Article

Entrapment of Phenylalanine Ammonia-Lyase in Nanofibrous Polylactic Acid Matrices by Emulsion Electrospinning

Gábor Koplányi 1, Evelin Santa-Bell 1,2, Zsófia Molnár 1,2, Gergő Dániel Tóth 3, Muriel Józó 3, András Szilágyi 3,*, Ferenc Ender 4,5, Béla Pukánszky 3,6, Beáta G. Vértessy 2,7, László Poppe 1,8, and Diána Balogh-Weiser 1,3,*, *

1 Department of Organic Chemistry and Technology, Budapest University of Technology and Economics, Műegyetem rkp. 3, 1111 Budapest, Hungary; koplanyig@edu.bme.hu (G.K.); santa-bell.evelin@vbk.bme.hu (E.S.-B.); molnar.zsofia@vbk.bme.hu (Z.M.); poppe.laszlo@vbk.bme.hu (L.P.)
2 ELKH Research Center for Natural Sciences, Institute of Enzymology, Magyar tudósok krt. 2, 1117 Budapest, Hungary; vertessy.beata@vbk.bme.hu
3 Department of Physical Chemistry and Materials Science, Budapest University of Technology and Economics, Műegyetem rkp. 3, 1111 Budapest, Hungary; tothgergodaniel@edu.bme.hu (G.D.T.); jozo.muriel@vbk.bme.hu (M.J.); szilagyi.andras@vbk.bme.hu (A.S.); pukanszky.bela@vbk.bme.hu (B.P.)
4 Department of Electron Devices, Budapest University of Technology and Economics, Műegyetem rkp. 3, 1111 Budapest, Hungary; ender.ferenc@vbk.bme.hu
5 SpinSplit LLC., Vend u. 17, 1025 Budapest, Hungary
6 Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, P.O. Box 286, 1519 Budapest, Hungary
7 Department of Applied Biotechnology and Food Science, Budapest University of Technology and Economics, Műegyetem rkp. 3, 1111 Budapest, Hungary
8 Biocatalysis and Biotransformation Research Centre, Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University of Cluj-Napoca, Arany János Str. 11, RO-400028 Cluj-Napoca, Romania

* Correspondence: balogh.weiser.diana@vbk.bme.hu; Tel.: +36-1-463-1265

Abstract: Immobilization of the recombinant, plant-derived Petroselinum crispum phenylalanine ammonia lyase (PcPAL) in electrospun matrices have the potential to create promising, easy-to-use biocatalysts. Polylactic acid (PLA) a biologically inert, commercial biopolymer, was chosen as the material of the carrier system. PLA could be electrospun properly only from water-immiscible organic solvents, which limits its application as a carrier of sensitive biological objects. The emulsion electrospinning is a proper solution to overcome this issue using non-ionic emulsifiers with different hydrophilic-lipophilic balance (HLB) values. The stabilized emulsion could protect the sensitive PcPAL dissolved in the aqueous buffer phase and improve fiber formation, plus help to keep the biocatalytic activity of enzymes. In this study, the first approach is described to produce PLA nanofibers containing PcPAL enzymes by emulsion electrospinning and to use the resulted biocatalyst in the ammonia elimination reaction from L-phenylalanine.

Keywords: electrospinning; recombinant enzyme; enzyme entrapment; emulsion electrospinning; phenylalanine ammonia-lyase

1. Introduction

Enantiopure amino acids have great importance in every aspect of our lives, especially as dietary supplements, essential chiral building elements of peptides, or pharmaceuticals, thus amino acid-related biotransformation has increasing significance [1,2]. In recent years, the application of recombinant enzymes has gained interest mainly in the fields of food and [3,4] the pharmaceutical industry [5–7], but also the production of renewable fuels and chemicals [8]. Simultaneously, heterologous expression systems and protein engineering strategies have made possible the high-level production of recombinant enzymes with improved performance and catalytic activity [9]. An industrial scale enzyme is commonly synthesized by a variety of host cells, often by the Escherichia coli (E. coli) bacterium, then isolated from the highly complex cellular environment [9,10]. These so-called downstream...
processes are expensive and have technological challenges in many aspects, including the formation of inclusion bodies, the structural sensitivity of proteins, and long-term storage [11–13].

As a member of the industrially relevant ammonia-lyase family, phenylalanine ammonia-lyase (PAL) is a well-studied recombinant enzyme expressed by *E. coli* and applied to perform the nonoxidative deamination of L-phenylalanine to form cinnamic acid and ammonia (Figure 1) [14].

![Figure 1. Ammonia elimination of L-phenylalanine and reverse ammonia addition to (E)-cinnamic acid catalyzed by PAL.](image)

In nature, PAL plays an essential role in primary and secondary metabolic processes in plants, as the formed cinnamic acid is a precursor of phenylpropanoids (flavonoids and lignin) [15]. The natural catalytic reaction of PAL—the ammonia elimination—is reversible, thus, enantiopure amino acids can be obtained by adding ammonia to the double bond of the corresponding amino acid derivative [16,17]. The recombinant, plant-derived homotrameric *Petroselinum crispum* phenylalanine ammonia-lyase (PcPAL) could be applied for the enantioselective synthesis of L-amino acids of pharmaceutical interest. Furthermore, the chemical syntheses based on classical organic chemistry principles often contain more steps and require harsher conditions as compared to the biocatalyzed processes [18].

The immobilization of the enzyme using suitable carrier systems contributes to enhancing the performance of the biocatalysts. The separation of the reaction medium and the biocatalyst can be enabled throughout the process and the multiple reuses of the immobilized biocatalysts could increase the overall effectiveness of the biocatalytic process [19–22]. The proper immobilization can improve the stability of enzymes towards harsh reaction conditions such as extreme pH, high temperature, and the presence of organic solvents. The physicochemical properties of carriers, such as nanostructured systems, can be advantageously adapted to the specific target protein [23]. The use of nanomaterials, such as nanoparticles, nanotubes, and nanofibers can maximize the surface area and increase the activity of immobilized enzymes [24–26]. Among nanostructured materials, nanofibers (or nanofibrous matrices) have become increasingly popular: the fibrous material with suitable surface functionalization can immobilize proteins onto the surface by covalent bonds or physical interactions. Enzymes can also be entrapped in situ within the forming nanofibers providing a unique way of rapid immobilization and stabilization. Nanofibrous matrices are usually fabricated by solvent-based electrospinning [27]. Electrospinning is a fiber production technique that is based on the electric force to draw charged threads of polymer-based precursor solutions, which leads to the formation of uniform fibers with micron and submicron scale diameters [28]. After the jet leaves the solution droplet, fiber formation starts with a whipping motion towards the grounded collector, simultaneously losing their solvent content rapidly, resulting in a solid nanofibrous membrane [29,30]. If a small or a large molecule (they are usually active pharmaceutical ingredients, APIs, such as small molecular drugs, proteins, and antibodies) is soluble in the precursor solution, it can be directly entrapped in the polymeric phase during fiber formation. For this reason, the electrospinning technique has recently gained more importance as the immobilization method for classic APIs or biological molecules, such as enzymes [31–34].

A suitable carrier system is of utmost importance for the industrial use of immobilization technology. Polylactic acid (PLA) is a commercially available, biologically inert, inexpensive, and biodegradable polymer, which is proved to be a promising material for the enzyme carrier system [35]. The insolubility of PLA in water, its chemical resis-
tance, and good stability as nanofibers against many solvents (e.g., buffered aqueous system, alcohols, aromatic and aliphatic organic solvents) can open new possibilities for synthetic biocatalysis. Nanoﬁbrous matrices from PLA could be prepared by solvent-based electrospinning technique commonly using halogenated organic solvents, such as dichloromethane and chloroform [36]. These solvents are toxic and incompatible with most biological macromolecules. The disadvantages can be overcome by aqueous-organic two-phase solvent systems. In such systems, the aqueous buffer containing the enzyme is immiscible with the organic phase, thereby compromising the fiber formation and the structural integrity of sensitive enzymes [37,38]. Emulsifiers strongly influence the type and structure of the dispersion formed from immiscible phases, so surfactants are widely used for such purposes due to their effectiveness in reducing surface tension, their ease of handling, and their compatibility with biomolecules. Emulsifiers can be characterized by their hydrophilic–lipophilic balance value (HLB), which describes the balance of size and strength of hydrophilic and lipophilic moieties of a molecule. According to the Grifﬁn’s method, the HLB value-based classiﬁcation indicates the function as follows: HLB < 10, oil soluble; HLB > 10, water soluble; 1 < HLB < 3, anti-foaming agents; 3 < HLB < 6, water in oil emulsifiers; 7 < HLB < 9, wetting and spreading agents; 13 < HLB < 16, detergents; 8 < HLB < 16, oil in water emulsifiers, 16 < HLB < 18, solubilizers [39]. However, the effect of emulsifiers on nanoﬁber formation by emulsion electrospinning is complex since the effectiveness of emulsification highly depends on surface tension, conductivity, and viscosity.

This study focuses on the entrapment of a recombinant PAL from Petroselinum crispum (PcPAL) in a nanofibrous matrix prepared from PLA by the emulsion electrospinning technique. To our best knowledge, electrospinning has not been reported as an immobilization technology for any PAL, yet. PLA is soluble in a solvent mixture of dichloromethane and N,N-dimethylformamide is immiscible with the aqueous enzyme solution causing limitation in enzyme-loading capacity the fiber formation. Expectedly, stabilization of the emulsion with emulsifiers can improve the electrospinning process since a “water in oil” emulsion can simultaneously provide an aqueous micro-environment for the enzyme while keeping the PLA dissolved in the organic phase. In addition, emulsifiers can also inﬂuence viscosity, conductivity and surface tension, which affect fiber morphology and continuous fiber formation as well. In this study, the effect of non-ionic emulsifiers with different HLB values is systematically investigated on the immobilization of a recombinant PcPAL. Characterization of the emulsions by rheological measurements, optical microscopy, and investigation of the nanoﬁbrous morphology of the biocatalyst by electron microscopy are reported. The biocatalytic performance of the immobilized PcPAL is characterized by the ammonia elimination reaction from 1-phenylalanine (Figure 2).

Figure 2. Entrapment of PcPAL in PLA nanofibers by emulsion electrospinning.
2. Results
2.1. Entrapment of PcPAL in PLA Nanofibers

First, the immobilization of PcPAL by entrapment in simple PLA nanofibers was investigated. The immobilization capacity is a critical issue for all immobilization systems, especially for entrapment within in situ forming supports such as electrospun nanofibers. Since the presence of proteins can directly influence the physico–chemical properties of precursor mixtures, the maximal enzyme loading within the forming immobilized biocatalyst is limited due to unfavorable aggregation and precipitation, even in ideally buffered systems. To find the optimal amount of PcPAL, different quantities from the aqueous solution of enzyme (representing five different enzyme loadings: 0.05, 0.10, 0.20, and 0.25% w/w%, PcPAL to PLA dry matter content) mixed to the PLA solution (PLA 8 w/w% in DCM:DMF 6:1 v/v) were applied in electrospinning experiments.

To compare the precursor systems with various PcPAL contents, the viscosity of precursors was determined by rotational rheometer, and the morphology of the final fibrous materials was characterized by scanning electron microscope (SEM). In addition, a numeric value was introduced, the so-called “spinability score” ($S$, defined on a scale from 0–3), to evaluate the fiber formation process. In the determination of $S$, the visual observation of the electrospinning process can provide important information, where the dosage of precursor, the stability of the Taylor cone (or jet), the continuity of the fiber production, and yield of fiber formation were qualified. The spinability score, $S$ was given according to the following consideration: 0 (no fiber formation), 1 (very limited fiber formation, unstable jet), 2 (non-continuous fiber formation with weak to moderate yield), 3 (continuous fiber formation with stable jet and high yield).

The highest enzyme loading (0.25%) during the electrospinning experiments resulted in insufficient fiber formation (spinability score: 0–1), thus, only a limited amount of fibrous material was produced (Table 1). This result is correlated with the result of the rheological investigations. The viscosity of the precursor mixture shows a continuous decrease with an increasing ratio of the aqueous to the organic phase. At the highest enzyme loading applied, viscosity decreases below a critical level, causing low-quality fiber formation and improper behavior in the electrospinning process. The analysis of the average fiber diameters ($d_f$) of the produced electrospun samples (calculation of $d_f$ is based on SEM images) showed that larger enzyme concentration of the PLA-based solvent mixture resulted in smaller fiber diameter (Table 1).

Table 1. The effect of the amount of enzyme solution (enzyme loading) on the viscosity of precursor mixture, fiber formation, and diameter of nanofibers.

| Enzyme Loading (w/w%) 1 | Viscosity (mPas) | S 2 (-) | Fiber Diameter (nm) |
|-------------------------|-----------------|---------|---------------------|
| -                       | 623 ± 53        | 3       | 780 ± 78            |
| 0.05                    | 683 ± 71        | 3       | 667 ± 87            |
| 0.10                    | 688 ± 17        | 2       | 672 ± 205           |
| 0.15                    | 561 ± 92        | 2       | 457 ± 84            |
| 0.20                    | 592 ± 33        | 2       | 451 ± 109           |
| 0.25                    | 470 ± 56        | 0–1     | -                   |

1 Amount of PcPAL solution in PLA precursor (protein concentration: 1.79 mg mL$^{-1}$) mixture (8 w/w% of PLA in DCM:DMF 6:1), which represent the enzyme loading in nanofibers, 2 Spinability score is given according to the following consideration: 0 (no fiber formation), 1 (very limited fiber formation, unstable jet), 2 (non-continuous fiber formation with weak to moderate yield), 3 (continuous fiber formation with stable jet and high yield).

Typical morphologies of PLA nanofibers with different enzyme loadings are illustrated by representative SEM images (Figure 3). The products with enzyme loading between 0.05 to 0.20% (Figure 3b–e) were similar to the pure PLA matrix (Figure 3a), having a relatively uniform fibrous structure. In accordance with the viscosity values, only the preparation with the highest enzyme loading (highest water concentration in the mixture) resulted in unsuccessful fiber formation (Figure 3f).
The lyase activity of PLA-based nanofibrous PcPAL (PcPAL-PLA) biocatalysts was also investigated. The specific biocatalytic activity \( [U_B; \text{shows the amount of product (\text{umol}) produced per one minute}] \) and the specific enzyme activity \( [U_E; \text{shows the amount of product (\text{umol}) produced per one minute by 1 g of immobilized enzyme}] \) were determined in the enzyme-mediated ammonia elimination from L-phenylalanine. Regarding either \( U_B \) (Figure 4a) or \( U_E \) (Figure 4b), the PcPAL-PLA biocatalyst with 0.15 w/w% enzyme loading provided the largest activity. These results indicate that the 0.15% PcPAL content is the optimal enzyme loading for PcPAL-PLA nanofibers. Above 0.15% enzyme loading, however, more enzyme results the smaller activity, which may caused by an unfavorable enzyme aggregation. In an ideal case, the values of specific enzyme activity should be the same at the different enzyme loadings according to its definition (see equations in Section 4.3). However, \( U_E \) shows a maximum at 0.15% enzyme loading. The weaker values of \( U_E \) at the smaller or larger enzyme loadings than 0.15% can be caused by complex effects, such as the shape and distribution of emulsified droplets. In addition, the concentration conditions of the enzyme molecules inside the water droplets can also influence the \( U_E \) of the biocatalysts.

**Figure 3.** Effect of PcPAL loading (w/w%, enzyme:dry mass of PLA) on the morphology of PLA nanofibers (a) without, and (b) with 0.05%, (c) 0.10%, (d) 0.15%, (e) 0.2% and (f) 0.25% of PcPAL.

**Figure 4.** The effect of enzyme loading on the (a) specific biocatalyst activity \( (U_B) \) and (b) specific enzyme activity \( (U_E) \) of PcPAL entrapped in PLA nanofibers in ammonia elimination from L-phenylalanine. Every measurement was made in triplicate. The difference between all samples was significant \( (p < 0.005) \) in the case of \( U_B \) and in also in the case of \( U_E \) except between enzyme loading 0.10 and 0.20.
2.2. Effect of Additives on the Enzymatic Activity of Native PcPAL

The optimization of the composition of the precursor mixture during the electrospinning process is a key issue and it is especially important if sensitive molecules, such as enzymes, are immobilized in the nanofibers. While the DCM and DMF-based PLA solution are immiscible with the aqueous solution of PcPAL, the behavior and stability of the emulsion could strongly influence spinability conditions, thereby affecting directly and indirectly the enzymatic activity. The nature and stability of the forming emulsion can be efficiently fine-tuned by the use of emulsifiers, which are typically non-ionic surfactants.

The hydrophilic/lipophilic balance (HLB) value can be used to characterize the emulsifiers. However, the applied emulsifiers can interact with the enzymes as well—commonly by secondary interactions to certain structural regions—thereby influence their properties. For example, polyethylene glycols and their analogues are commonly used as a stabilizing agent during protein crystallization and as cryoprotective agents [40]. Moreover, the emulsifiers can also change the viscosity of the media, which affects fiber formation. In this study, emulsifiers with a wide range of HLB values (Tween 80 (HLB = 15.0), Tween 85 (HLB = 11.0), Brij 30 (HLB = 9.7), Span 60 (HLB = 8.6), Span 40 (HLB = 6.7), Span 20 (HLB = 4.7)) were investigated. First, their effects on the native, solubilized PcPAL were investigated in the aqueous phase by assaying the PcPAL-mediated ammonia elimination from L-phenylalanine. In each case, 2-propanol (IPA) was added to reaction mixtures as a commonly used co-solvent to improve the solubility of the emulsifiers. The specific enzyme activity (U_E) values indicate that IPA decreases the activity of PcPAL and the presence of Tween 80, Tween 85, or Brij 30 (HLB ≥ 9.7) led to further loss of the specific enzyme activity. However, emulsifiers with lower HLB values (HLB ≤ 8.6) could compensate this negative effect of IPA. In the case of Span 40 and Span 60, U_E reached the initial value achieved with solvent- and additive-free PcPAL (Figure 5). Based on these experiments, there are some differences in the effect of investigated additives, all of them were applied for further enzyme immobilization studies.

Figure 5. The effect of the emulsifiers (10 mM) with different HLB values on the specific enzyme activity (U_E) of native PcPAL in ammonia elimination from L-phenylalanine. Every measurement was made in triplicate, the difference between native enzyme (colored with blue) and samples with IPA (colored with green) and emulsifier (colored with purple) is significant (p < 0.005) in all cases except samples with IPA (20 v/v%) + Span 40 and IPA (20 v/v%) + Span 60.

2.3. Effect of the Emulsifiers with Different HLB Values on the Entrapment of PcPAL in PLA Nanofibers

The entrapment of PcPAL in the presence of emulsifiers with different HLB values was performed by electrospinning similarly to the additive-free cases. In Section 2.1, we showed that low enzyme loadings result in decreased specific enzyme activity and overloading causes difficulties in fiber fabrication, therefore, further immobilization experiments were carried out with the three different enzyme loadings in the mid-loading range (0.1, 0.15,
and 0.2 w/w%). The comprehensive analysis of these precursor mixtures included rheological measurements, the determination of spinability, the optical examination of the formed emulsions, and the morphological analysis of the resulted fibrous PcPAL biocatalysts (Table 2). It should be noted that the investigated emulsifiers were applied in the concentration of 0.1 M, which is much larger than the critical micelle concentration (CMC) of each additive (CMC values are in the range 0.1–10 µM) [41,42].

Table 2. The effect of the emulsifiers with different HLB values on the characteristic of precursor mixtures and the nanofibers.

| Additive | HLB | PcPAL Loading (w/w%) ¹ | Viscosity (mPas) | Droplet Diameter (µm) | S ² |
|----------|-----|-------------------------|-----------------|-----------------------|-----|
| Tween 80 | 15  | 0.10                    | 278 ± 15        | 148 ± 73              | 3   |
|          |     | 0.15                    | 218 ± 25        | 143 ± 36              | 3   |
|          |     | 0.20                    | 234 ± 27        | 169 ± 37              | 3   |
| Tween 85 | 11  | 0.10                    | 225 ± 28        | 74 ± 16               | 2   |
|          |     | 0.15                    | 228 ± 21        | 86 ± 31               | 3   |
|          |     | 0.20                    | 233 ± 34        | 109 ± 30              | 3   |
| Brij 30  | 9.7 | 0.10                    | 357 ± 86        | 91 ± 43               | 2   |
|          |     | 0.15                    | 372 ± 109       | 109 ± 53              | 2   |
|          |     | 0.20                    | 385 ± 40        | 94 ± 46               | 2   |
| Span 20  | 8.6 | 0.10                    | 421 ± 94        | u.d. ³                | 3   |
|          |     | 0.15                    | 354 ± 69        | u.d.                  | 2   |
|          |     | 0.20                    | 360 ± 38        | u.d.                  | 2   |
| Span 40  | 6.7 | 0.10                    | 439 ± 21        | 36 ± 16               | 3   |
|          |     | 0.15                    | 452 ± 28        | 44 ± 15               | 2   |
|          |     | 0.20                    | 432 ± 84        | 39 ± 32               | 1   |
| Span 60  | 4.7 | 0.10                    | 281 ± 52        | 44 ± 19               | 2   |
|          |     | 0.15                    | 306 ± 53        | 47 ± 25               | 1   |
|          |     | 0.20                    | 362 ± 42        | 20 ± 10               | 2   |

¹ Amount of PcPAL solution (protein concentration: 1.79 mg mL⁻¹) in the PLA precursor mixture (8 w/w% of PLA in DCM:DMF 6:1), which represent the enzyme loading in nanofibers. ² S: Spinability score is given according to the following consideration: 0 (no fiber formation), 1 (very limited fiber formation, unstable jet), 2 (non-continuous fiber formation with weak yield), 3 (continuous fiber formation with stable jet and high yield), ³ below the detection limit, diameter smaller than 1 µm.

The different emulsifiers reduce the viscosity to similar values (typically to the range of 200–450 mPas), the viscosities of the emulsifier-free PcPAL-PLA mixtures are in the range of 600–700 mPas, see in Table 1). Remarkably, the viscosity of additive-containing mixtures being lower than that of the non-spinable additive-free mixture with 0.25% enzyme loading could be used for fiber fabrication, probably due to overbalancing effects of other factors. The typical shape and diameter of emulsion droplets are key parameters influencing not just the precursor stability but also the electrospinning conditions and the final structure of nanofibrous products. Thus, droplets forming from the aqueous enzyme solution dispersed in the organic phase (PLA in a mixture of DCM and DMF) were investigated by digital optical microscope (DOM). DOM images revealed a decreasing diameter of aqueous droplets with decreasing HLB value of the emulsifiers. Similarly, the spinability score was also decreased, which can be caused by the stabilizing effect of the additives with smaller HLB values.

DOM images indicate the effect of emulsifiers on the emulsion droplets: while the emulsifier-free precursor mixtures led to polymorphic droplets (Figure 6a; the average diameters for 0.1, 0.15, and 0.2 w/w% enzyme loading were 103 ± 25, 167 ± 51, and 125 ± 49 µm, respectively), in the presence of the emulsifiers more uniform, and mainly spherical droplets were formed (Figure 6b–d). The larger degree of uniformity is beneficial for the reproducibility of precursor preparation, for the stability and handling of
the forming emulsion, and for the reproducibility and quality of nanofiber production (Figure 6b–d).

![DOM images of PcPAL-PAL precursor mixtures](image)

**Figure 6.** Representative DOM images of PcPAL-PAL precursor mixtures (a) without emulsifier, and PcPAL-PAL precursor mixtures with various emulsifiers (0.1 M): (b) Tween 80, (c) Brij 20, and (d) Span 40.

SEM images (Figure 7) show that all electrospun matrices have fibrous structure, and the fibers containing Tween 80 (HLB = 15.0) or Tween 85 (HLB = 11.0) (Figure 7a,b, respectively) are thicker than the emulsifier-free fibers (Table 1 and Figure 7c–e) and the fibers with the other emulsifiers used (Figure 7b–f).

![SEM images of PLA nanofibers](image)

**Figure 7.** The effect of (a) Tween 80, (b) Tween 85, (c) Brij 30, (d) Span 20, (e) Span 40, and (f) Span 60 on the morphology of PLA nanofibers containing 0.1 w/w% PcPAL.

Regarding the average diameter of nanofibers, it can be concluded, that the emulsifiers with HLB values smaller than 9.7 (Brij 30, HLB = 9.7; Span 20, HLB = 8.6; Span 40, HLB = 6.7; Span 60, HLB = 4.7) resulted in thinner fibers and the size distribution of average fiber diameter are also became smaller (Figure 8). These results can depend on the viscosity and droplet diameter in organic/aqueous emulsion. In the presence of Tween 80 or Tween 85 the viscosity of the precursor is remarkably smaller, however the droplet of emulsions are bigger than in the case of other investigated emulsifiers (see in Table 2). It can be examined with stronger cohesion of PLA chains resulting thinner and more uniform fibers during the electrospinning process.

The activity of the PcPAL enzyme entrapped in PLA nanofibrous membranes was determined, as described before, by assaying the ammonia elimination from L-phenylalanine. The effect of the emulsifiers on specific enzyme activity ($U_E$) was compared to the emulsifier-free PcPAL-PLA biocatalysts with three different enzyme loadings (0.1, 0.15, and 0.2 w/w%, Figure 9a–c, respectively). Most of the emulsifiers increased the specific enzyme activity of PcPAL. The most significant improvement can be observed in the presence of Tween 80, Tween 85, and Brij 30 at all PcPAL loadings. At the smallest examined enzyme loading...
(0.1 w/w%, Figure 9a) Tween 85, while at 0.15 w/w% and 0.2 w/w% PcPAL content, Brij 30 gave the largest improvement (Figure 9b,c). Consequently, it seems that HLB values of the surfactants have an optimum regarding the activity of entrapped PcPAL. The best specific enzyme activity of PcPAL can be observed at 0.15%. Notably, the specific enzyme activity of PcPAL entrapped in PLA nanofibers containing Brij 30 at 0.15% enzyme loading ($U_E = 117 \pm 5 \text{ Ug}^{-1}$) is larger than that of the native, non-immobilized PcPAL ($U_E = 96 \pm 5 \text{ Ug}^{-1}$; Figure 5).

**Figure 8.** The effect of the surfactants (Tween 80, Tween 85, Brij 30, Span 20, Span 40, and Span 60) with different HLB values on the diameter of PcPAL loaded PLA nanofibers at different PcPAL loadings: (a) 0.10, (b) 0.15, and (c) 0.20 w/w%. Size distribution of fibers was calculated from $n = 50$ measurements, the difference between emulsifier-free (colored with blue bars on Panel (a-c) and samples with emulsifier is significant ($p < 0.005$) in all cases, except samples with Span 20 at PcPAL loading 0.10 w/w% and Span 40 at PcPAL loading 0.20 w/w%.

**Figure 9.** The effect of the surfactants (Tween 80, Tween 85, Brij 30, Span 20, Span 40, and Span 60) with different HLB values on the specific enzyme activity ($U_E$) of PcPAL entrapped in PLA nanofibers (colored with green bars), and PcPAL entrapped in surfactant-free PLA nanofibers (colored with blue bars) at (a) 0.1 w/w%, (b) 0.15 w/w%, and (c) 0.2 w/w% PcPAL contents in ammonia elimination from L-phenylalanine. Every measurement was made in triplicate, the difference between PcPAL entrapped in PLA nanofibers at different enzyme loading without emulsifiers (colored with blue bar) and samples with emulsifier is significant ($p < 0.005$) in all cases, except samples Span 60 at 0.1 and 0.15 w/w% enzyme loading and Span 40 and Span 60 at 0.2 w/w% enzyme loading.
3. Discussion

In this study, the recombinant enzyme, PcPAL was successfully entrapped in PLA nanofibers. The immiscible phases of PLA dissolved in organic solvents (DCM and DMF) and the buffered aqueous solution of PcPAL form an unstable emulsion. Emulsifiers (Tween 80, Tween 85, Brij 30, Span 20, Span 40, Span 60) with different HLB values were selected to stabilize the emulsion, and their effect on precursor properties, the electrospinning process, activity of native and immobilized PcPAL in the PLA nanofibers was investigated. The results showed the emulsifiers have a significant role in fiber formation and they also influence the enzymatic function. In general, all of the additives were successfully applied for fiber formation and enzyme immobilization. The viscosity of the precursor, the diameter of droplets in the emulsions, the spinability of the precursor mixtures, and the average diameter of fibers also decrease as a function of the decreasing HLB values. As an important observation, the effect of HLB values on the specific enzyme activity needs to be highlighted. Regarding the three different investigated enzyme loadings (0.1, 0.15 and 0.2 w/w%), Tween 85 (HLB = 11.0), Brij 30 (HLB = 9.7), and Span 20 (HLB = 8.6) showed a positive effect on the specific enzyme activity of PcPAL. The application of Brij 30 emulsifier caused a more than six-fold increase in specific enzyme activity at the two larger enzyme loadings.

4. Materials and Methods

4.1. Materials

Polyactic acid (PLA, $M_w = 68,000 \text{ g mol}^{-1}$, $d_p$: 3–5 mm) is the product of GoodFellow (Cambridge, UK). 2-propanol (IPA), $N,N$-dimethylformamide (DMF), dichloromethane (DCM), polyoxyethylene sorbitan monooleate (Tween 80), polyoxyethylene sorbitan trioleate (Tween 85), polyethylene glycol monododecyl ether (Brij 30), sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), and sorbitan monostearate (Span 60) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Hydrogen chloride (cc. aq. solution) and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Merck (Darmstadt, Germany). L-Phenylalanine (L-Phe) and cinnamic acid are the products of Honeywell Fluka (Seelze, Germany). Maxcolor® Blue food grade coloring solution (E131 in water/ethanol solution) was obtained from MaxAroma Llc (Kerepes, Hungary). Ultrapure water ($p > 18.2 \text{ M} \Omega \text{ cm}$, Millipore, Bradford, MA, USA) was used for the preparation of aqueous solutions. Syntheses and measurements were carried out at 22 ± 2 °C unless otherwise noted.

4.2. Production and Purification of Recombinant PcPAL

Phenylalanine ammonia-lyase from parsley (Petroselinum crispum, PcPAL) carrying an $N$-terminal His10-tag was overexpressed in E. coli Rosetta™ host. The enzyme was purified with immobilized metal affinity chromatography (IMAC) and dialyzed according to the method described in our previous work [16]. The purity of the enzyme was examined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, purity >95%) and enzyme concentration was determined using Nanodrop2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

4.3. Determination of Biocatalytic Activity of Native PcPAL

For the investigation of the biocatalytic activity of native enzymes, TRIS-base buffer (85 µL, 50 mM, pH 8.8) and freshly dialyzed enzyme solution of PcPAL in TRIS-base buffer (15 µL, 50 mM, pH 8.8, the concentration of PcPAL: 1.79 mg mL$^{-1}$) was mixed in a 96-well plate (UV-Star® 96 W microplate, Greiner Bio One Gmbh, Kremsmünster, Austria) and incubated for 5 min at 37.0 °C in an orbital shaker at 450 rpm (Titramax 1000, Heidolph, Schawbach, Germany).

For determining the specific enzyme activity of native PcPAL in the presence of emulsifiers, the corresponding emulsifier solution (10 mM; Tris bufferIPA 20 v/v%) was prepared and sonicated (45 kHz) for 15 min at 25 °C (in Sonorex Digitec DT 255 ultrasonic...
bath, Bandelin Electronic Gmbh, Berlin, Germany). Then this emulsifier containing solution (85 µL, each) and freshly dialyzed enzyme solution of PcPAL in TRIS-base buffer (15 µL, 50 mM, pH 8.8, dialyzed PcPAL solution concentration: 1.79 mg mL⁻¹) was mixed in the 96-well plate and incubated for 5 min at 37.0 °C at 450 rpm applying the orbital shaker. L-phenylalanine in TRIS-base buffer (100 µL, 10 mM, 50 mM, pH 8.8) was added to each PcPAL containing emulsifier solution in the 96-well plate. The specific enzyme activity of each sample was calculated by the determination of cinnamic acid formation at λ = 290 nm for 5 min at 37.0 °C by the “kinetics method” using a Multiskan Sky 96-well plate reader machine by Thermo Fisher Scientific (Waltham, MA, USA).

To characterize the productivity of the native PcPAL, the enzyme activity was calculated using the equations for specific enzyme activity

\[ U_E = \frac{n_P}{m_E t} \]

where \( m_E [g] \) is the mass of the PcPAL, \( n_P [\mu \text{mol}] \) is the amount of the product, \( t [\text{min}] \) is the time, and for specific biocatalytic activity

\[ U_B = \frac{n_P}{m_B t} \]

where \( m_B [g] \) is the total mass of the applied biocatalyst. The amount of the product, cinnamic acid, was calculated based on calibration at its characteristic absorption wavelength of λ = 290 nm, extinction coefficient, \( \varepsilon = 9650 \, \text{M}^{-1} \text{cm}^{-1} \). All the measurements were performed in triplicate. Statistical significances were calculated by a two-sample T-test, where the difference was significant if \( p < 0.05 \).

4.4. Entrapment of PcPAL in PLA Nanofibers

First, the precursor mixtures were prepared; different quantities from freshly dialyzed enzyme solution of PcPAL in TRIS-base buffer (PcPAL 1.79 mg mL⁻¹, 50 mM, pH 8.8) according to the enzyme loading (0.05, 0.1, 0.15, 0.2, and 0.25 w/w%, where the mass of the enzyme was calculated according to the dry mass of PLA in its solution). The mixture containing the PcPAL and the additive (0.1 M, Tween80, Tween85, Brij30, Span60, Span40, or Span20) was homogenized for 2 min by vortex (IKA Mixer Vortex Shaker MS 2, IKA Gmbh, Staufen im Breisgau, Germany). Then this mixture was added to the PLA solution (1.00 g, 8% w/w in DCM—DMF 6:1 v/v), and it was homogenized for 2 min by vortex. A SpinCube Electrospinning machine (SpinSplit Llc, Budapest, Hungary) was used for the preparation of PLA matrices. The precursor mixture was fed to the emitter from a syringe (3.0 mL) at an optimal flow rate (8–100 µL min⁻¹) by a syringe pump. The distance between the collector and the emitter (with 0.7 mm internal diameter) was 10–15 cm. Constant voltage ranging from 10.0 to 20.0 kV was applied to the emitter using a direct current power supplier. Electrospun fibers were collected on an aluminum foil. The electrospun samples were dried at room temperature for 1 h, then stored at 4 °C before use.

4.5. Rheological Investigation of the PcPAL-PLA-Based Precursor System

The rheological properties of precursor mixtures were measured at 25 °C by an Anton Paar Physica MCR 301 rheometer (Austria) equipped with a cone geometry (diameter of 25 mm / 1° cone plate). Every measurement was performed in triplicate.

4.6. Investigation of Precursor Emulsions by Digital Optical Microscopy (DOM)

The structure of the emulsions was investigated by a Keyence VHX 5000 digital optical microscope (DOM). Samples were placed onto a microscope slide (IDL Gmbh., 18 mm × 18 mm borosilicate glass) and observed in transmission mode with partial top and full bottom illumination. For better imaging, Maxcolor® blue food coloring solution (20 µL) was added to the emulsions (0.5 g). The size distribution of emulsion droplets was determined by ImageJ-1.53a (2020) Image Analyzer.

4.7. Morphological Characterization of Nanofibrous Membranes by SEM

The morphology of different nanofibrous membranes was analyzed by a JEOL JSM-5500 LV scanning electron microscope (SEM). Samples were placed on a copper grid; then they were coated with a gold nano-film layer for better imaging by a nebulizer (using Ar-plasma, at 10 mA for 180 s). The size distribution of fiber diameter was determined
4.8. Determination of Biocatalytic Activity of Entrapped PcPAL in PLA Nanofibrous Matrices

To determine the enzymatic activity of the immobilized PcPAL biocatalysts, 5.0 mg of the nanofibrous membranes was measured into a 4.0 mL glass vial, then L-phenylalanine in TRIS-base buffer (2.0 mL, 10 mM, 50 mM, pH 8.8) was added to it. The mixture was incubated at 37 °C and shaken in a flat shaker at 600 rpm for 24 h. After incubation, the amount of cinnamic acid was determined and the specific enzyme activity (UE) was calculated as described in Section 4.3. Statistical significances were calculated by a two-sample T-test, where the difference was significant if $p < 0.05$.

5. Conclusions

Electrospinning provides a unique opportunity for the immobilization of sensitive recombinant enzymes, such as PcPAL. The PLA dissolved in an organic medium (DCM:DMF 6:1, v/v) and the aqueous solution of PcPAL are immiscible phases, which can be applied for emulsion electrospinning. In this study, the enzyme loading has been found optimal in the range of 0.10–0.20% (w/w%, enzyme:PLA mass) in the PLA nanofibers. Non-ionic emulsifiers were selected in a wide range of HLB values (4.7–15.0) to systematically compare the entrapment of PcPAL. The results showed that the applied emulsifiers can influence fiber formation, fiber diameter, and the specific biocatalytic activity of PcPAL. The use of Tween 85 and Brij 30 caused a remarkable increase in the biocatalytic activity compared to emulsifier-free samples.

By applying a well-selected polymer, an optimized emulsified precursor system, a suitable media can be ensured for the enzymes, thus, their biocatalytic activity can be conserved or enhanced. The physicochemical properties of the precursor mixture determined by the type of polymer and its solvent, co-solvents, and additives, determine the most significantly the final conditions of electrospinning and the character of the produced fibrous materials. Surfactants as additives have a complex effect on the fiber formation process and the immobilized enzyme as well. HLB values can be a good prognostic factor in the optimization procedure. The nanofibrous membrane provides an easy-to-handle carrier material, which can be integrated into batch or continuous-flow reactors.

Author Contributions: Conceptualization, G.K. and D.B.-W.; methodology, G.K., G.D.T. and F.E.; analytical support, G.K., G.D.T., E.S.-B., A.S. and M.J.; enzyme production, Z.M.; writing—original draft preparation, G.K.; writing—review and editing, B.G.V., B.P., L.P. and D.B.-W.; visualization, D.B.-W.; supervision, L.P. and D.B.-W.; funding acquisition, A.S., L.P. and D.B.-W. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been supported by the National Research Development and Innovation (NRDI) Fund (TKP2020 IES, Grant No. BME-IE-BIO) based on the charter of bolster issued by the NRDI Office under the auspices of the Ministry for Innovation and Technology, Hungary. Further support was provided by the NRDI Office via grant PD-131467 (D.B.W.), SNN-125637 (L.P.), K135231, and NKP-2018-1.2.1-NKP-2018-00005 (L.P., B.G.V.). B.W.D. acknowledges the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00175/21) and the ÚNKP-21-5 (ÚNKP-21-5-BME-386) New National Excellence Program of the Ministry of Human Capacities.

Acknowledgments: Gergő Dániel Tóth thanks the support of Servier-Beregi fellowship. The authors thank Melitta Gedai for the preliminary investigation of nanofiber fabrication and thank Anna Vincze and György T. Balogh for UV-Vis plate reader measurements.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. de Lange, B.; Hyett, D.J.; Maas, P.J.D.; Mink, D.; van Assema, F.B.J.; Sereinig, N.; de Vries, A.H.M.; de Vries, J.G. Asymmetric synthesis of (S)-2-indolinecarboxylic acid by combining biocatalysis and homogeneous catalysis. *ChemCatChem* **2011**, *3*, 289–292. [CrossRef]
2. Ferrazzano, L.; Corbisiero, D.; Martelli, G.; Tolomelli, A.; Viola, A.; Ricci, A.; Cabri, W. Green solvent mixtures for solid-phase peptide synthesis: A dimethylformamide-free highly efficient synthesis of pharmaceutical-grade peptides. ACS Sustain. Chem. Eng. 2019, 7, 12867–12877. [CrossRef]

3. Peng, Q.; Wang, X.; Shang, M.; Huang, J.; Guan, G.; Li, Y.; Shi, B. Isolation of a novel alkaline-stable lipase from a metagenomic library and its specific application for milkfat flavor production. Microb. Cell Fact. 2014, 13, 1. [CrossRef]

4. He, L.; Mao, Y.; Zhang, L.; Wang, H.; Alias, S.A.; Gao, B.; Wei, D. Functional expression of a novel α-amylase from Antarctic psychrotolerant fungus for baking industry and its magnetic immobilization. BMC Biotechnol. 2017, 17, 22. [CrossRef] [PubMed]

5. Hydery, T.; Coppenrath, V.A. A comprehensive review of pegvaliase, an enzyme substitution therapy for the treatment of phenylketonuria. Drug Target Insights 2019, 13, 1–8. [CrossRef]

6. Li, X.-J.; Zheng, R.-C.; Ma, H.-Y.; Zheng, Y.-G. Engineering of Thermomyces lanuginosus lipase lip: Creation of novel biocatalyst for efficient biosynthesis of chiral intermediate of pregabalin. Appl. Microbiol. Biotechnol. 2014, 98, 2473–2483. [CrossRef]

7. Siódiáik, T.; Mangelings, D.; Vander Heyden, Y.; Ziegler-Borowska, M.; Marszall, M.P. High enantioselective Novozym 435-catalyzed esterification of (R,S)-flurbiprofen monitored with a chiral stationary phase. Appl. Biochem. Biotechnol. 2015, 175, 2769–2785. [CrossRef] [PubMed]

8. Lambertz, C.; Ece, S.; Fischer, R.; Commandeur, U. Progress and obstacles in the production and application of recombinant lignin-degrading peroxidases. Bioengineered 2016, 7, 145–154. [CrossRef]

9. Trono, D. Recombinant enzymes in the food and pharmaceutical industries. In Biomass, Biofuels, Biochemicals: Advances in Enzyme Technology; Elsevier: Amsterdam, The Netherlands, 2019; pp. 349–387, ISBN 978044461144.

10. Kainer, F.W.; Pletzenauer, R.; Rossetti, L.; Herwig, C.; Glieder, A.; Spadiut, O. Purification and basic biochemical characterization of 19 recombinant plant peroxidase isoenzymes produced in Pichia pastoris. Protein Expr. Purif. 2014, 95, 104–112. [CrossRef] [PubMed]

11. Saraswat, M.; Musante, L.; Ravidá, A.; Shortt, B.; Byrne, B.; Holthofer, H. Preparative purification of recombinant proteins: Current status and future trends. BioMed Res. Int. 2013, 2013, 312709. [CrossRef]

12. Conner, J.; Wuchterl, D.; Lopez, M.; Minshall, B.; Prusti, R.; Bocclair, D.; Peterson, J.; Allen, C. The biomanufacturing of biotechnology products. In Biotechnology Entrepreneurship: Starting, Managing, and Leading Biotech Companies; Academic Press: Cambridge, MA, USA, 2014; pp. 351–385, ISBN 9780124047303.

13. Puetz, J.; Wurm, F.M. Recombinant proteins for industrial versus pharmaceutical purposes: A review of process and pricing. Processes 2019, 7, 476. [CrossRef]

14. Parmeggiani, F.; Weise, N.J.; Ahmed, S.T.; Turner, N.J. Synthetic and therapeutic applications of ammonia-lyases and aminomutases. Chem. Rev. 2017, 118, 73–118. [CrossRef] [PubMed]

15. Ritter, H.; Schulz, G.E. Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase. Plant Cell 2004, 16, 3426–3436. [CrossRef] [PubMed]

16. Poppe, L.; Païzis, C.; Kovács, K.; Irhimie, F.-D.; Vértesy, B. Preparation of unnatural amino acids with ammonia-lyases and 2,3-aminomutases. In Methods in Molecular Biology: Unnatural Amino Acids (Methods and Protocols); Humana Press: Totowa, NJ, USA, 2012; Volume 794, pp. 3–19.

17. Tork, S.D.; Nagy, E.Z.A.; Cserepes, L.; Bordea, D.M.; Nagy, B.; Toșa, M.I.; Païzis, C.; Bence, L.C. The production of L- and D-phenylalanines using engineered ammonia-lyases with Pseudomonas crista. Biochem. Eng. J. 2019, 139, 202–219. [CrossRef]

18. Patel, R.N. Biocatalysis for synthesis of pharmaceuticals. Bioorg. Med. Chem. 2018, 26, 1252–1274. [CrossRef]

19. Cai, C.; Gao, Y.; Liu, Y.; Zhong, N.; Liu, N. Immobilization of Candida antarctica lipase B onto SBA-15 and their application in glyceraldehyde for diacylglycerols synthesis. Food Chem. 2016, 212, 205–212. [CrossRef]

20. Krisch, E.; Balogh-Weiser, D.; Klimkó, J.; Gyarmati, B.; László, K.; Poppe, L.; Szilágyi, A. Composite beads of silica gel, alginate and poly(aspartic acid) for the immobilization of a lipase enzyme. Express Polym. Lett. 2019, 13, 512–523. [CrossRef]

21. Gihaz, S.; Weiser, D.; Dror, A.; Sátorhelyi, P.; Jerabek-Willemsen, M.; Poppe, L.; Fishman, A. Creating an efficient methanol-stable biocatalyst by protein and immobilization engineering steps towards efficient biosynthesis of biodiesel. ChemSusChem 2016, 9, 3161–3170. [CrossRef]

22. Weiser, D.; Nagy, F.; Bánočzi, G.; Oláh, M.; Farkas, A.; Szilágyi, A.; László, K.; Gellért, Á.; Marosi, G.; Kemény, S.; et al. Immobilization engineering—How to design advanced sol–gel systems for biocatalysis? Green Chem. 2017, 19, 3927–3937. [CrossRef]

23. Bilal, M.; Asgher, M.; Cheng, H.; Yan, Y.; Iqbal, H.M.N. Multi-point enzyme immobilization, surface chemistry, and novel platforms: A paradigm shift in biocatalyst design. Crit. Rev. Biotechnol. 2019, 39, 202–219. [CrossRef]

24. Gupta, M.N.; Kaloti, M.; Kapoor, M.; Solanki, K. Nanomaterials as matrices for enzyme immobilization. Artif. Cells Blood Substit. Biotechnol. 2011, 39, 98–109. [CrossRef]

25. Bai, C.; Liu, M. From chemistry to nanoscience: Not just a matter of size. Angew. Chem. Int. Ed. 2013, 52, 2678–2683. [CrossRef]

26. Sheldon, R.A. Enzyme immobilization: The quest for optimum performance. Adv. Synth. Catal. 2007, 349, 1289–1307. [CrossRef]

27. Islam, M.S.; Ang, B.C.; Andriyana, A.; Afifi, A.M. A review on fabrication of nanofibers via electrospinning and their applications. SN Appl. Sci. 2019, 1, 1248. [CrossRef]

28. Rutledge, G.C.; Fridrikh, S.V. Formation of fibers by electrospinning. Adv. Drug Deliv. Rev. 2007, 59, 1384–1391. [CrossRef]
29. Pillay, V.; Dott, C.; Choonara, Y.E.; Tyagi, C.; Tomar, L.; Kumar, P.; Du Toit, L.C.; Ndesendo, V.M.K. A review of the effect of processing variables on the fabrication of electrospun nanofibers for drug delivery applications. *J. Nanomater.* 2013, 789289. [CrossRef]

30. Haider, A.; Haider, S.; Kang, I.K. A comprehensive review summarizing the effect of electrospinning parameters and potential applications of nanofibers in biomedical and biotechnology. *Arab. J. Chem.* 2018, 11, 1165–1188. [CrossRef]

31. Tran, D.N.; Balkus, K.J. Enzyme immobilization via electrospinning. *Top. Catal.* 2012, 55, 1057–1069. [CrossRef]

32. Balogh-Weiser, D.; Németh, C.; Ender, F.; Gyarmati, B.; Szilágyi, A.; Poppe, L. Electrospun nanofibers for entrapment of biomolecules. In *Electrospinning Method Used to Create Functional Nanocomposites Films*; IntechOpen: London, UK, 2018; ISBN 978-1-78923-581-4.

33. Weiser, D.; Sóti, P.L.; Bánozzi, G.; Bódai, V.; Kiss, B.; Gellért, Á.; Nagy, Z.K.; Koczka, B.; Szilágyi, A.; Marosi, G.; et al. Bioimprinted lipases in PVA nanofibers as efficient immobilized biocatalysts. *Tetrahedron* 2016, 72, 7335–7342. [CrossRef]

34. Sóti, P.L.; Weiser, D.; Vigh, T.; Nagy, Z.K.; Poppe, L.; Marosi, G. Electrospun polylactic acid and polyvinyl alcohol fibers as efficient and stable nanomaterials for immobilization of lipases. *Bioprocess Biosyst. Eng.* 2016, 39, 449–459. [CrossRef] [PubMed]

35. Casalini, T.; Rossi, F.; Castrovinci, A.; Perale, G. A perspective on polylactic acid-based polymers use for nanoparticles synthesis and applications. *Front. Bioeng. Biotechnol.* 2019, 7, 259. [CrossRef]

36. Casasola, R.; Thomas, N.L.; Trybala, A.; Georgiadou, S. Electrospun poly lactic acid (PLA) fibres: Effect of different solvent systems on fibre morphology and diameter. *Polymer* 2014, 55, 4728–4737. [CrossRef]

37. Kumar, A.; Dhar, K.; Kanwar, S.S.; Arora, P.K. Lipase catalysis in organic solvents: Advantages and applications. *Biol. Proced. Online* 2016, 18, 2. [CrossRef]

38. Stepankova, V.; Bidmanova, S.; Koudelakova, T.; Prokop, Z.; Chaloupkova, R.; Damborsky, J. Strategies for stabilization of enzymes in organic solvents. *ACS Catal.* 2013, 3, 2823–2836. [CrossRef]

39. Griffin, W.C. Classification of surface-active agents by “HLB”. *J. Cosmet. Sci.* 1949, 1, 311–326.

40. Sadhukhan, N.; Muraoka, T.; Ui, M.; Nagatoishi, S.; Tsumoto, K.; Kinbara, K. Protein stabilization by an amphiphilic short monodisperse oligo(ethylene glycol). *Chem. Commun.* 2015, 51, 8457–8460. [CrossRef]

41. Hait, S.K.; Moulik, S.P. Determination of critical micelle concentration (CMC) of nonionic surfactants by donor-acceptor interaction with iodine and correlation of CMC with hydrophile-lipophile balance and other parameters of the surfactants. *J. Surfactants Deterg.* 2001, 4, 303–309. [CrossRef]

42. Peltonen, L.; Hirvonen, J.; Yliuruusi, J. The behavior of sorbitan surfactants at the water-oil interface: Straight-chained hydrocarbons from pentane to dodecane as an oil phase. *J. Colloid Interface Sci.* 2001, 240, 272–276. [CrossRef]