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Ionic liquid supported hydrogel–lipase biocatalytic systems in asymmetric synthesis of enantiomerically pure S-ibuprofen

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ABSTRACT

Novel hydrogel biocatalysts with immobilized lipase, stabilized by ionic liquids (ILs) of different hydrophobicity, were synthesized and evaluated. Variations of the time of immobilization and ratio of substrates during hydrogel synthesis were considered to obtain the most stable biocatalyst with the highest activity. Physicochemical characterization proved the success of the hydrogel synthesis and enzyme deposition on the surface of the support. Nevertheless, the key objective was to produce a biocatalyst for further application in ibuprofen methyl ester resolution, with the aim of obtaining an enantiomerically pure product. The hydrogel biocatalysts obtained in the presence of 5 wt% ILs after 8 h of immobilization achieved the highest activity recovery of 62 %. After 10 reaction cycles, enzymatic activity was still above 60 %, and the negative effect of pH and temperature on the activity of immobilized lipase was much lower than in the case of the free enzyme. After application of the catalyst in the resolution of ibuprofen methyl ester, the enantiomeric excess and conversion rate of the process were obtained for the dynamic kinetic resolution in isooctane. A conversion rate of 95 % was achieved due to the stabilization of the biocatalyst with IL and its resulting high catalytic activity. The study thus provides the pharmaceutical industry with a new potential approach with a strong scientific foundation.

1. Introduction

Hydrogels are water-insoluble polymeric networks with hydrophilic properties, and may be made from one or more monomer units, polymers, or cross-linking agents. Hydrogels exhibit multiple unique features, depending on the compounds used for their preparation. Their important advantages include the possibility of designing the composition, pore size, or surface groups. Due to their three-dimensional structure, they have the tendency to retain high amounts of fluid, such as water. They offer the desired functionality, stability, and biocompatibility for a variety of applications in science and industry [1,2]. The characteristics of hydrogels enable their use in the immobilization of enzymes, to improve operational stability and reusability and to reduce the cost of using enzymatic catalysts [3]. In the aqueous environment of hydrogels, enzymes are protected from denaturation and their catalytic activity is significantly enhanced. Additionally, hydrogel materials offer an intelligent response to environmental factors such as temperature or pH, allowing their physicochemical properties to be adjusted [4]. The addition of appropriate chemical groups enables further improvement of the properties of the hydrogel, such as the degree of swelling and stiffness [5].

With a three-dimensional porous structure, hydrogels are permeable to metabolites or small molecules forming as products of an enzymatic reaction. Moreover, hydrogels can immobilize a variety of enzymes using different methods (e.g. entrapment, absorption, covalent binding). Hydrogels for immobilization are currently synthesized using a variety of synthetic, semi-synthetic, and natural polymers [4]. Alginate and chitosan are two natural polymers that are commonly used in the production of hydrogels. Pirozzi et al. [6] immobilized lipase from *Candia rugosa* on chitosan hydrogel by adsorption and entrapment, achieving immobilization yields of 48 % and 65 %, respectively. In polyacrylamide-based gels, on the one hand charges may form, while on the other the side chains of the acrylamide units may react with the environment to form acrylic acid, which is sufficient to produce ionized carboxyl groups. It has been demonstrated that encapsulating an enzyme in a structure that is strongly chemically charged enhances its activity

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[7].

Over the past few years, biocatalysis has gained increasing interest as an alternative to traditional organic chemistry, particularly in the pharmaceutical industry, for synthesizing active pharmaceutical ingredients (API). Notably, biocatalysis meets the growing demand for sustainable, eco-friendly and safe industry [8,9], and aligns with the principles of Green Chemistry [10]. For instance, enzymes facilitate and accelerate biochemical reactions, eliminating the need to raise the reaction temperature, thereby enhancing energy efficiency [11]. The chemo-, regio- and stereoselectivity of enzymes enable the elimination of certain reaction steps, reducing energy consumption and by-product formation. This characteristic makes biocatalysis an environmentally friendly process, highlighting its superiority over the traditional synthesis methods of organic chemistry [12].

Biocatalysis also offers a range of possibilities for asymmetric synthesis of pharmaceutically active compounds, since a large number of drugs contain chiral active pharmaceutical ingredients. Chiral compounds are those with two enantiomers (R- and S-) that are identical in chemical formula but cannot be superimposed on each other. These enantiomers often have different biological and pharmaceutical properties. For example, (*S*)-ibuprofen is 160 times more pharmaceutically active than the (*R*)-enantiomer [13].

Ionic liquids are becoming increasingly popular as means of supporting the immobilization of enzymes on hydrogels. Low volatility, chemical stability, and biocompatibility are just a few of the many features of ionic liquids. It is possible to improve enzyme–carrier bonding by adding a suitable ionic liquid during enzyme immobilization, due to the formation of bridges on the surface of the carrier. Additionally, ionic liquids can be used as additives during the immobilization process in order to extend enzyme life [14]. Moreover, hydrophobic ionic liquids facilitate modification of the conformation of the enzyme's active center, by promoting the opening of the lipase lid via interfacial activation to increase its activity [15]. A study by Cabrera-Padill et al., who used various hydrophobic ionic liquids in the immobilization of lipase from *Candida rugosa*, also confirmed improved functional properties such as thermal stability and the retention of activity for a longer period of time [16].

The main goal of the present study was to develop a novel biocatalyst composed of lipase immobilized using polymeric hydrogels, supported by ionic liquids characterized by different hydrophobicity to enhance enzyme activity for the asymmetric synthesis of (*S*)-ibuprofen. The work consisted of three major stages: synthesis of the hydrogel support platform, production of biocatalysts through immobilization and their characterization, and the resolution of racemic ibuprofen methyl ester. Physicochemical analyses were carried out to provide a detailed characterization of the materials and biocatalytic systems, and the products were analyzed using HPLC to evaluate the enantioselectivity and conversion rate under varying process conditions. This study had the aim of determining novel protocols for the efficient, sustainable and eco-friendly production of API compounds.

2. Materials and methods

2.1. Reagents and chemicals

Ibuprofen methyl ester was purchased from Chemat. Organic solvents such as hexane and isooctane were supplied by POCH. Lipases from *Candida rugosa*, the ionic liquids [BMIM]Cl and [BMIM]PF_6, ammonium persulfate (APS), acrylamide (AM), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), *N,N′*-methylenebisacrylamide (MBAm), *para*-nitrophenyl palmitate (pNPP), sodium carbonate, and buffers (acetate, phosphate and TRIS buffers at various pH and a molarity of 50 mM) were purchased from Sigma Aldrich (Merck Group, Poland).

2.2. Hydrogel synthesis

The first experimental stage included the synthesis of a polyacrylamide (PAM) hydrogel via free radical polymerization of acrylamide and the cross-linking reagent *N,N′*-methylenebisacrylamide (MBAm) (Fig. 1). PAM hydrogels with four different ratios of AM to MBAm were obtained and examined: 8:1 (15 wt%), 8:1 (20 wt%), 9:1 (15 wt%), and 9:1 (20 wt%). Two solutions were prepared for this purpose. For the 9:1 ratio (15 wt%), mixture A was prepared by mixing 38.84 mg of MBAm (crosslinking reagent) with 1.9 mL of water and adding 161.16 mg of AM (monomer) and 0.01 mL of *N,N,N′,N′*-tetramethylethylenediamine (TEMED), which acts as a catalyst for the polymerization. Mixture B was prepared by adding 0.03 g of ammonium persulfate (APS, radical initiator) to 0.1 mL of water and placing the mixture in an ultrasonic bath for 30 s. Then mixture B was added to mixture A with vigorous stirring for about 10 s. After a 10 min reaction time, the resulting hydrogel was kept for 24 h at a temperature of −20 °C. The water present in the hydrogel increases in volume when it freezes, which favors the formation of a porous structure. The hydrogel samples were then freeze-dried using a Martin Christ Alpha 1–2 LD Plus freeze dryer to remove water residues.

2.3. Enzyme immobilization

The next step involved immobilization of the enzyme on the hydrogel support. Enzyme screening using different strains of lipase, such as *Pseudomonas cepacia, Aspergillus oryzae, Pseudomonas fluorescens* and *Candida rugosa*, was performed during preliminary studies. Because lipase derived from *Candida rugosa* displayed the highest catalytic activity, it was applied in the further experiments. An enzyme solution was prepared for immobilization at a concentration of 5 mg/mL in 50 mM phosphate buffer at pH 7. 5 mL of lipase solution. This was transferred to falcons, and then a certain amount of ionic liquid, [BMIM]Cl or [BMIM]PF_6 (1, 5, 10 wt% calculated based on the volume of the enzyme solution) was added to each falcon to test the effect of the amount of IL on immobilization performance and activity recovery. For comparison, control samples without ILs were also produced. The last step was to add approximately 30 mg of hydrogel to each falcon. The immobilization process was carried out in an Eppendorf Thermomixer laboratory incubator for 8 h at a temperature of 40 °C and a mixing speed of 200 rpm. The samples obtained were assayed using pNPP (see Section 2.4.1) to determine the immobilization yield (Eq. (1)). The post-immobilization filtrates were tested with Bradford reagent to calculate the amount of immobilized enzyme, taking into account the amount of the support material used.

$$\textit{Immobilization yield}\ (\%) = \frac{A_i - A_f}{A_i} \cdot 100\% \quad (1)$$

A_i – initial activity of lipase added to the immobilization medium,

A_f – total activity of the enzyme in the supernatant and washing solution after immobilization.

2.4. Characterization of the biocatalyst produced

2.4.1. Activity recovery, effect of process conditions and thermal stability

To select suitable process parameters for the immobilized lipase with the addition of ILs (samples without addition of ILs were found to have significantly lower activity recovery, hence were not analyzed in detail), the effect of temperature and pH on the activity was investigated. For specific pH values, different buffers were used: 50 mM acetate buffer for pH 4 and 5, 50 mM phosphate buffer for pH from 6 to 8, and 50 mM TRIS buffer for pH 9 and 10. For investigation of the effect of pH, 2 mL of buffer at a specific pH was added to Eppendorf tubes containing 30 mg of biocatalyst (mass of hydrogel with immobilized enzyme; the mass of immobilized enzyme was 6.5 mg), followed by addition of 1 mL of pNPP

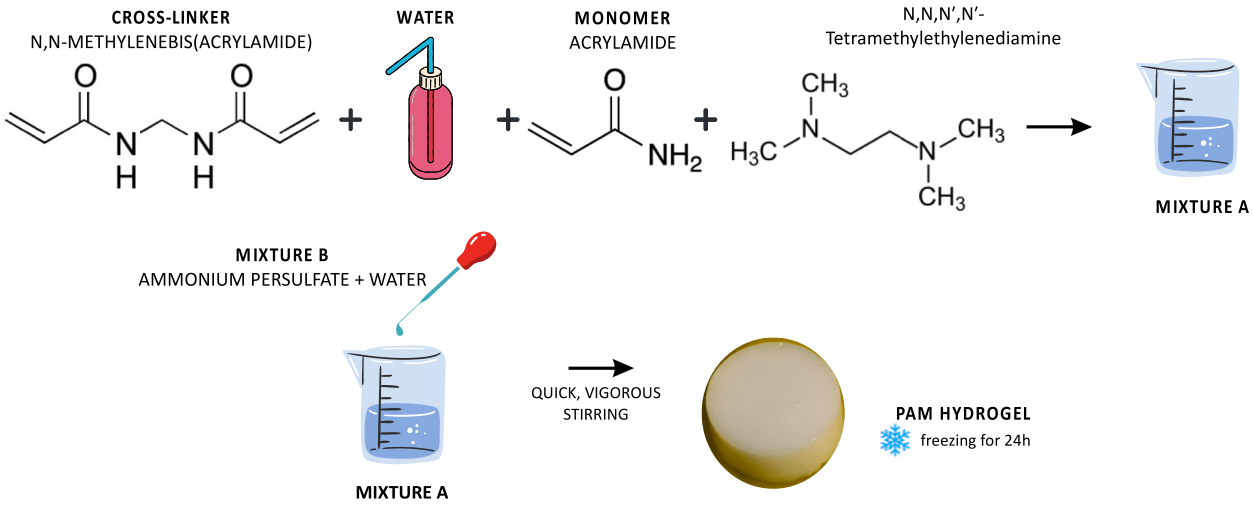

Fig. 1. Scheme of polyacrylamide (PAM) hydrogel synthesis.

solution at a concentration of 15 mM and reaction for 5 min at pH values 4, 5, 6, 7, 8 and 9, in an Eppendorf ThermoMixer C (Hamburg, Germany). Reactions were conducted at a temperature of 40 °C. To determine the influence of temperature on enzymatic activity, processes were conducted as described previously, in a buffer solution at pH 7 in varying temperatures: 30, 40, 50, 60 °C. Thermal stability was investigated under heat stress. At first the samples were incubated at a specific temperature (30, 40, 50, 60 °C) at pH 7 for 2 h. Afterwards the relative activity was measured according to the model reaction described above. After 5 min of model pNPP reaction, samples were subjected to UV–Vis measurements at λ = 410 nm and the concentration of the product was determined based on the calibration curves of para-nitrophenol. Lipase activity recovery was calculated according to Eq. (2).

$$\textit{Activity recovery}\ (\%) = \frac{A_t}{A_i} \cdot 100\% \tag{2}$$

A_i – initial activity of lipase added to the immobilization medium,

A_t – activity of the immobilized lipase.

All measurements were repeated three times and the results are presented as mean value ± standard deviation.

2.4.2. Reusability

For the reusability study, 10 reaction cycles of pNPP hydrolysis were performed at pH 7 and a temperature of 40 °C, where 30 mg of the biocatalyst, 2 mL of 50 mM phosphate buffer at pH 7 and 1 mL of 15 mM pNPP were added to an Eppendorf tube. The reaction was continued for 5 min in an Eppendorf ThermoMixer C; afterwards, the biocatalyst was washed a few times with the use of phosphate buffer (pH 7) and used in the next reaction. After the samples were collected, UV–Vis spectroscopy was performed and absorbance was measured at λ = 410 nm. Based on the results, the activity was calculated. In these experiments, initial enzyme activity was defined as 100 % activity. All measurements were repeated three times, and the results are presented as mean value ± standard deviation.

2.4.3. Kinetic parameters

Kinetic parameters – the Michaelis–Menten constant (K_M) and maximum reaction rate (V_{max}) – of the free lipase and lipase immobilized with and without ILs were evaluated based on the hydrolysis reaction of pNPP described in Section 2.4.1, by measuring the initial reaction rates, using various concentrations of pNPP (0.1–1 mM). The progress of the reaction was followed spectrophotometrically at λ = 410 nm (Jasco V750, Jasco, Japan). The kinetic parameters were calculated using Hanes–Woolf plots under optimum assay conditions. A double reciprocal chart for all tested samples is given in the Supplementary Materials as Fig. S2. All measurements were repeated three times and the results are presented as mean value ± standard deviation.

2.5. Analytical procedures

To characterize the hydrogel material before and after immobilization, the FTIR (Fourier transform infrared spectroscopy) technique was used in attenuated total reflectance (ATR) mode (Bruker, Vertex 70) in the wavenumber range 400–4000 cm^{-1}. The morphologies of the hydrogels before and after lipase immobilization were evaluated based on confocal laser scanning microscopy (CLSM) photographs (LSM710, Zeiss, Germany), and SEM photographs were obtained using an EVO40 scanning electron microscope (SEM, Zeiss, Germany). During the characterization of the products, the parameters of their porous structure were also assessed. The specific surface area of the tested material was determined using the BET (Brunauer–Emmett–Teller) algorithm (ASAP 2020 porosimetry analyzer, Micromeritics Instrument Co.), while the pore volume and pore size were determined based on the BJH (Barett–Joyner–Halenda) algorithm. X-ray energy dispersion microanalysis (EDS) was used to verify the effectiveness of the enzyme immobilization and to characterize the biocatalytic systems before and after reaction; this was done using the EDS analyzer in the scanning electron microscope. Before the analysis, samples were attached to the base with a carbon tape. A thermogravimetric analyzer (TGA) (Jupiter STA 449F3, Netzsch, Germany) was used to investigate the thermal stability of samples. The measurements were carried out under liquid nitrogen (10 mL/min) at a heating rate of 10 °C/min over a temperature range of 25–1000 °C, with an initial sample weight of approximately 5 mg. Wetting angle was determined using a KRÜSS DSA100 Drop Shape Analyzer with a micropipette for applying droplets of constant volume and a system for drop image analysis. The parameter was determined for drops applied to the hydrogel surface using two liquids: polar (water – Θw) and dispersive (diiodomethane – Θd). The determination of the wetting angle was based on the average of ten successively recorded values. Further, TLC was used to determine progress in the asymmetric synthesis of (*S*)-ibuprofen, using silica gel plates with fluorescent indicator (Sigma Aldrich) and a combination of toluene:ethyl acetate:acetic acid (17:13:1) as a developing solvent. The chromatographic separation process (HPLC) was carried out using the 1290 Infinity II LC System from Agilent. To separate the enantiomers of ibuprofen, a chiral Poroshell 120 Chiral-T column with dimensions of 4.6 × 150 mm and a packing particle size of 2.7 μm was used. The separation of analytes was carried out in a reversed phase system – the mobile phase was a 15 mM solution of ammonium formate in water (A), adjusted to pH = 4 with formic acid,

and methanol (B). The analysis was performed for 12 min in isocratic conditions with a mobile phase composition of 40:60 (A:B) and a flow of 0.4 mL/min. The chromatographic column was maintained at 22 °C during the analysis, and the injection volume was 0.25 μL. The detection of analytes was enabled by a diode array detector (DAD) using a wavelength of 220 nm. To determine the concentrations of ibuprofen enantiomers and ibuprofen methyl ester racemate, standard curves were prepared for each of these compounds. Three injections were made for each test sample and for the standard solutions to evaluate the repeatability of the results.

2.6. Resolution of ibuprofen – batch process

2.6.1. Kinetic enzymatic resolution of ibuprofen methyl ester

An appropriate amount of the prepared biocatalytic system consisting of a hydrogel carrier and 5 mg or 25 mg of immobilized lipase was introduced into a conical flask. Next, 5 mL of 50 mM phosphate buffer at pH 7 and 5 mL of a solution of ibuprofen methyl ester in isooctane with a concentration of 45.45 mM (10 mg/mL) were added to the system. To determine the influence of the type of organic solvent on the process conversion rate, additional hydrolysis reactions of ibuprofen ester were carried out in the presence of hexane. The reaction medium formed two phases – an organic phase containing an ibuprofen ester solution, and an aqueous phase. The flask was tightly closed and then placed in the Eppendorf Thermomixer laboratory incubator. The kinetic resolution process was carried out for 144 h (6 days) at a temperature of 40 °C and a mixing speed of 200 rpm. The hydrolysis reaction of ibuprofen methyl ester took place only at the interface of the two phases. During the process, 0.1 mL samples were taken from the organic phase at specified intervals, and the course of the reaction was monitored using chiral HPLC methods. Preparation of reaction samples for chromatographic analysis involved evaporation of the organic solvents hexane and isooctane. For this purpose, samples were placed in chromatographic vials, and an Eppendorf concentrator (Concentrator plus/Vacufuge®-plus) was used. The evaporation process was carried out at 30 °C and under reduced pressure. Complete evaporation of hexane was achieved after 3 h, while 6 h was needed for the evaporation of isooctane. After evaporation of hexane and isooctane, 1 mL of methanol was added to the chromatography vials to redissolve their contents, and then each solution was further diluted.

2.6.2. Dynamic kinetic enzymatic resolution of ibuprofen methyl ester

To increase the conversion rate of the process, an attempt was also made to carry out dynamic kinetic separation of ibuprofen methyl ester, in which the enantioselective hydrolysis of the (*S*)-ester occurs simultaneously with the racemization of the unreacted substrate (*R*)-ester. In this process, the reaction medium was $NaHCO_3$-NaOH buffer at pH 9.5 without any addition or with the addition of 3 mL of DMSO as an in situ racemization agent and isooctane/hexane as an organic solvent. The remaining process conditions, including the amount of immobilized enzyme, were the same as in the case of kinetic enzymatic separation. A list of the prepared samples with an indication of the process conditions is given in Table S1 in the Supplementary Materials. Afterwards, the samples were analyzed with HPLC to determine the conversion rate and the enantiomeric excess of the conversion process. The degree of conversion of the racemic mixture of ibuprofen methyl ester was determined based on the results of HPLC analysis using Eq. (3):

$$c = 1 - \frac{[E_1]}{[E_2]} \cdot 100\% \quad (3)$$

c – degree of conversion of ibuprofen methyl ester (%),

$[E_1]$ – ibuprofen methyl ester concentration at $t = 0$ h (mg/mL),

$[E_2]$ – concentration of ibuprofen methyl ester after the reaction (mg/mL).

The enantiomeric excess of the obtained (*S*)-ibuprofen over (*R*)-ibuprofen was calculated from Eq. (4):

$$ee = \frac{|[S] - [R]|}{[S] + [R]} \cdot 100\% \quad (4)$$

ee – enantiomeric excess (%),

[S] – concentration of (*S*)-ibuprofen obtained (mg/mL),

[R] – concentration of (*R*)-ibuprofen obtained (mg/mL).

3. Results

3.1. Characterization of hydrogels and selection for further experiments

Prior to the synthesis of the biocatalyst, various hydrogel materials underwent characterization. Initially, four different ratios of monomer to cross-linker in PAM hydrogel were investigated: 8:1 (15 wt%), 8:1 (20 wt%), 9:1 (15 wt%), and 9:1 (20 wt%).

After the synthesis, platforms were lyophilized and their stabilities in phosphate buffers were established. The evaluation of hydrogel stability in PBS, with accompanying photographs, is given in the Supplementary Materials (Fig. S1). Since the stability of the support material is crucial for a successful immobilization process, the selection of a suitable hydrogel was determined by this factor. The lyophilization process can significantly change the material's structure and durability, hence all of the hydrogels produced using different ratios were subjected to freeze-drying. The most suitable hydrogel for further use in enzyme deposition was found to be the 9:1 (15 wt%) product, due to its stable structure after lyophilization and lack of defragmentation during incubation. SEM and CLSM photographs were taken to establish the structure and possible deposition of the enzyme on the surface and in the materials' pores (Figs. 2 and 3, respectively). From the images, it can be concluded that the porous structure of the hydrogel material is well-developed, which increases the likelihood of enzyme deposition because of the larger surface area of the material. What is more, clusters of enzymes adsorbed on the material are visible (Fig. 2b). Fig. 3b shows a cross-section of hydrogel after immobilization, illustrating the deposition of the lipase inside the hydrogel bulk. The biocatalysts' penetration through the pores, deeper into the porous hydrogel, is visible in the CLSM photographs taken in fluorescence mode (Fig. 3b), which confirm the successful deposition of enzyme not only on the surface but also inside the hydrogel's pores.

The prepared hydrogel materials are used as supports in the enzyme immobilization process. Thus, following characterization of the materials, the influence of ionic liquids on the immobilization process and on the properties of the materials themselves was investigated. The produced biocatalytic systems were tested for activity to determine the most suitable anions and the amount of IL to be used. Table 1 contains values of the activity recovery of the produced systems. It is seen that the addition of 1 wt% IL during the immobilization process leads to no significant difference in the activity of the enzyme compared with

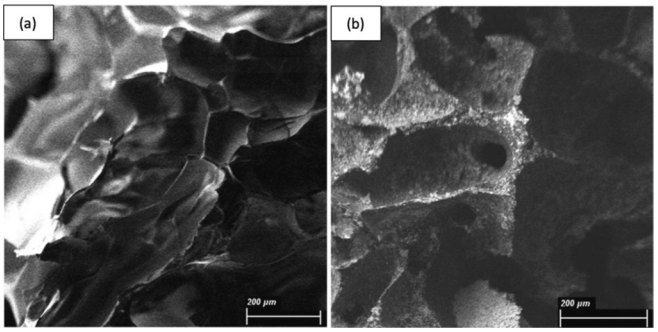

Fig. 2. SEM photographs of: (a) pristine hydrogel material, (b) hydrogel with deposited enzymes. Both images show PAM hydrogels at an AM to MBAm ratio of 9:1 (15 wt%).

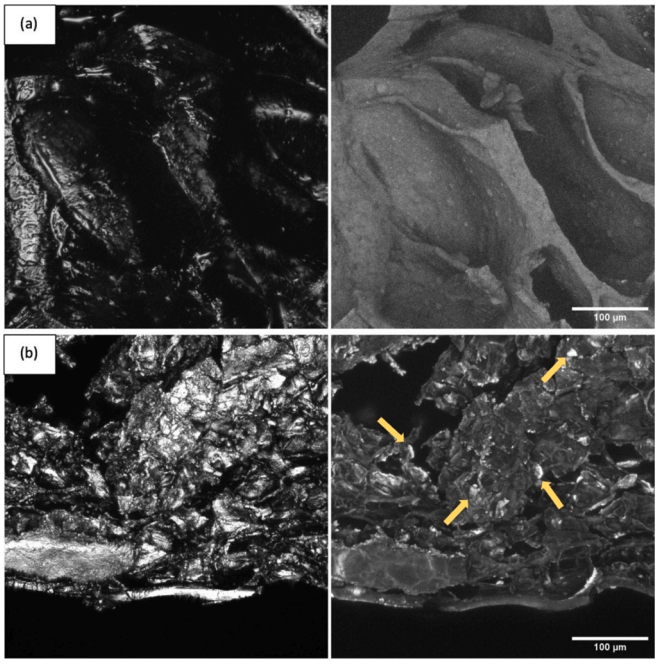

Fig. 3. CLSM photographs of (a) pristine hydrogel material and (b) hydrogel with deposited enzymes in reflection mode (left) and fluorescence mode (right). Yellow arrows indicate enzyme molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Activity recovery of immobilized enzyme depending on immobilization time and the type and dosage of IL.

Immobilization time (h)	Activity recovery without IL (%)	Activity recovery with [BMIM]Cl (%)	Activity recovery with [BMIM]PF_6 (%)
1 wt% of IL			
2	28 ± 0.85	43 ± 1.6	47 ± 1.2
8	39 ± 1.7	26 ± 1.2	45 ± 0.9
16	35 ± 0.05	32 ± 0.7	18 ± 0.3
24	32 ± 1.5	28 ± 1.3	32 ± 0.4
5 wt% of IL			
2	28 ± 1.1	43 ± 0.1	49 ± 1
8	39 ± 1.2	57 ± 0.4	62 ± 0.3
16	35 ± 0.8	34 ± 0.3	43 ± 0.1
24	32 ± 0.75	33 ± 0.7	32 ± 0.15
10 wt% of IL			
2	28 ± 0.12	34 ± 1	36 ± 0.05
8	39 ± 1.4	39 ± 0.1	45 ± 1
16	35 ± 0.7	35 ± 1.4	41 ± 0.9
24	32 ± 0.3	33 ± 0.3	27 ± 0.1

immobilization without IL. With the addition of greater amounts of IL, the changes are more significant. The highest activity recovery (62 %) was obtained for the system immobilized for 8 h with 5 wt% of [BMIM]PF_6. Based on the presented data on immobilization efficiency and activity recovery, this system was applied in further experiments. There are several reasons why ILs may improve an enzyme's properties. First, the proposed amount of IL may have a stabilizing effect on the biocatalyst's structure, resulting in increased activity recovery [17]. It is notable that anions have a large impact on structure stability. Through the use of two different ILs, hydrophobic [BMIM]PF_6 and hydrophilic [BMIM]Cl, we found that the PF_6^- anion is more favorable to lipase activity, due to the hydrophobic environment it creates. [BMIM]Cl also increases the amount of deposited enzyme on the hydrogel, but the catalytic activity is inhibited [18]. What is more, a mechanism specific to lipases is lid opening, called interfacial activation. This phenomenon occurs in a less polar environment, for instance in the presence of hydrophobic ILs, and ensures easier access of the substrate to the active enzyme center, which translates directly into a higher activity recovery. The lipase structure contains a mobile polypeptide chain known as the lid. When the lid is closed, it isolates the active center from the surrounding medium. Movement of the lid exposes a large hydrophobic pocket and the active center to the medium, causing strong adsorption on any hydrophobic surface.

Consequently, lipases can target insoluble substrate droplets. This type of catalytic process is referred to as interfacial activation, because lipase activity generally increases as the substrate forms insoluble droplets. Since [BMIM]PF_6 is much more hydrophobic, the interfacial activation phenomenon may be intensified in comparison with the other tested ionic liquid. The hydrophobic nature of the ILs can mimic the lipid–water interface, promoting lid opening and thus increasing the activity of the lipase, determined as the activity recovery of immobilized lipase in a tested sample [19]. Moreover, in hydrophobic environments, besides classic adsorption interactions, hydrophobic interactions were also reported. This phenomenon, also observed in our study, is referred to as immobilization via interfacial activations, and improves both the catalytic activity of the immobilized lipase and its binding to the support. The applied immobilization conditions ensure strong interactions, and the enzyme's relatively large lid leads to robust hydrophobic interactions, resulting in the enzyme being embedded with an open lid and maintaining high activity. On the other hand, in samples immobilized with 10 wt% of IL, the viscosity of the additive may influence the free transfer of a substrate to the active center of the enzyme, resulting in a system with slightly lower activity [20].

The influence of ionic liquids was clearly visible for the deposited enzyme (Table 2). The addition of the ionic liquid increased the amount of immobilized enzyme almost threefold. Irrespective of the type of ionic liquid added, the efficiency of enzyme immobilization is significantly greater, at over 50 %, compared with the system without ILs. A possible explanation is that the enzyme might be dissolved partially in the ionic liquid, which additionally improves the efficiency of binding to the material's surface [21].

In Fig. 4, a broad band with a maximum at a wavenumber of approximately 3100–3500 cm^{-1} corresponds to stretching vibrations characteristic of the N—H group, indicating the presence of repeating acrylamide units in the hydrogel. The chemical structure of the obtained hydrogel is also confirmed by an intense band with a maximum at 1650 cm^{-1}, resulting from the stretching vibrations of the C═O moiety present in the amide group of acrylamide. Moreover, the band at 1537 cm^{-1} corresponding to N—H deformation vibrations is also characteristic of the amide group. Further, a band of low intensity is observed, with a maximum at 2930 cm^{-1}, resulting from C—H stretching vibrations in the aliphatic chain of the hydrogel. The effectiveness of the polymerization process is also confirmed by the presence of bands in the range 1410–1450 cm^{-1} resulting from deformation vibrations of C—N bonds. The analyzed spectrum confirms the successful synthesis of a hydrogel platform that possesses functional groups capable of forming bonds and reacting with different compounds – for example, bonding with enzymes.

Table 2
Amount of enzyme immobilized on PAM hydrogels with and without addition of ILs.

Type of biocatalyst	Amount of immobilized enzyme (mg/g)	Immobilization yield (%)
With [BMIM]Cl	215 ± 6	86 ± 2.8
With [BMIM]PF_6	217 ± 3	87 ± 1.2
Without IL	79 ± 3	32 ± 3.8

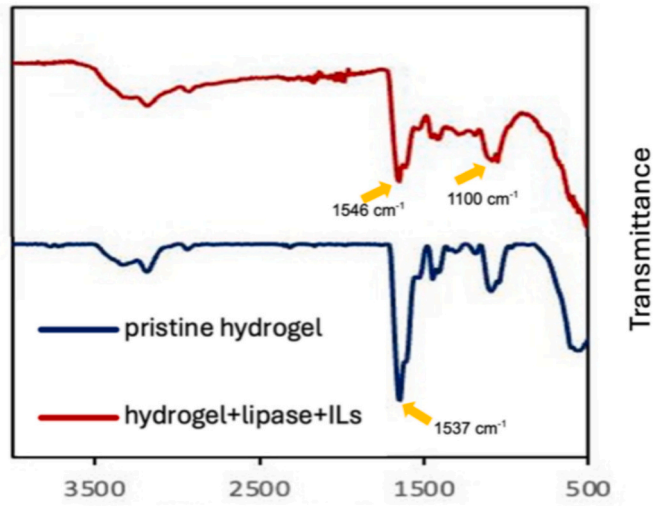

Fig. 4. FTIR spectra of pristine hydrogel and hydrogel with immobilized lipase with the addition of [BMIM]PF_6 IL.

In the spectrum of hydrogel with immobilized lipase, in addition to the bands characteristic of the polyacrylamide hydrogel, there is an intense absorption band at approximately 1100 cm^{-1}, resulting from stretching vibrations of the C–O–C bridge, which is characteristic of the lipase structure. Additionally, the success of immobilization is confirmed by deformation bands with maxima at 844 cm^{-1}, 752 cm^{-1} and 619 cm^{-1}, which are characteristic of proteins rich in amide bonds and were not visible in the spectrum of the pure hydrogel [22]. The fact that the hydrogel and lipase have common functional groups (e.g., C═O, N—H) results in similarity of the FTIR spectra of the hydrogel before and after immobilization. Nevertheless, subtle but important differences in shape, intensity and the maxima of the signals for C–O–C bonds (peak maximum at 1100 cm^{-1}) and a slight shift of the signal characteristic for amide II bonds (from 1537 cm^{-1} in pristine hydrogel to 1546 cm^{-1} in the spectrum of the material after immobilization) allow us to conclude that the lipase from *Candida rugosa* was immobilized efficiently on the polymer hydrogel, as reported previously [23].

TGA (Fig. 5) and BET analyses were performed to understand better the changes resulting from enzyme immobilization and the addition of the ionic liquid. The measurements showed that the mass loss for all tested samples exceeded 60 %, and the TGA curves of the hydrogel before and after immobilization follow a similar trend. The 15 % mass loss at around 100 °C is attributed to the evaporation of water present on the surface and in the pores of the samples [24]. At higher temperatures,

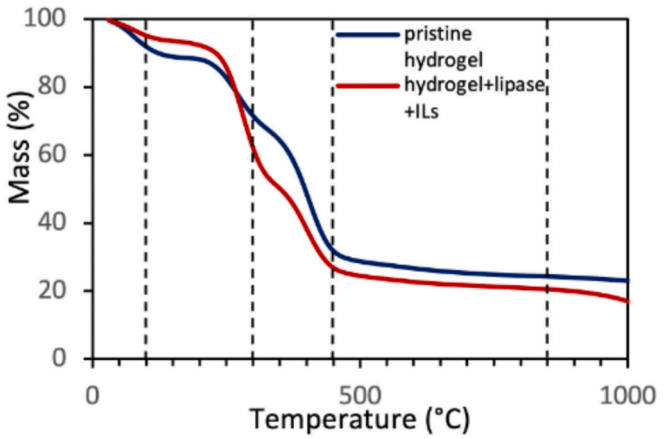

Fig. 5. TGA curves of pristine hydrogel and biocatalytic systems with the addition of [BMIM]PF_6 ILs.

the biocatalytic systems exhibit greater decomposition than the pristine hydrogel due to the enzyme present within the material. In the range 300–450 °C there is a rapid mass loss related to the partial decomposition of the hydrogel material [25]. A slight reduction in mass above 850 °C is attributed to the breakdown of the enzyme's carbon structure [26]. To investigate the structural properties of the prepared materials, nitrogen physisorption analysis was used. For the pristine hydrogel, the surface area is 3.98 m^2/g with an average pore size of 15.04 nm. Following immobilization of the enzyme, the surface area of the material decreased to 2.22 m^2/g, and the pore size fell to 12.78 nm. The lowest surface area (1.61 m^2/g) was obtained for the material following enzyme immobilization with an IL, which may indicate greater deposition of the enzyme on the surface and in the pores of the material [27]. The results show a close correlation with the amount of enzyme deposited (Table 3). Enzyme deposition increased threefold with the addition of IL, resulting in a decrease in pore size (10.58 nm) compared with the pristine hydrogel and the biocatalyst obtained without IL support.

EDS analysis provided information about the elemental composition of the surfaces of the produced systems. Fig. 6a shows the spectrum revealing the elemental composition of the PAM-based hydrogel. It is characteristic of this type of carrier that the contents of carbon, oxygen and nitrogen result from the functional groups of the substrates used in the hydrogel synthesis process, indicating the successful synthesis of a hydrogel material that should possess amino or carbonyl surface groups, for example, capable of enzyme binding [28]. For the hydrogel after enzyme immobilization (Fig. 6b) there were changes in the percentages of elements on the surface. The carbon-to-nitrogen ratio decreased, which provides confirmation of the success of the immobilization process. What is more, successful immobilization is indicated by the increase in the percentages of carbon and oxygen present in the sample, as these are among the main components of the functional groups of the enzyme [29]. Additionally, the immobilization of the enzyme on the hydrogel surface is confirmed by the increase in the percentage of sulfur, which results from the presence of cysteine bridges in the enzyme structure. The elemental composition of the enzyme–hydrogel system surface after the enzymatic process (Fig. 6c) differs significantly from the composition of the system before that process. The high oxygen content may indicate the adsorption of substrates and/or products of the enzymatic reaction of the racemic mixture of ibuprofen methyl ester. Additionally, the high sulfur content is related to the use of DMSO in selected process samples.

The wetting angle of the synthesized hydrogel and post-immobilization materials was also tested. The results gave the contact angle of all tested materials as 0°, which reflects the very high hydrophilicity of the hydrogel material. This property is directly related to the structure of the material. While the addition of ionic liquids has an impact on lipase activity, increasing the catalytic properties, the support itself retains the properties of the original material. It can be concluded that the amounts of deposited enzyme and ionic liquid may be too small to produce a significant change in the material's properties. Thus the ionic liquid has a direct impact on the enzyme, while not significantly altering the carrier material's surface.

3.2. Biocatalytic characterization

The resulting biocatalytic systems were analyzed to determine the

Table 3
Kinetic parameters of free enzymes and of enzymes immobilized without and with the addition of [BMIM]PF_6.

Sample	K_M (mM)	V_{max} (mM/mg·min)
Free enzyme	0.572 ± 0.029	0.927 ± 0.032
Without IL	0.665 ± 0.027	0.173 ± 0.008
[BMIM]PF_6	0.475 ± 0.014	1.519 ± 0.068

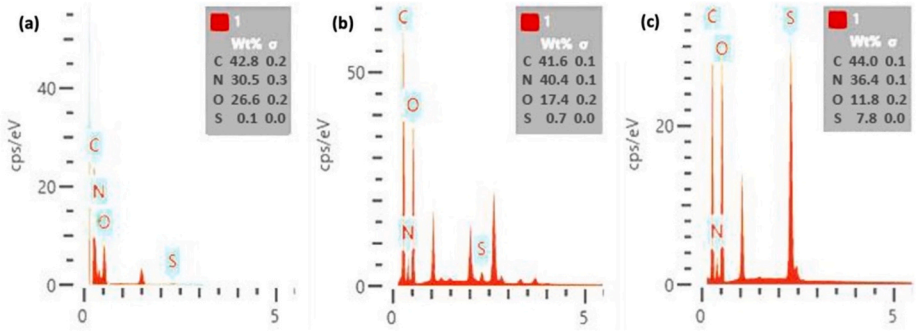

Fig. 6. Elemental composition of the surface of: (a) hydrogel, (b) the enzyme–hydrogel system, and (c) the enzyme–hydrogel system post-reaction.

effect of the use of ionic liquid, the immobilization efficiency, and the influence of process parameters on the enzyme's relative activity and stability.

After immobilization, a thorough characterization of the biocatalyst produced with the addition of [BMIM]PF_6 was performed to determine relative activity at different temperatures and pH, as well as its reusability and thermal stability. Fig. 7a shows that the most suitable temperature was 40 °C, where the enzyme displayed 100 % relative activity – this is linked to the properties of this strain of lipase, and similar results are obtained for its native form. Nevertheless, at lower temperatures, the free enzyme becomes inactive, and at higher temperatures it is more susceptible to denaturation, retaining only around 30 % of its activity at 60 °C (data not shown). As regards the pH parameter (Fig. 7b), the native form of the enzyme remains most active at pH 7. On the other hand, immobilized biocatalysts can offer increased stability and prolonged activity over a broad temperature and pH range. The hydrogel-based catalyst has higher relative activity at more basic pH values, which may be related to the slight change in the conformation of the enzyme's structure upon immobilization [30]. The hydrophobic IL provides a suitable environment for the lid opening mechanism in the lipase structure, leading to greater activity and easier access to the active center of the enzyme over a wide pH range. What is more, the addition of ionic liquids reduces the deteriorating effect of organic solvents on the enzyme structure, resulting in increased catalytic performance. Qiu et al. noticed a significant improvement in biocatalyst performance after applying amino-functionalized ionic liquid as a surface modifier. In their study, not only did the IL prevent magnetite particles from agglomerating in aqueous systems, but more importantly, it enhanced the affinity of the immobilized enzyme toward the substrate [31].

The thermal stability profiles obtained for the immobilized enzymes are in agreement with the results on the effect of temperature on enzyme activity (Fig. 7c). The native form of the enzyme becomes inactive at higher temperatures after incubation for 2 h (data not shown) due to thermal dissociation followed by denaturation. On the other hand, immobilized enzymes remain active, with relative activity mostly above 70 % and sometimes up to 90 %, due to the protective influence of the

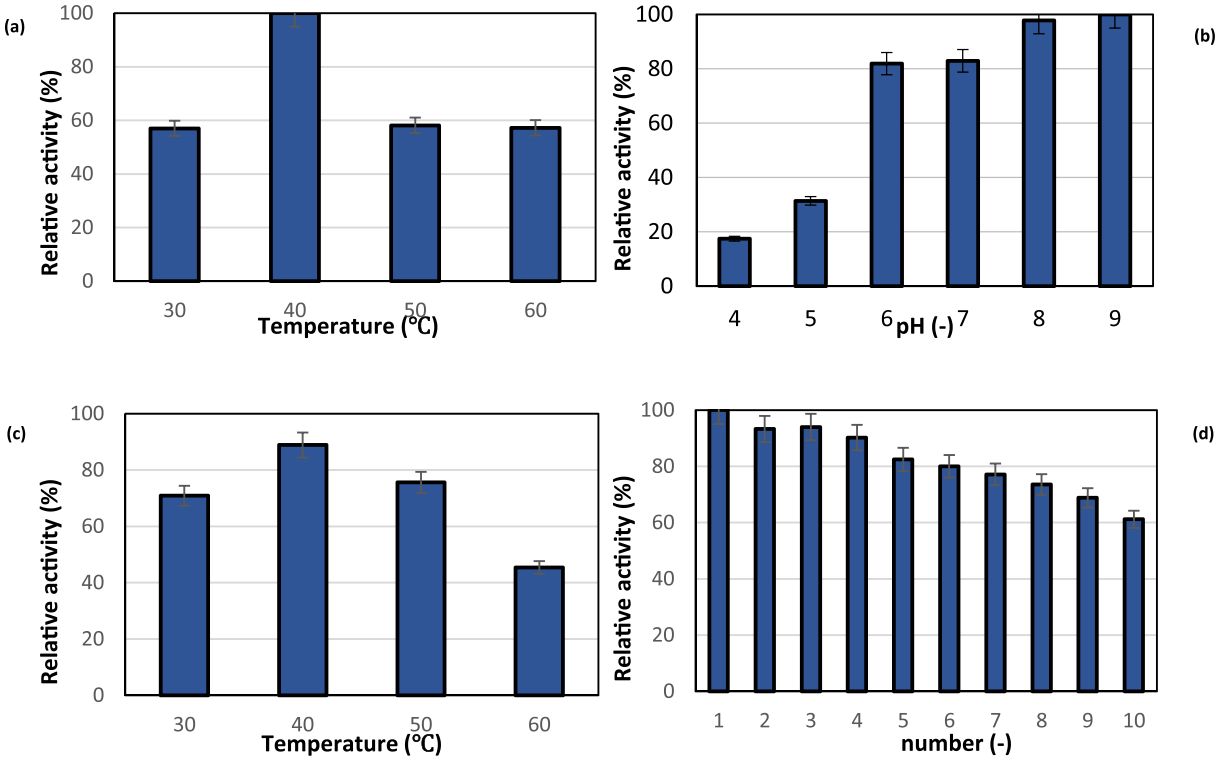

Fig. 7. Relative activity of the biocatalyst at varying (a) temperature and (b) pH; (c) thermal stability; and (d) reusability of lipase immobilized on hydrogel material with the addition of [BMIM]PF_6. For pH and temperature effects 100 % relative activity is defined as the highest activity obtained during the tests; for thermal stability 100 % activity is defined as the starting activity before incubation; for reusability, 100 % activity is defined as the activity of immobilized lipase in the first cycle. The error bars represent the mean value ± standard deviation from three measurements.

support material and IL. In the tests of reusability, the system with immobilized enzyme exhibited high durability, retaining over 60 % relative activity after the 10th cycle. The protective effect and the compatibility of the support and the ILs with the immobilized lipase results in the observed high stability of the biocatalytic system produced.

Interactions occurring between the material, the IL and the enzyme might limit the elution and reduce the inactivation of the enzyme, resulting in higher activity. Another advantage of the system produced is the possibility of its fast and easy removal from the reaction medium. Due to the solid one-piece structure of the hydrogel, it can be removed by separation using, for example, simple filtration or centrifugation. In another study, Quin et al. obtained comparable reusability for hydrogel biocatalytic systems, recording around 60 % and 70 % relative activity after the 10th cycle [32].

The Michaelis–Menten constant (K_M) and maximum reaction rate (V_{max}) of the free lipase and enzymes immobilized with and without ILs were determined and compared, as they reflect the substrate affinity and its changes upon immobilization (Table 3). When comparing the kinetic parameters of tested lipases, it is clear that the presence of ILs in the system strongly improves lipase–substate affinity, also resulting in the higher activity of this system when compared with free lipase or lipase immobilized without ILs. For the system with the addition of 5 wt% [BMIM]PF_6 during immobilization, K_m is lower than in the case of the native form of the enzyme, while V_{max} is higher, suggesting a greater affinity toward the substrate and improved reaction rate [33]. The improved affinity may be related to the increased hydrophobicity of the environment around the lipase's active center, increasing the catalytic activity [20]. The introduction of ionic liquid into the system may also increase the solubility of the substrates, as well as reducing the probability of a reduction in diffusion resistance or limitations in accessing active sites. On the other hand, the performance of the system after immobilization without any stabilizers indicates a significant drop in enzyme affinity toward the substrate compared with the native enzyme, which may be due to changes in the conformation of the enzyme structure and to diffusional limitations [34].

3.3. Resolution/hydrolysis of racemic ibuprofen ester in batch reactor

The enzymatic resolution of ibuprofen methyl ester racemate included enantioselective hydrolysis of that compound in the presence of lipase from *Candida rugosa*. The reaction products were (*S*)-ibuprofen and (*R*)-ibuprofen methyl ester. The resolution of the racemic ester mixture was conducted in two different modes in a batch reactor. To increase the racemization of the sample, which is followed by a higher conversion rate and a greater amount of (*S*)-ibuprofen as the final product, the dynamic racemic resolution was performed with the addition of DMSO to increase the pH value for higher racemization of undesired (*R*)-ibuprofen methyl ester (Fig. 8b). Classic kinetic resolution was performed at a lower pH, without the addition of DMSO (Fig. 8a). The concentration of each sample according to HPLC analysis is presented in the Supplementary Material (Table S2).

Reactions using immobilized enzymes were conducted using biocatalysts produced with the addition of 5 wt% ionic liquid ([BMIM]PF_6), but also without the addition of ILs, to investigate the IL's impact on the stabilization and activity of the enzyme in real reactions. For the samples with biocatalysts without added ionic liquid (data not shown), the conversion rates and enantiomeric excesses were significantly lower and did not exceed 35 % conversion (E) and 60 % enantiomeric excess (ee).

Table 4
Enantiomeric excess and conversion rate of the obtained (*S*)-ibuprofen methyl ester after enzymatic asymmetric hydrolysis of racemic ibuprofen methyl ester with the use of biocatalytic systems with the addition of [BMIM]PF_6.

Sample designation	Process duration (h)	Conversion rate (%)	Enantiomeric excess (%)
Isooctane/PBS pH 7	48	27 ± 0.5	89 ± 0.7
	96	35 ± 0.3	95 ± 1
	144	48 ± 0.1	96 ± 1
Isooctane/Buffer	48	37 ± 0.2	97 ± 1.1
$NaHCO_3$-NaOH pH 9.5	96	68 ± 0.2	98 ± 1.8
	144	83 ± 0.4	99 ± 0.05
Isooctane/Buffer	48	54 ± 1	98 ± 1
$NaHCO_3$-NaOH pH 9.5/	96	77 ± 0.4	98 ± 1.3
DMSO	144	95 ± 0.1	98 ± 1
Hexane/PBS pH 7	48	24 ± 2.1	88 ± 0.05
	96	26 ± 3	94 ± 2
	144	36 ± 0.1	94 ± 2.5
Hexane/Buffer $NaHCO_3$-	48	35 ± 0.7	95 ± 0.9
NaOH pH 9.5	96	43 ± 0.5	96 ± 0.4
	144	59 ± 2.1	97 ± 0.3
Hexane/Buffer $NaHCO_3$-	48	33 ± 1	96 ± 0.6
NaOH pH 9.5/DMSO	96	45 ± 0.9	97 ± 1
	144	71 ± 0.4	98 ± 1

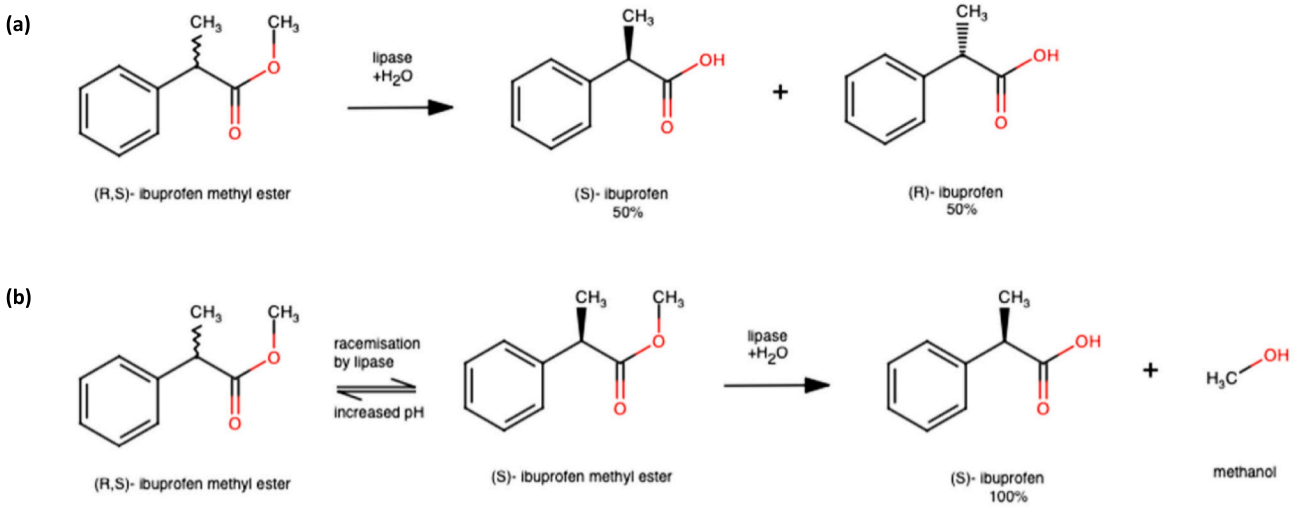

Fig. 8. Theoretical process of resolution of racemic ibuprofen methyl ester with the use of lipase as a catalyst: (a) kinetic resolution and (b) dynamic kinetic resolution.

Table 4 shows that the use of the kinetic separation process (process carried out in a phosphate buffer with pH 7) made it possible to obtain 48 % and 36 % conversion, respectively, for the reaction carried out in isooctane and hexane after 144 h. Since the maximum conversion rate of the kinetic separation process is 50 %, these values are satisfactory. Higher yields of the enzymatic process were achieved with dynamic kinetic enzymatic separation carried out in an alkaline environment. For processes carried out in the $NaHCO_3$-NaOH buffer without the addition of DMSO, maximum yields of 83 % (in isooctane) and 59 % (in hexane) were obtained after 144 h of the enzymatic conversion process. This proves that the addition of a base to enable racemization of the unreacted R-enantiomer can increase the degree of substrate conversion. The use of a buffer with pH 9.5 and the addition of a small amount of DMSO made it possible to obtain even higher conversion rates for the process carried out in isooctane, at a level of 95 %, and for the process with the addition of hexane, at a level of 71 %. This is due to the nature of DMSO, which when added to the reaction medium increases the alkalinity of the mixture, this being necessary for racemization. Suo et al. also investigated the influence of IL on lipase performance and proved the increased affinity between lipase and substrate immobilized on the prepared supports [35]. In their studies, the specific activity of the immobilized lipase system PPL-IL-MCMC was 1.43 and 2.81 times higher than that of free PPL and PPL-MCMC, respectively. It should also be added that in the study low DMSO concentration was applied, resulting in negligible effect of this solvent on lipase activity (see Table S3).

Analyzing the effect of the solvent, it should be noted that the reaction carried out in isooctane leads to a higher degree of conversion than when hexane is used as an organic solvent, and this is observed regardless of the other process conditions. Analysis of control samples after 48 h or 96 h and after the end of the process at 144 h confirms that the time of the process affects the degree of conversion of ibuprofen methyl ester. For all samples, the degree of conversion increased with time. However, the most significant increase, between 48 h and 96 h, was observed for the process carried out in isooctane and at pH 9.5, as the conversion rate increased from 37 % to 83 %. In turn, between 96 h and 144 h of the process, the highest increase in the degree of conversion was obtained for the reaction sample with the addition of hexane, a buffer at pH 9, and DMSO (an increase from 45 % to 71 %). These results indicate that time is an important parameter that should be considered when designing enzymatic processes.

Referring in turn to the enantiomeric excess of the obtained (*S*)-ibuprofen (desired product) over (*R*)-ibuprofen in the tested samples, it should be noted that the excess is high (in most cases >90 %) regardless of the process variables used (organic solvent, buffer pH, presence of DMSO, process time). This indicates the high stereospecificity of the enzymatic separation process catalyzed by lipase obtained from *Candida rugosa*, and confirms the validity of using enzymes in the separation processes of racemic mixtures of pharmaceutically active substances. It should also be highlighted that in all cases, the produced immobilized lipase supported by the ILs showed robustness and should be considered a promising alternative for future applications in the pharmaceutical industry.

4. Conclusions

In this study, the obtained PAM hydrogel support materials and the prepared biocatalyst were fully characterized. One of the main aims was to evaluate the effect of ILs on the lipase's catalytic performance and stability. The highest catalytic activity of the biocatalysts was obtained at a temperature of 40 °C and pH 7. What is more, the biocatalysts were catalytically effective and stable, and their activity remained above 60 % even after 10 reaction cycles. Biocatalysts produced with or without ILs were applied in the resolution of racemic methyl ibuprofen ester in two-phase systems using different organic solvents and two aqueous phases with different pH values. It was established that the highest conversion rate and enantiomeric excess were obtained for the system supported by IL, in the presence of isooctane, $NaHCO_3$-NaOH buffer at pH = 9.5 and DMSO, resulting in a conversion rate of 95 % and an enantiomeric excess of 98 %. The technologies proposed show good potential as a prospective solution, although in their present state they require further scientific testing and development. The results of the present research demonstrate significant applicability and can serve as a solid base for future studies. This study has confirmed that ionic liquids have a substantial impact on enzyme activity, leading to a desired improvement in lipase's catalytic performance.

CRediT authorship contribution statement

Oliwia Degórska: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Daria Szada:** Writing – original draft, Validation, Methodology. **Qiang Fu:** Writing – review & editing, Methodology, Funding acquisition. **Long Duc Nghiem:** Writing – review & editing. **Andrzej Biadasz:** Formal analysis, Data curation. **Teofil Jesionowski:** Writing – review & editing, Supervision, Resources. **Jakub Zdarta:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.136221.

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