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Short Communication

A biocatalytic approach for resolution of 3-hydroxy-3-phenylpropanonitrile with the use of immobilized enzymes stabilized with ionic liquids

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ABSTRACT

Due to the growing importance of synthesizing active pharmaceutical ingredients (APIs) in enantiomerically pure form, new methods of asymmetric synthesis are being sought. Biocatalysis is a promising technique that can lead to enantiomerically pure products. In this study, lipase from *Pseudomonas fluorescens*, immobilized on modified silica nanoparticles, was used for the kinetic resolution (via transesterification) of a racemic mixture of 3-hydroxy-3-phenylpropanonitrile (3H3P), where the obtaining of a pure (S)-enantiomer of 3H3P is a crucial step in the fluoxetine synthesis pathway. For additional stabilization of the enzyme and enhanced process efficiency, ionic liquids (ILs) were used. It was found that the most suitable IL was [BMIM]Cl; a process efficiency of 97.4 % and an enantiomeric excess (ee%) of 79.5 % were obtained when 1 % (w/v) of that IL in hexane was applied and the process was catalyzed by lipase immobilized on amine-modified silica.

1. Introduction

Biocatalysis has become increasingly important as an alternative to conventional methods of organic synthesis, providing processes that are sustainable and in line with the principles of Green Chemistry [1]. Today, the rapidly growing pharmaceutical industry is becoming the main area in which enzymes are used. Enzymes are suitable candidates for catalyzing the synthesis of active pharmaceutical ingredients (APIs) and their key intermediates [2]. The most important features of enzymes are high stereoselectivity, regioselectivity and chemoselectivity. These are particularly important for the synthesis of chiral APIs, which represent most commercially available drugs (e.g., fluoxetine, naproxen) [3,4]. They have many advantages over classic chemical synthesis, due to the high enantioselectivity that is required in pharmaceutical industry. Moreover, using biocatalysts, also costs reduction is possible.

Despite the many benefits of enzyme catalysis, there are certain limitations relevant to the use of their free forms, as enzymes in native form have limited stability under harsh reaction conditions [5]. To improve the properties of free enzymes, immobilization – the binding of an enzyme to a solid carrier – is frequently used [6]. The use of immobilized enzymes facilitates the separation of the biocatalyst from the product, improves downstream processing and reduces enzyme losses, and improves the stability (a solid carrier often has a protective effect on the enzyme) and reusability of the immobilized enzymes, which is economically advantageous [7].

Among many groups of enzymes, lipases are the most widely applied in the synthesis of APIs. A common example is the enantioselective synthesis of (S)-propranolol, a drug used to treat high blood pressure [8]. Duloxetine, sertraline, fluoxetine (antidepressants) and pregabalin (antipsychotic agent) are significant examples of substances successfully synthesized via an enzymatic approach [9,10]. One of the most important APIs in psychiatry is fluoxetine, a drug with antidepressant and anxiety-reducing properties. Fluoxetine (Prozac) is commercially available as a racemic mixture of (R)-fluoxetine and (S)-fluoxetine, as both enantiomers have a similar effect on serotonin reuptake inhibition, which is reflected in the pharmacological effect [11]. However, the metabolites of (S)-fluoxetine and (R)-fluoxetine – (S)-norfluoxetine and (R)-norfluoxetine, respectively – exhibit striking discrepancy in activity; indeed, (S)-norfluoxetine has an approximately 20-fold higher capacity to inhibit serotonin reuptake than (R)-norfluoxetine [12].

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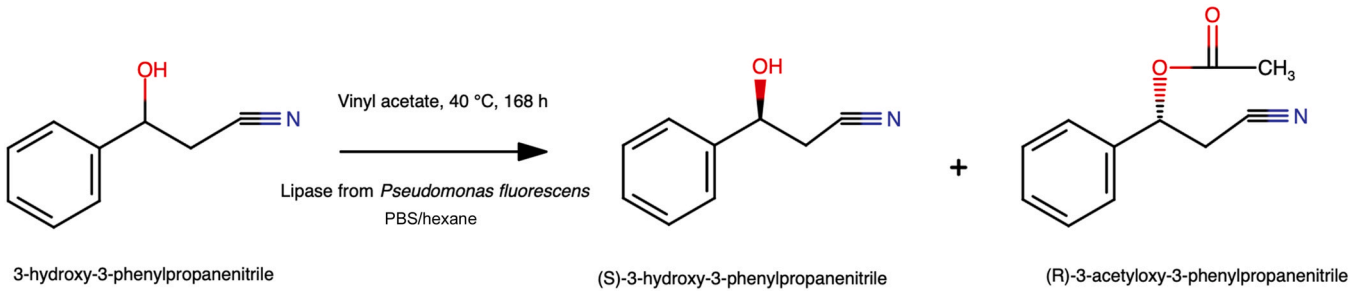

Fig. 1. Enzymatic resolution of 3H3P with the use of immobilized lipase.

In this study, lipase from *Pseudomonas fluorescens*, immobilized on modified silica nanoparticles, was used for the kinetic resolution of a racemic mixture of 3-hydroxy-3-phenylpropanonitrile (3H3P) to obtain a pure (S)-enantiomer of 3H3P. Transesterifications were carried out with the use of a novel approach based on the application of imidazolium ionic liquids with Cl^- and NTf_2 anions to stabilize the enzymes and to increase their activity and stereoselectivity. A detailed analysis of enzyme stability and the results of tests of reaction performance indicate that this is a promising approach in fluoxetine synthesis.

2. Experimental

2.1. Support modification, enzyme immobilization and biocatalyst characterization

The silica support was modified according to the methodology presented previously [13] using 3-(aminopropyl)triethoxysilane (APTES), 3-(mercaptopropyl)trimethoxysilane, triethoxy(octyl)silane and vinyltrimethoxysilane. Modification has been conducted with use of 2 g of silica, which was placed in the crystallizer. Then, solution consisting of 40 mL of methanol:water (4:1) mixture was prepared in volumetric flasks, to which 0.6 g of the appropriate modifier was added. The next step was to evenly wet the silica weighing with a specific modifier solution, which was sprayed over the material using an atomizer. The final step in the modification process was to dry the obtained samples at 80 °C for 24 h. The dried material was transferred to falcons and stored at room temperature. Next, lipase from *Pseudomonas fluorescens* was immobilized (5 mg/mL, pH 7, T = 30 °C, t = 24 h) on the surface of the modified supports.

The resulting biocatalysts were characterized with the use of a 15 mM solution of *para*-nitrophenyl palmitate (pNPP) according to the methodology reported previously [14], to determine the relative activity of the immobilized enzymes. Biocatalysts were tested in appropriate buffer solution over a range of pH values (5–10) and temperatures (30–70 °C), and reusability (10 cycles) and thermal stability (3 h incubation at 30–70 °C) were also determined [15]. To each of the reaction system 25 mg of free lipase or corresponding amount of the system after immobilization containing 25 mg of the biocatalyst was used. The biocatalysts activities is presented as relative activity, where 100 % relative activity is defined as the highest optimal activity for tests showing effect of pH, temperature and thermal stability, whereas for reusability test, 100 % relative activity is defined as the initial value of enzyme activity at the beginning of the test.

2.2. Racemic mixture resolution: biocatalysts with the addition of ILs

The produced biocatalysts were used in the resolution of two enantiomers of 3H3P that were obtained according to a previously published method [16]. Kinetic resolution of 3H3P (Fig. 1) was conducted with the addition of two ionic liquids separately, [BMIM]Cl and [BMIM]NTf_2, to test how the amount and type of ILs affect the enzymes' activity and process efficiency. At this stage, a proper amount of freshly prepared biocatalyst with APTES-modified support containing 25 mg of the immobilized lipase was added to the mixture containing 0.1 g of 3H3P racemic mixture, 0.26 g of vinyl acetate, 1 %, 5 % and 10 % (w/v, relative to the solvent) of each ionic liquid separately, and 10 mL of phosphate buffer at pH 7 or 10 mL of hexane, for comparison. Each process was continued for 7 days (168 h) at a temperature of 40 °C. All samples were analyzed using High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS) according to the methodology presented in the Supplementary materials.

3. Results and discussion

3.1. Characterization of the biocatalyst

Surface modifiers with various functional groups were tested to determine their effect on the activity and stability of the immobilized biocatalysts over a wide range of process conditions. The effect of temperature and pH, thermal stability, and reusability were evaluated for all of the obtained systems, and for free lipase as a control. The tests were performed to evaluate which biocatalyst had the highest tolerance to the process conditions to be used in API synthesis.

3.1.1. Catalytic activity recovery

The main parameter tested was catalytic activity recovery (Table 1). The highest catalytic activity recovery of immobilized lipase was obtained for the support modified with APTES, where 93 % of catalytic activity was retained. This result may be related to the presence of the most suitable functional groups on the surface of modified silica ($-NH_2$), which are most compatible with enzyme surface groups, creating stable bonds between the material and the lipase. For the other modified materials, the catalytic activity was

Table 1
Catalytic activity recovery of lipase immobilized on modified silica nanoparticles.

Modifier	Activity recovery (%)	Amount of immobilized enzyme (mg/g)
3-(aminopropyl)triethoxysilane	93	105
3-(mercaptopropyl)trimethoxysilane	91	98
triethoxy(octyl)silane	88	94
vinyltrimethoxysilane	89	97

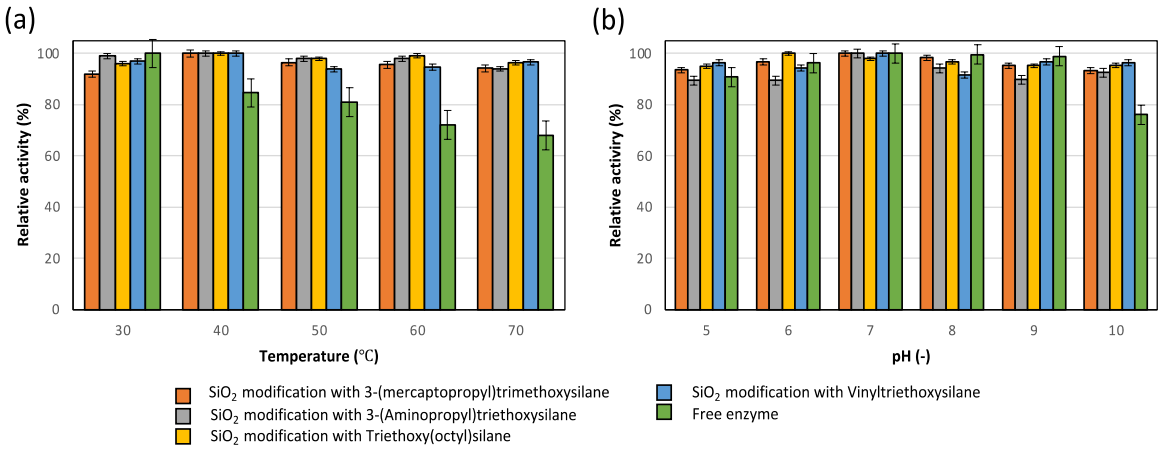

Fig. 2. Characterization of free lipase and lipase immobilized on modified silica supports effect of: (a) temperature, (b) pH.

lower, at around 90 %. Additionally, the amount of immobilized enzyme was also investigated. As it's been shown in the table the amount of immobilized enzyme corresponds with the activity recovery for each biocatalyst. The great amount of deposited enzyme on the silica surface did not lead to creation of the diffusional limitations resulting in high relative activity of the developed biocatalyst.

3.1.2. Effect of pH and temperature

It was found that immobilization increased the enzyme's stability under varying temperature. The native enzyme was characterized by decreased relative activity in harsh conditions, exhibiting less than 70 % activity at 70 °C, whereas all immobilized enzymes showed over 90 % relative activity at the same conditions (Fig. 2a). Further, at 40 °C, where all immobilized enzymes achieved their highest activity, the free enzyme retained around 85 % relative activity.

The highest relative activity for all biocatalysts, including free lipase, was obtained at pH 7 (Fig. 2b). This is related to the optimal conditions for the strain of lipase used and suggests that no significant changes in enzyme structure occurred during immobilization [20]. However, all immobilized samples showed improved pH tolerance and retained higher activity over a wide pH range. For the enzyme immobilized on a support modified with APTES, the highest relative activity was observed at 40 °C and pH 7; nevertheless, at different temperatures and pH values the relative activity remained higher than 90 %. In the case of native lipase, the highest activity was observed at a temperature of 30 °C and pH 7, but the relative activities were much lower (65–90 %) when broad ranges of process parameters were applied. This confirms the stabilizing effect of the support on the enzyme's structure; the free enzyme is directly exposed to the process conditions and is more sensitive to them, as the results clearly show.

3.1.3. Reusability and thermal stability

Tests of reusability showed that the relative activity dropped continuously with repeated use (Fig. 3a). The best performance was recorded for the biocatalyst on a support modified with APTES, where after the 10th cycle the relative activity was above 80 %. By contrast, the reuse of free lipase is impossible. Further, a study of thermal stability (Fig. 3b) showed that for all immobilized biocatalysts the relative activity was similar over a range of temperatures, but the activity of the native enzyme fell significantly at higher temperatures. For example, a decrease in activity by more than 40 % was observed at 70 °C.

The results show that immobilization significantly increases the enzyme's stability and durability in changing process conditions, due to the protective effect of the support on the enzyme's structure, and the formation of stable enzyme–support interactions that rigidize the enzyme structure and limit its denaturation in harsh conditions and with repeated use [17].

Based on the collected data, the biocatalyst obtained by immobilizing lipase on silica modified with APTES was selected for use in the process of 3H3P conversion, as it exhibited the highest relative activity and durability, which were expected to result in the highest possible efficiency of API synthesis [18].

3.2. Resolution of racemic mixture

After evaluation of their properties, the system based on APTES-modified silica was applied in the resolution of a racemic mixture of 3H3P in various solvents and in the presence of various ionic liquids. The latter served to improve the enzyme's stability and activity, due to the protective and activation effect of ILs on the enzyme structure (Table 2). The addition of ionic liquids to the reaction medium supports enzyme recovery and allows the interfacial activation to occur. Because of the hydrophobic property of the IL, enzyme is more stable and its performance in terms of efficiency and enantiomeric excess is increased [19]. Nevertheless, in synthesis of various compounds, the high viscosity of ILs might be a problem when they are used as main solvents, that's why only an addition of ionic liquids that did not exceed 10 % w/v, was performed in our study [20].

Different reaction media were used to test the enzyme's performance in aqueous and aprotic solvents. As predicted, in the presence of organic solvent the lipase catalyzed the transesterification reaction required in the tested process, whereas in the buffer medium low efficiencies of transesterification were obtained (the highest efficiency did not exceed 31.6 %). For reactions conducted in hexane the highest efficiency (97.4 %) and an ee% of 79.5 % were obtained when 1 % (w/v) of [BMIM]Cl was added to the reaction medium. It was found that for the IL with a chlorine anion, with an increase in the concentration of the IL the efficiency and ee% decreased by more than 20 %, irrespective of whether the reaction was performed in aquatic or organic solvent. The enantiomeric excess followed an opposite trend with the addition of an IL with the NTf_2 anion, where the ee% increased with increasing concentrations of the ionic liquid, reaching 73.3 % for reactions conducted in hexane. Further, significant differences in process efficiency were observed when increasing amounts of ILs, irrespectively by the type, were added to the system: with higher amounts of ILs the efficiencies were lower.

This may be related to the increased ionic strength and viscosity of the reaction medium, which reduce enzyme activity. By contrast, in the tests with free lipase the best performance was obtained using

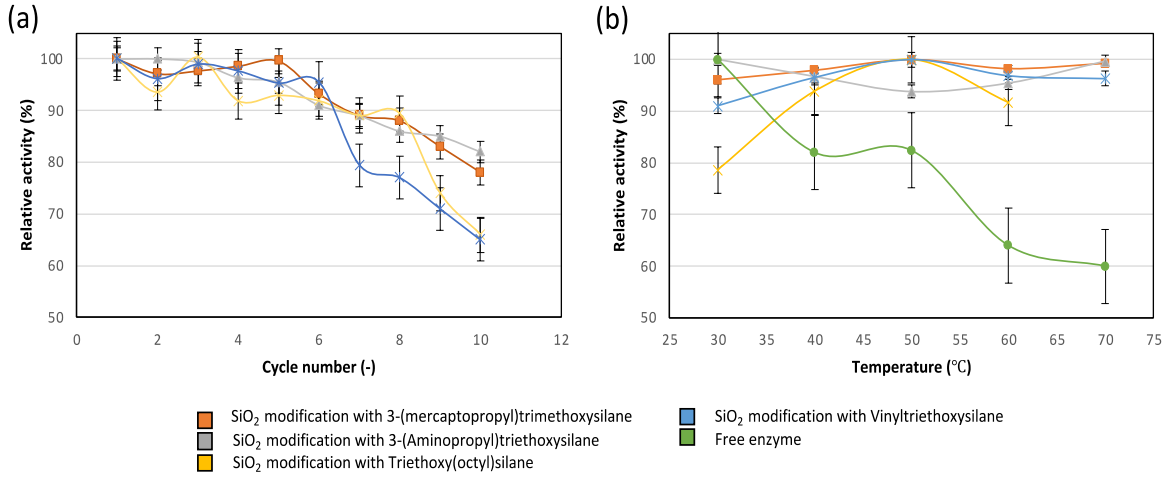

Fig. 3. Characterization of free lipase and lipase immobilized on modified silica nanoparticle supports: (a) reusability, (b) thermal stability.

Table 2
Effect of type of solvent and type and concentration of ionic liquid on the enantiomeric excess and efficiency of resolution of a racemic mixture of 3H3P.

Type of IL	Amount of IL (%, w/v)	Type of solvent	Efficiency (%)	ee (%) of S-alcohol
[BMIM]Cl/immobilized enzyme	1	hexane	97.4	79.5
[BMIM]Cl/immobilized enzyme	5	hexane	75.6	64.3
[BMIM]Cl/immobilized enzyme	10	hexane	72.4	52.0
[BMIM]NTf_2/immobilized enzyme	1	hexane	82.5	61.2
[BMIM]NTf_2/immobilized enzyme	5	hexane	56.0	74.1
[BMIM]NTf_2/immobilized enzyme	10	hexane	52.0	73.3
[BMIM]Cl/immobilized enzyme	1	PBS	12.7	68.5
[BMIM]Cl/immobilized enzyme	5	PBS	3.9	27.9
[BMIM]Cl/immobilized enzyme	10	PBS	3.9	26.4
[BMIM]NTf_2/immobilized enzyme	1	PBS	31.6	58.2
[BMIM]NTf_2/immobilized enzyme	5	PBS	26.3	74.1
[BMIM]NTf_2/immobilized enzyme	10	PBS	14.5	83.0
[BMIM]Cl/free enzyme	1	hexane	91.2	78.1
[BMIM]Cl/free enzyme	1	PBS	22.7	32.7
[BMIM]NTf_2/free enzyme	1	hexane	77.1	68.3
[BMIM]NTf_2/free enzyme	1	PBS	25.4	43.7
-	-	hexane	68.2	53.7
-	-	PBS	21.3	41.4

the IL with the Cl^- anion and hexane as solvent; in this case the efficiency and ee% were 91.2 % and 78.1 %, respectively. In case of samples without ILs addition, lower efficiency and ee% were reached. The better performance of the biocatalysts in the presence of ILs is related to the interfacial activation of lipases occurring at the boundary of two phases (aprotic–aqueous). Ionic liquids also strongly influence enzyme activity due to their over-stabilizing action. This is related to the protective effect and hydrophobic environment that promote activity [21]. Using ILs it is possible to maintain the enzyme's conformation in the ionic net, and the addition of ionic liquid can be considered as providing both a reaction medium and an immobilization support.

Table 3
Comparison of chemical synthesis and biocatalysis in the synthesis/resolution of 3-hydroxy-3-phenylpropanonitrile.

Type of reaction	Catalyst	Reaction medium	Time (h)	S-alcohol		R-acetate		Ref.
				Yield (%)	ee (%)	Yield (%)	ee (%)	
Chemical synthesis	RuCl[(S,S)-N-(p-toluenesulfonyl)-1,2-diphenylethylenediamine](p-cymene)	formic acid/ triethylamine (3.1:2.6)	24	100	98	-	-	[21]
	RuCl[(S,S)-N-(p-toluenesulfonyl)-1,2-diphenylethylenediamine](p-cymene)	2-propanol	24	< 1	-	-	-	[21]
	RuCl[(S,S)-N-(p-toluenesulfonyl)-1,2-diphenylethylenediamine](p-cymene	water	18	< 3	-	-	-	[22]
Free lipase	Lipase from *Pseudomonas cepacia*	diisopropyl ether	67	53	61	39	73	[23]
	Lipase from *Pseudomonas cepacia*	diisopropyl ether	180	46	> 99	44	> 99	[24]
	Candida rugosa lipase	diisopropyl ether	139	65	33	28	78	[24]
Immobilized lipase	*Pseudomonas* sp. lipase immobilized on Hyflo Super-Cel	diisopropyl ether	19	42	> 99	45	> 99	[23]
	Pseudomonas cepacia lipase immobilized on modified ceramic particles	diisopropyl ether	13	46	> 99	46	> 99	[24]
	Pseudomonas cepacia lipase immobilized on diatomite	diisopropyl ether	75	45	> 99	46	> 99	[24]
This study	Lipase from *Pseudomonas fluorescens* immobilized on modified silica nanoparticles	hexane [BMIM]Cl	168	97.4	79.5	-	-	-

4. Conclusions

It has been demonstrated that immobilized enzymes can be successfully used in the kinetic resolution of 3H3P. The influence of ionic liquids on the stabilization and improvement of lipase activity in this process has been proved for the first time. It was shown that the most suitable IL was [BMIM]Cl; in this case a process efficiency of 97.4 % and an ee% of 79.5 % were obtained when 1 % (w/v) of IL in organic solvent was used and the process was catalyzed by lipase immobilized on APTES-modified silica. Studies without addition of ILs show that enzymes' activity is improved only in the presence of ionic liquids. These studies confirm the highly important role that enzymes can play in the pharmaceutical industry, due to high efficiency and enantioselectivity and the reduction of reaction steps. Nevertheless, the use of ionic liquids with immobilized enzymes for the resolution of APIs or their intermediates is still a novel and ongoing field of research, and further tests are very much needed. Below (Table 3) we also present the comparison of three different techniques applied for the synthesis/resolution of 3-hydroxy-3-phenylpropanonitrile. It is clear that enzymatic conversion allows the synthesis of specific enantiomers, without the need of using harmful catalysts. What is more yield of reaction after biocatalysis is relatively high comparing to chemical synthesis, showing key benefits of using enzymes. In our study, reactions conducted in hexane with the addition of ionic liquids proves the great advantage of immobilized lipases in enzymatic resolutions, by not only the high yield (above 97 %) of reaction but also high enantiomeric excess (almost 80 %). It is worth mentioning that comparing to the chemical synthesis enzymes are much safer for the environment and processes can be designed to obtain specific enantiomer.

CRediT authorship contribution statement

Oliwia Degórska: Conceptualization, Investigation, Visualization, Writing – original draft. **Daria Szada:** Investigation, Formal analysis, Visualization. **Teofil Jesionowski**: Writing – review & editing, Supervision. **Jakub Zdarta:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.02.026.

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