-opening-polymerizations

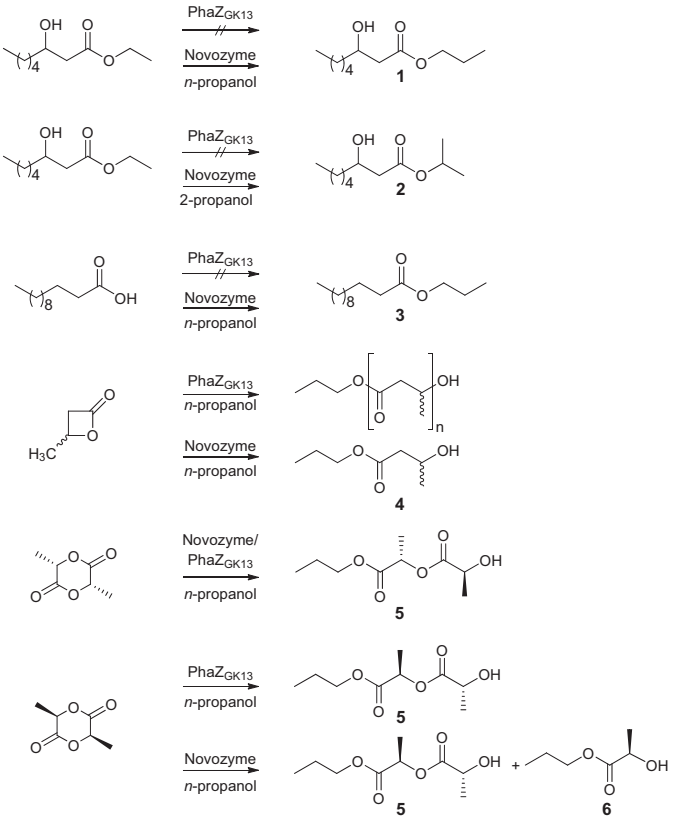
The general procedure used for the enzymic ring-opening polymerization of lactones and L- and D-lactides was as follows. A mixture of monomer (100 mg) and the enzyme (1 wt%) was stirred under a N_2 atmosphere in a capped vial placed in an oil bath at 80 °C. Unless otherwise specified all reactions were performed for 24 h. After the reaction, the mixture was dissolved in deuterated chloroform (1 mL), and the insoluble enzyme was removed by filtration. The chloroform was then evaporated *in vacuo* to quantitatively obtain the product mixture.

The molecular weight and the molecular weight distribution of the polymer fraction were determined by SEC. Depending on the monomers used the reactions were performed in bulk or in toluene.

Monomer conversion was determined by ^{1}H NMR spectroscopy, comparing the integration intensities of protons of the monomers with those of the repeating unit in the polymer. For β-PL, ε-CL and PDL the position of the methylene protons adjacent to the oxygen atom in the monomer were $\delta = 4.2$ (C-3 of β-PL), 4.23 (C-6 of ε-CL) and 4.15 ppm (C-15 of PDL) and in the polymer were $\delta = 4.3$ [C-3 of P(3HP)], 4.06 [C-6 of P(6HH)] and 4.05 ppm [C-15 of P(PDL)]. In the case of (*R*,*S*)-β-BL the monomer conversion was determined by comparison of the integration intensities for the peak at $\delta = 1.58$ ppm corresponding to the methyl protons of the monomer with the corresponding methyl protons of the repeating unit of P(3HB) at $\delta = 1.28$ ppm.

Conversion of lactides to poly(lactic acid) (PLA) was determined by comparison of the integration intensities of the peak at $\delta = 4.76$ ppm corresponding to the methyne protons of the monomeric lactide with the peak at $\delta = 5.11$ ppm corresponding to PLA.

In all cases, control reactions without enzyme were performed.



Scheme 1. Transacylation reactions.

3. Results and discussion

The potential of mcl-PHA depolymerase from *P. fluorescens* GK13 as a new catalyst for esterification reactions was investigated. In order to evaluate the potential use of this new biocatalyst, a direct comparison with the reactivity of commercially available Novozyme 435 for different ester forming reactions was performed. All reactions were carried out simultaneously under the same conditions using both catalysts. However, the fact that $PhaZ_{GK13}$ was used as free enzyme will ultimately influence comparison between these two protein catalysts.

3.1. Preparation of $PhaZ_{GK13}$

The $PhaZ_{GK13}$ was purified as described by Gangoiti et al. [18]. SDS-PAGE analysis showed a major double band at about 25 kDa, which corresponded to the enzyme (data not shown). The enzyme solution in phosphate buffer (5 mM, pH 8.0) was then subjected to lyophilisation. The enzyme retained 87% of its initial activity after lyophilisation. Routinely, preparations of $PhaZ_{GK13}$ with a specific activity of approximately 52.7 U/mg for P(3HO) were used as catalyst in ester-bond forming reactions.

3.2. Transacylation reactions

The natural substrate hydrolyzed by mcl-PHA depolymerases in aqueous media are enantiopure (*R*)-P(3HO) or structurally related polyhydroxyalkanoates. Therefore, ethyl-3-hydroxy-octanoate was selected as a substrate for the transesterification reaction to determine the activity of $PhaZ_{GK13}$ for polyester synthesis *via* condensation in a non-aqueous medium. However, under the studied conditions, no monomer conversion was achieved after 48 h. Similarly, $PhaZ_{GK13}$ did not promote the transacylation of ethyl-3-hydroxy-octanoate with *n*-propanol and 2-propanol (Scheme 1) after 2 days of reaction, while 41% and 10% of conversion was obtained, respectively, when the reaction was conducted by the Novozyme 435.

Additionally, the potential of using $PhaZ_{GK13}$ to catalyze the esterification of lauric acid was studied. As expected, after 72 h high conversions (96%) were obtained using Novozyme 435, whereas low conversions (5%) were observed in the case of $PhaZ_{GK13}$.

Previous studies described that the scl-PHA depolymerase from *P. lemoignei* catalyzed alcoholysis of different cyclic esters in organic solvents [14] obtaining best results with L-LA (*SS*), *meso*-LA (*RS*), D-LA (*RR*) and racemic β-BL. Lactides and β-BL have secondary hydroxyl groups attached to the chiral centre that participate in the formation of ester bonds. Furthermore, the substituents attached to the chiral centres of both lactide and β -BL are methyl groups. In contrast to β-BL, which has the hydroxyl group in β position to the carbonyl group, lactides have the hydroxyl group in α-position. Motivated by the above, reaction of *n*-propanol with racemic β-BL, and D- and L-lactides was studied (Scheme 1). As shown in Table 1, when β-BL was used as acyl donor, 19% of *n*-propanol was transacylated by $PhaZ_{GK13}$ in toluene after 72 h as revealed by the presence of the signal ($\delta = 3.97$ ppm) corresponding to the acylated alcohol in the ^{1}H NMR spectra. However, 63% of β-BL conversion was determined. Besides, SEC analysis confirmed the presence of a polymer with an estimated M_n of 770 g/mol indicating that β-BL polymerization also occurred. When this reaction was conducted in bulk instead of toluene, 6% of transacylated product was estimated by ^{1}H NMR and 99% of β-BL conversion was observed. In this case a polymer of a M_n of 1800 g/mol was obtained.

Table 1
Enzyme catalyzed esterification of *n*-propanol using different acyl-donors.[a]

Monomer	Enzyme	Solvent	Time (h)	Conversion (%)	Product (%)	M_n[b]
β-BL	$PhaZ_{GK13}$	In bulk	24	99	Polymer (93)	1800
	$PhaZ_{GK13}$	Toluene (1.8 mL)	72	63	Polymer (44)	770
	Novozyme	Toluene (1.8 mL)	72	54	**4** (54)	–
LLA	$PhaZ_{GK13}$	Toluene (0.6 mL)	24	99	Oligomer (11)	nd[c]
					5 (88)	–
	Novozyme	Toluene (0.6 mL)	24	78	**5** (78)	–
DLA	$PhaZ_{GK13}$	Toluene (0.6 mL)	24	99	Oligomer (19)	nd[c]
					5 (80)	–
	Novozyme	Toluene (0.6 mL)			**5** (74)	–
			24	99	**6** (25)	–

Numbers 4, 5 and 6 correspond to the products represented in Scheme 1.
[a] Reaction was performed at 80 °C (mol ratio of alcohol/acyl donor = 1:1) using 1% (w/w) of $PhaZ_{GK13}$ or Novozyme 435.
[b] Molecular weight (M_n) determined by SEC using narrow distributed poly(methyl methacrylate) standards and chloroform as eluent.
[c] nd: not determined.

As a consequence of β-BL polymerization, the number of free terminal carboxyl groups able to react with *n*-propanol was limited. Based on the ^{1}H NMR results we supposed we have obtained a functionalized polymer. We assume that *n*-propanol acts as initiator and once the β-BL ring is opened the rate of polymerization is higher than the rate of initiation and consequently oligomers are the preferred product. In contrast, the absence of P(3HB) in the product mixture when Novozyme 435 catalyzed the transacylation of *n*-propanol indicated that the immobilized CALB promoted only the transacylation of the *n*-propanol to propyl-3-hydroxybutanoate reaching a 54% of conversion (Table 1). In the case of Novozyme 435, it is assumed that the rate of initiation is higher and propagation by the secondary alcohol is inefficient. Similar to immobilized CALB, Kumar et al. [14] reported that scl-PHA depolymerase of *P. lemoignei* catalyzed the *n*-propylation of racemic β-BL leading to propyl-3-hydroxybutanoate with 50% of conversion.

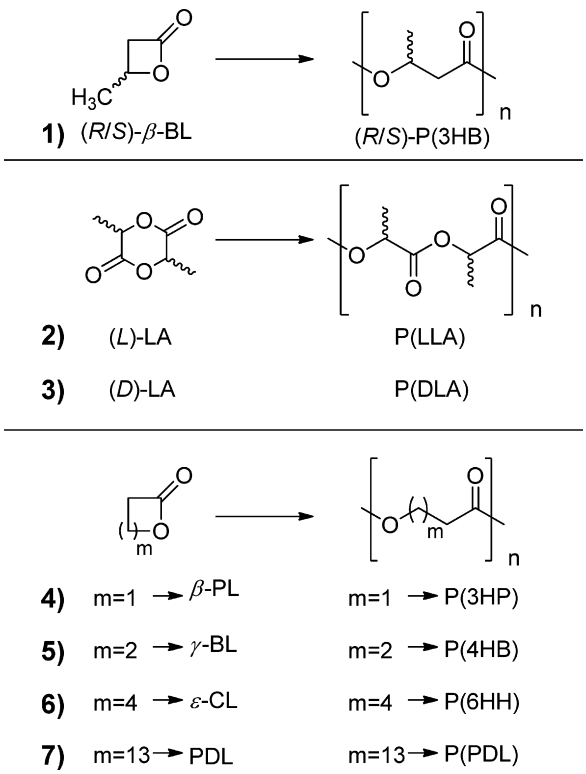
On the other hand, $PhaZ_{GK13}$ catalyzed the *n*-propylation of LLA and DLA to *n*-propyl lactide. After 24 h of reaction using $PhaZ_{GK13}$, more than 80% of *n*-propanol was transacylated (Table 1). However, 99% of lactide conversion was observed, indifferently of the stereochemistry of the lactide used. This fact and the presence of a weak but detectable signal at $\delta = 4.97$ ppm correspondent to unreacted α methyne proton of the carboxyl end group suggested that lactide oligomers have been formed as a side product (Table 1). When the molar ratio monomer/alcohol was increased from 1:1 to 1:3, 100% of lactide was converted to propyl lactide. On the other hand, LLA ring-opening and esterification by Novozyme 435 yielded 78% of propyl lactide as the only product. In contrast, in the case of DLA esterification catalyzed by Novozyme 435 yielded beside to *n*-propyl D-lactide (74%), *n*-propyl D-lactate (25%), as revealed by the presence of signal at $\delta = 4.23$ ppm (data not shown). Similar results were obtained with the scl-PHA depolymerase from *P. lemoignei* [14]. However, based on our results, $PhaZ_{GK13}$ is unable to further cleave the internal ester bond of the *n*-propyl-lactide to form *n*-propyl-lactate indifferently of the stereochemistry of the monomer.

It seems that $PhaZ_{GK13}$ catalyzes ester-bond-formation showing different reactivity than the one determined for Novozyme 435. In fact, in contrast to the immobilized CALB, $PhaZ_{GK13}$ is unable to catalyze the transacylation of ethyl-3-hydroxy-octanoate and to propylate lauric acid. Both of these substrates present an alkyl moiety that might impede the recognition of their functional group by the active centre. However, $PhaZ_{GK13}$ catalyzed transacylation of *n*-propanol when using lactides as acyl donors producing *n*-propyl lactide as the major product with high yield. On the contrary, $PhaZ_{GK13}$ catalyzed ring opening polymerization of β-BL in the presence of a functional alcohol and not transacylation leading to propyl-3-hydroxybutanoate, while in the case of Novozyme 435 no P(3HB) polymer was synthesized.

3.3. Ring-opening polymerization reactions

Based on our previous results, $PhaZ_{GK13}$ might be a potential catalyst for the ring opening polymerization of the four-membered β-methyl substituted lactone, β-BL (Scheme 2).

As shown in Table 2, $PhaZ_{GK13}$ catalyzed the ring opening polymerization of racemic β-BL with a monomer conversion of 98% to yield P(3HB) with a M_n of 1500 after 24 h. Similarly, the scl-PHA depolymerase of *P. stutzeri* catalyzed the polymerization of β-BL with 95% of monomer conversion obtaining a P(3HB) polymer with a M_n ~1250 g/mol. However, in this study the reaction was performed for 3 days at 80 °C in the presence of 5 wt% of enzyme [15]. Under the same conditions (Table 2), β-BL was hardly polymerized by Novozyme 435 and only 8% of conversion was observed. The lipase-catalyzed ring-opening polymerization of this monomer was first reported by Nobes et al. [19]. P(3HB) having



Scheme 2. Ring opening polymerization reactions. $PhaZ_{GK13}$ catalyzed reactions 1, 2, 3 and 4. Novozyme 435 catalyzed reactions 1, 3, 6 and 7.

Table 2
Enzyme catalyzed ring-opening polymerization of cyclic esters: monomer, conversion and data from SEC analysis.[a]

Monomer	Enzyme	Solvent	Time (h)	Conversion (%)	M_n[b]	M_w/M_n[b]
β-BL	$PhaZ_{GK13}$	In bulk	24	98	1500	1.52
	Novozyme 435			8	760	1.05
LLA	$PhaZ_{GK13}$	Toluene (0.6 mL)	24	98	900	1.03
	Novozyme 435			0	–	–
DLA	$PhaZ_{GK13}$	Toluene (0.6 mL)	24	93.4	900	1.01
	Novozyme 435			14.3	nd[c]	nd[c]
β-PL	$PhaZ_{GK13}$	In bulk	6	100	9200	2
	None (Control)			3	960	1.15
γ-BL	$PhaZ_{GK13}$	In bulk	24	0	–	–
	Novozyme 435			0	–	–
ε-CL	$PhaZ_{GK13}$	In bulk	48	0	–	–
	Novozyme 435			98.1	6500	1.9
PDL	$PhaZ_{GK13}$	In bulk	48	0	–	–

[a] Reaction was performed with 1 wt% of enzyme to monomer and at 80 °C in all cases except of β-PL reaction which was carried out at 60 °C.
[b] Molecular weight (M_n) and molecular weight distribution (M_w/M_n) determined by SEC using narrow distributed poly(methyl methacrylate) standards and chloroform as eluent.
[c] nd: not determined.

weight average molecular weights ranging from 260 to 1050 g/mol were obtained after several weeks of polymerization. Recent work reported by Gorke et al. [20] using Novozyme 435 as catalyst in ionic liquids gave oligomers of β-BL with a degree of polymerization of 5. Consequently, based on these results the mcl-PHA depolymerase of *P. fluorescens* GK13 might be a promising catalyst for β-BL polymerization.

It has been reported that, P(3HB) obtained from the lipase catalyzed ring-opening polymerization of (*R*,*S*)-β-BL presented three different kinds of structural isomers, cyclic, hydroxyl- and crotonate-terminated P(3HB) [21]. The ^{1}H NMR spectrum of P(3HB) revealed small peaks corresponding to the hydroxymethyne (δ = 4.2 ppm) and crotonate (δ = 1.86, 5.75 and 6.9 ppm) terminal groups of P(3HB). However, by ^{1}H NMR spectroscopy we were unable to confirm the presence of cyclic oligomers.

Additionally, studies were performed to assess the $PhaZ_{GK13}$ activity toward ring-opening polymerization of lactides of different configuration (Scheme 2). Lactide, a six-membered ring monomer, is highly reactive in chemical ring-opening polymerization and results in biocompatible and biodegradable polymers that are used in a variety of biomedical applications. This makes this monomer interesting for exploration in $PhaZ_{GK13}$-catalyzed ring-opening polymerization. As shown in Table 2, $PhaZ_{GK13}$ showed excellent polymerization activities toward LLA and DLA. In fact, after 24 h the conversion obtained with LLA and DLA is very high in both cases. However, the molecular weight of the synthesized polymers was low ($M_{n,SEC}$ ~900). On the other hand, under the same conditions Novozyme 435 did not promote the polymerization of the monomer in (*S*)-configuration while 14% conversion was obtained with DLA. Although enzymes have been successfully used for the synthesis of many classes of polyesters, yet no enzyme has shown any high activity for the *in vitro* polymerization of D,L-lactide. Several hydrolases, such as *Burkhordelia cepacia* lipase (lipase PS), *Humicola insolens* cutinase, *Candida cylindracea* lipase, Proteinase K and lipase CALB have been evaluated for the ring-opening polymerization of lactides. However, long reaction time, high background activity and high enzyme concentration have characterized these studies [22]. By the moment, best results were reported by Matsumura et al. [23] using 3 wt% of lipase PS relative to D,L-lactide. After 7 days of reaction at 130 °C poly(D,L-lactide) with a M_n of 115,000 was obtained. In recent years, Hans et al. [24] reported that the polymerization of DLA catalyzed by Novozyme 435 resulted in 80% of conversion at 50 °C after 2 days, while no polymer was obtained when using LLA. Two problems that limited the application of CALB in the ring-opening polymerization of lactides have been identified so far. First, CALB is known to be a slow catalyst when ester substrates have a substituent at α-position next to the carbonyl group [25] and, secondly, CALB is highly enantioselective for *R*-secondary alcohols [26].

In contrast, as occurred in lipase PS [23], $PhaZ_{GK13}$ can polymerize LLA and DLA, indicating that both enzymes are not enantiospecific for lactides. This is the first report on *in vitro* ring-opening polymerization of L- and D-LA into PLA, by a PHA depolymerase. Taking into account that $PhaZ_{GK13}$ catalyzed not only the ring-opening polymerization of β-BL, but also of D- and L-LA monomers, the possibility of copolymerizing these substrates might be explored. Interestingly, $PhaZ_{GK13}$ does not catalyze the hydrolysis of P(3HB) and PLA polymers in aqueous media [9]. Therefore, the enzyme might show different specificity for the hydrolysis and formation of ester bonds of these substrates.

In order to understand in detail the interaction between the enzyme and lactones, the activity and specificity of $PhaZ_{GK13}$ toward ring-opening polymerization of unsubstituted lactones of various ring-sizes was evaluated and compared with that of Novozyme 435 under the same conditions. In this study, β-PL, γ-BL, ε-CL, and PDL were used as monomers and subjected to the ring-opening polymerization reaction (Scheme 2). It was found that lactones showed various characteristic reactivities (Table 2).

$PhaZ_{GK13}$ catalyzed β-PL ring-opening polymerization after 6 h at 60 °C with a 100% of monomer conversion. The M_n of the resulting P(3HP) was 9200 g/mol. Similar results were obtained when 1 wt% of the wild-type scl-PHA depolymerase of *A. faecalis* T1 was used as catalyst. In this case, after 2 days at 60 °C a P(3HP) polymer with a M_n of 8200 g/mol was obtained [17]. On the other hand, as expected, both $PhaZ_{GK13}$ and Novozyme 435 were not able to polymerize γ-BL because of the thermodynamic stability of the 5-membered ring lactone. In fact, there are few reports on the enzyme-catalyzed ring-opening polymerization of the γ-BL, except for Nobes et al. [19] and Dong et al. [27]. However, in both cases enzymatic polymerization by using γ-butyrolactone as monomer only lead to low molecular weight products and monomer conversions. Indeed, best results were obtained by Nobes et al. [19] after 18 days of reaction yielding P(4HB) in up to 42% yield with a M_w of ~900. Moreover, ε-CL (Table 2) and PDL [28], which show excellent polymerization activities by Novozyme 435, were not polymerized by $PhaZ_{GK13}$ (Table 2).

In conclusion, $PhaZ_{GK13}$ is able to catalyze ester-bond-formation using not only primary, but also secondary alcohols in α and β

positions. In fact, $PhaZ_{GK13}$ catalyzed the polymerization of β-PL, β-BL and LLA and DLA, with ring sizes of 4 and 6, respectively. Interestingly, $PhaZ_{GK13}$ does not show enantioselectivity toward secondary alcohols, while Novozyme 435 polymerizes only DLA under the reaction conditions used. According to Uppenberg et al. [29], the active site of CALB is composed of two channels; one hosting the acyl- and the other hosting the alcohol-moiety of the substrate, with the first channel being the more spacious one, showing a high enantioselectivity toward secondary alcohols and a broad substrate tolerance. Although, there is no information about the quaternary structure of mcl-PHA depolymerases, based on our results it can be suggested that $PhaZ_{GK13}$ may show a different organization being less restrictive to alcohol moieties.

Furthermore, contrary to Novozyme 435, $PhaZ_{GK13}$ does not show polymerization activity toward lactones with ring size higher than 6. Similar results were reported for scl-PHA depolymerase from *A. faecalis T1* by Suzuki et al. [17] who based on a structural model proposed that these substrates seemed to be over size limit for the molecular recognition of the narrow active site cleft of scl-PHA depolymerases.

4. Conclusions

This report demonstrates the potential of using mcl-PHA depolymerase of *P. fluorescens* GK13 as a novel catalyst for ester-bond forming reactions in non-aqueous media. This is the first time that a member of the family of mcl-PHA depolymerases has been identified as a new enzyme for polymer synthesis showing different specificity and other valuable attributes in comparison to Novozyme 435. The introduction of this new family of enzymes for bond formation in anhydrous media might give the opportunity to explore reactions that thus far were not successfully performed. However, further research is required to evaluate the possible benefits of the mcl-PHA depolymerase over other extensively studied enzymes.

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References

[1] D.Y. Kim, H.W. Kim, M.G. Chung, Y.H. Rhee, J. Microbiol. 45 (2007) 87–97.
[2] L.L. Madison, G.W. Huisman, Microbiol. Mol. Biol. Rev. 63 (1999) 21–53.
[3] G.Q. Chen, Adv. Biochem. Eng. Biotechnol. 125 (2011) 29–45.
[4] G.Q. Chen, Q. Wu, Appl. Microbiol. Biotechnol. 67 (2005) 592–599.
[5] D. Jendrossek, R. Handrick, Annu. Rev. Microbiol. 56 (2002) 403–432.
[6] A. Schirmer, D. Jendrossek, J. Bacteriol. 176 (1994) 7065–7073.
[7] L.I. de Eugenio, J.L. García, P.L. García, M.A. Prieto, J.M. Sanz, Eng. Life Sci. 8 (2008) 260–267.
[8] A. Schirmer, D. Jendrossek, H.G. Schlegel, Appl. Environ. Microbiol. 59 (1993) 1220–1227.
[9] K.E. Jaeger, A. Steinbüchel, D. Jendrossek, Appl. Environ. Microbiol. 61 (1995) 3113–3118.
[10] R.A. Gross, M. Ganesh, W. Lu, Trends Biotechnol. 28 (2010) 435–443.
[11] A.C. Albertsson, R.V. Srivastava, Adv. Drug Deliv. Rev. 60 (2008) 1077–1093.
[12] R.A. Gross, A. Kumar, B. Kalra, Chem. Rev. 101 (2001) 2097–2124.
[13] U.T. Bornscheuer, C. Bessler, R. Srinivas, S.H. Krishna, Trends Biotechnol. 20 (2002) 433–437.
[14] A. Kumar, R.A. Gross, D. Jendrossek, J. Org. Chem. 65 (2000) 7800–7806.
[15] Y. Suzuki, T. Ohura, K.I. Kasuya, K. Toshima, Y. Doi, S. Matsumura, Chem. Lett. 29 (2000) 318–319.
[16] Y. Suzuki, S. Taguchi, T. Saito, K. Toshima, S. Matsumura, Y. Doi, Biomacromolecules 2 (2001) 541–544.
[17] Y. Suzuki, S. Taguchi, T. Hisano, K. Toshima, S. Matsumura, Y. Doi, Biomacromolecules 4 (2003) 537–543.
[18] J. Gangoiti, M. Santos, M.J. Llama, J.L. Serra, Appl. Environ. Microbiol. 76 (2010) 3554–3560.
[19] G.A.R. Nobes, R.J. Kazlauskas, R.H. Marchessault, Macromolecules 29 (1996) 4829–4833.
[20] J.T. Gorke, K. Okrasa, A. Louwagie, R.J. Kazlauskas, F. Srienc, J. Biotechnol. 132 (2007) 306–313.
[21] Y. Osanai, K. Toshima, S. Matsumura, Macromol. Biosci. 1 (2001) 171–177.
[22] M. Takwa, M.W. Larsen, K. Hult, M. Martinelle, Chem. Commun. 47 (2011) 7392–7394.
[23] S. Matsumura, K. Mabuchi, K. Toshima, Macromol. Rapid. Commun. 18 (1997) 477–482.
[24] M. Hans, H. Keul, M. Moeller, Macromol. Biosci. 9 (2009) 239–247.
[25] P.O. Syrén, K. Hult, ChemBioChem 11 (2010) 802–810.
[26] F. Haeffner, T. Norin, K. Hult, Biophys. J. 74 (1998) 1251–1262.
[27] H. Dong, H.D. Wang, S.G. Cao, J.C. Shen, Biotechnol. Lett. 20 (1998) 905–908.
[28] M.L. Focarete, M. Scandola, A. Kumar, R.A. Gross, J. Pol. Sci. 39 (2001) 1721–1729.
[29] J. Uppenberg, N. Oehrner, M. Norin, K. Hult, G.J. Kleywegt, S. Patkar, V. Waagen, T. Anthonsen, T.A. Jones, Biochemistry 34 (1995) 16838–16851.