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REVIEW

Recent Advances in Engineering of Media for Enzymatic Catalysis with Lipase

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Most lipases display good solubility and catalytic activity in non-aqueous media. In many types of non-aqueous media, such as ionic liquids, supercritical fluids, fluorous solvents *etc*., substrates and products are soluble and are therefore able to diffuse during reactions, which might often be beneficial to the lipase activity. Lipases often exhibit higher activity and stability in non-aqueous media than in aqueous environments. In addition, after reactions are carried out in novel media, a simple separation procedure might be included with the reaction process to facilitate improved purification of the products without using chromatographic separation or liquid-liquid extraction. This review focuses on novel medium for lipase catalysis, such as fluorous solvents, ionic liquids, supercritical fluids, *etc*. Some future perspectives and challenges facing in the media for enzymatic synthesis with lipase are also proposed.

Keywords: Medium engineering, Non-aqueous media, Ionic liquids, Supercritical fluids, Fluorous solvents, Promiscuity catalytic.

INTRODUCTION

Lipases, which are the most important biocatalysts, have become increasingly popular in recent years because they can catalyze novel biotransformations and green chemistry reactions, both of which may be operated in either aqueous or non-aqueous media[1-3]. Lipases are an extremely versatile class of enzymes and are already the most commonly used enzymes for industrial processes, such as food/dairy (cheese ripening, personal care products and flavor enhancers), pharmaceutical (naproxen and ibuprofen), biofuels, agrochemical (insecticide, pesticide) and oleochemical (fat and oil hydrolysis and biosurfactant synthesis) industries[4,5]. This versatility is that primarily due to lipases' ability to accept a wide variety of substrates, operate under mild reaction conditions, remain stable toward non-aqueous media and catalyze transformations with excellent chemo-, regio- and enantioselectivity.

Over the last decade, lipases have gained particular importance within organic synthesis, even surpassing other enzymes at times. They are used to catalyze a number of useful reactions, including esterification[2,6], ester hydrolysis[7], transesterification[8], regioselective acylation of glycols[9] and multiple alcohols[10], synthesis of peptides[11], amidation[12] and so forth. In most lipases, the hydrophobic catalytic centers are often covered by a lid. The lid structure opens in the presence of a hydrophobic/hydrophilic interface. Therefore, solvent properties play an important role in catalytic activity and facilitate the solubility of lipase. Importantly, catalytic promiscuity often occurs with lipase in organic media, initiating the discovery of new chemical reactions with the enzyme, such as the Knoevenagel condensation reported by our research group[13-16].

Additionally, the enhanced solubility of substrates and the absence of water eliminate the hydrolysis reactions that normally compete with esterification and transesterification processes in anhydrous media. Lipase biocatalysis in non-aqueous media, such as organic solvents, supercritical fluids, ionic liquids, reverse micelles, fluorous solvents and multiphase system, has been an active and important area of research in recent decades.

Enacting lipase catalysis in non-aqueous media is advantageous if the enzyme retains its activity. For example, organic substrates displaying good solubility enable the use of moderate concentrations in reactions that carried out in non-aqueous media. The concentration affects the reaction equilibrium, generating a shift toward product formation in the case above. Additionally, in non-aqueous systems, product separation might be more straightforward than in water, side product formation might be hindered and microbial contamination of reaction processes might be precluded. However, using different media to influence enzyme activity, stabilization and

selectivity, which depend on the hydrophobicity/hydrophilicity of the reaction medium, modulates the activity of lipases with different origins. Several novel non-aqueous neoteric solvents have been developed for use with biotransformations[17], such as ionic liquids, supercritical fluids, fluorous solvents and liquid polymers (Fig. 1). In this review, it is focused on the growing library of novel media for lipase catalysis, such as fluorous solvents, ionic liquids and supercritical fluids. Multiphase systems, such as the non-aqueous media used for lipase promiscuity catalysis, will be also introduced and discussed. Some future perspectives and challenges for the development of new media for enzymatic synthesis with lipase were also proposed.

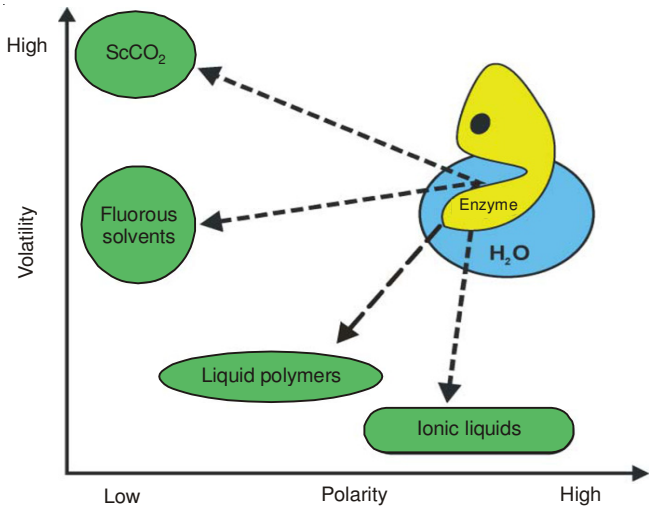

Fig. 1. Scheme of typical volatility and polarity characteristics of non-aqueous neoteric solvents suitable for enzyme catalysis[18]

Fluorous solvents: Fluorous solvents, which is a term coined by Horvath and Rabai[19], include perfluoro-substituted alkanes, dialkyl ethers and trialkylamines and are a class of non-polar, hydrophobic, chemically inert, slightly volatile, easily recyclable and nontoxic solvents; additionally, these solvents are more dense than the corresponding non-fluorinated solvents. Increased attention has been paid to this solvent family because of the novel properties listed above[20]. For example, fluorous solvents' miscibility with classical organic solvents, such as hexane, depends on the temperature, which is a property unique to fluorous solvents, such as perfluorohexane. This behaviour can be exploited for easy separation of the products and substrates of completed reactions and as the cornerstone for the development of multiphase synthetic processes. Because substrates and products may display different solubility profiles, biphasic fluorous/organic solvent mixtures (Fig. 2) might be heated to convert a heterogeneous reaction mixture into a homogeneous one, which would improve mass-transfer rates. After the completion of the reaction, the system can be returned to a heterogeneous mixture, facilitating product separation, by cooling to room temperature[21,22].

A highly fluorinated acyl donor has been applied to a lipase-mediated enzymatic kinetic resolution of a racemic alcohol to easily partition the fluorinated and non-fluorinated enantiomers between a fluorous and non-fluorous organic solvent, as reported by Hungerhoff *et al.*[23]. They confirmed

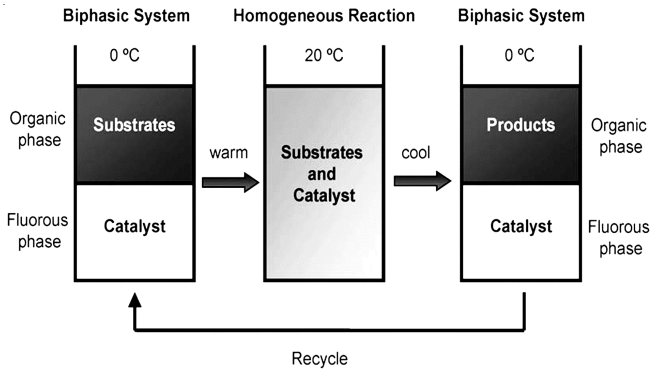

Fig. 2. Fluorous biphasic system[21]

that the enzyme in the fluorous biphasic system could be recovered easily by filtration for reuse without appreciable loss of activity. Beier and O'Hagan[24] have also described the lipase-catalyzed (trans)esterification of racemic 'organic' esters with 'fluorous' alcohols in homogenous perfluorocarbon-hydrocarbon solvent system. This biphasic system enabled the direct recovery, which was achieved *via* liquid-liquid separation, of the 'fluorous' ester products with ee up to 95 % from the fluorous phase. The reaction can also be carried out at gram scale, allowing potential applications to be explored in industry.

In addition to the biphasic system, fluorous triphasic system has also been developed and used by Luo and co-workers in a lipase-mediated enzymatic kinetic resolution of a fluorous racemic compound[25]. In their work, *Candida antarctica* B-derived lipase catalyzed the addition or removal of a fluorous tag from a racemic ester in a triphasic reaction system. The key technological advancement for this system is that the fluorous ester can only pass in one direction through the phases. This reaction provides an efficient method for separating a product with a high ee from a reaction mixture, which is achieved without chromatographic or extractive separation with organic solvents.

Moreover, to construct macromolecular frameworks for lipase in fluorous solvents, Maruyama *et al.*[26] utilized poly (ethylene glycol) (PEG) with lipase PL to form a complex during the lipase-catalyzed alcoholysis with vinyl cinnamate and benzyl alcohol. The results showed that the PEG-lipase PL complex exhibited reaction activity that was more than 16-fold higher than the unmodified lipase powder in a fluorous solvent. The higher activity is achieved for lipase complex-catalyzed systems in fluorous solvents because the substrates absorb to the PEG layer, placing the substrates in close proximity to the lipase molecules.

To enhance the solubility of lipase in fluorinated solvents, an anionic fluorinated surfactant, which was named Kryto Development Product (KDP) 4606, was used to form non-covalent complexes with lipase in a system reported by Shipovskov[22]. In this work, lipase collected from *Burkholderia cepacia* was used to catalyze the esterification of 1-phenyl ethanol and vinyl acetate in perfluoro(methylcy-clohexane) (PFMC). The lipase displayed good catalytic activity and stability toward storage. One of the advantages of using this enzymatic catalytic system is that the solubilized lipase can be separated and recovered three times by modulating the

temperature, which in turn affects the miscibility, of the fluorous (PFMC)/hexane biphasic system (FBS).

The temperature-dependent miscibility of fluorous media with other organic solvents can generate fluorous biphasic solvent systems that might be used as an alternative to aqueous biphasic solvent systems. Using a fluorous biphasic system makes both the efficient performance of enzymatic catalysts and convenient separation of products possible. However, some problems remain to be unresolved for lipase reactions performed in fluorous solvents. An optimal system that efficiently partitions the 'fluorous' esters into hexane without post-reaction washing has not been developed. Additionally, the fluorous solvents are also relatively cost-prohibitive, which is a major disadvantage for large-scale applications. As synthetic technological progresses, we will continuously observe the development of new methods for preparing inexpensive fluorous media.

Ionic liquids: Ionic liquids were discovered at the beginning of the 20th century, but their potential as reaction media and extraction solvents remained unexplored until recently. These compounds are liquid salts composed entirely of ions; their melting temperatures fall below 100 °C or even approach room temperature[27]. As a new generation of non-aqueous solvents, ionic liquids have many favorable properties compared to conventional solvents, such as a low vapor pressure, a wide liquid range, low flammability, good recyclability, high thermal conductivity, good solubility in both organic and inorganic materials, high thermal and chemical stability, *etc*.[28-30]. Because of these unique properties, ionic liquids have been widely recognized replacements for the volatile and hazardous organic solvents currently used in a wide range of fields, including biocatalysis and biotransformation[31]. In these non-aqueous reaction media, some enzymes retain their catalytic activity and stable conformation. Ionic liquids are non-miscible with water and most hydrophobic organic solvents, providing a non-aqueous polar alternative for two-phase systems. This attribute has been widely applied to the extraction of products from reaction mixtures. For the ionic liquids used in enzymatic catalysis, the functional groups of the ionic liquids could be altered to generate task-specific ionic liquids (TSILs) for each enzymatic reaction system[32]. The physicochemical and thermal properties of ionic liquids, such as their hydrophobicity, density, viscosity, melting point, polarity and solvent miscibility, can be tuned by choosing a suitable combination of cations and anions[33].

A systematic study concerning the effects of the concentration and composition (alkyl chain length and type of anion) of hydrophilic ionic liquids on the activity of *Candida antarctica* lipase B has been reported by Ventura's research group[34]. Increasing the ionic liquids alkyl chain length affects the lipase activity in a manner related to the hydrophobicity of the alkyl chain, which promotes the obstruction of the enzyme's non-polar active site by the ionic liquid. The strength of these interactions between the enzyme and the different anionic portion of the ionic liquids, which are dominated by dispersion forces and hydrogen bonding, are the major driving force behind any observed loss of activity. Cations with longer alkyl chains could also decrease the enzyme activity by obstructing the non-polar active site. Moreover, [C16tma][NTf2] ionic liquids[35], which has cations with long alkyl side chains, can also generate a switchable ionic liquid/solid phase that can be changed between liquid and solid phases based on the temperature. Because these hydrophobic ionic liquids have such unique properties, lipase catalysis for the production of flavor esters using these ionic liquids (Fig. 3) can be utilized in a two-step protocol; the tunable phase changes allow for the extraction processes to immediate follow the reaction, conveniently producing and separating the products. The phase during the enzymatic processes in [C16tma][NTf2] was a fully homogeneous liquid at 50 °C, with solid systems forming at room temperature. The product yield was improved up to 100 % at 50 °C and the enzyme/ionic liquid system can be easily recovered and reused with very little decrease in activity. Ternary systems containing an ionic liquid, *n*-hexane and an organic compound were used in the kinetic resolution of rac-2-pentanol; using an ionic liquid for the extraction of organic compounds from *n*-hexane medium has also been reported[36]. Using convenient and green methodologies for biotransformations and their subsequent clean separation procedures would open up new opportunities to develop green industrial processes.

In addition, the viscosity of ionic liquids affects the reaction rate by limiting mass transfer and the size of anionic portion of the ionic liquids majorly influences the enzyme in solubleness. Imidazolium-based ionic liquids ([Bmim][TfO]) with moderate physicochemical properties have been reported to accelerate mass transfer and dissolve large amounts of enzyme[37]. During the butyl acetate biosynthesis with *Candida antarctica* lipase B in [Bmim][TfO], the initial reaction and conversion rate were approximately 1.4 and 1.1 times higher, respectively, in [Bmim][TfO] than in a conventional solvent (*e.g*., *tert*-butanol). These improvements can be occurred because the cation $[Emim]^+$ is smaller than the cation of $[Bmim]^+$ and is therefore insufficient to offset the strong coordination of the $[TfO]^-$; ionic liquids containing strongly coordinating anion groups can change the secondary structure of enzyme by interrupting the intra-molecular hydrogen bonding, deactivating the enzyme. Although $[Hmim]^+$ and $[Omim]^+$ have larger cations that might efficiently counter the strong coordination of $[TfO]^-$, their high viscosity (124 cP and 190 cP, respectively) slows the mass-transfer rate in the reaction mixture. Therefore, $[Bmim]^+$ might be the most suitable cation because it can effectively counterbalance the strongly coordinating anion without overly limiting (due to the proper viscosity of 90 cP) the mass transfer.

Proton activity, which is the ability to transfer protons, has also been used as a parameter to select appropriate Pionic liquids for the solvation of biomolecules[38]. PILs are a subclass of the ionic liquid family formed by neutralizing a Bronsted base with a Bronsted acid. PILs have an additional tunable feature due to their proton activity. Compared to the analogous quaternary ionic liquids, PILs prepared directly from simple tertiary amines and carboxylic acids have many advantages, such as low cost, low ecotoxicity and good biodegradability. Because of the unique characteristics, PILs are widely accepted as green solvents for chemical and biocatalytic reactions. The hydrolytic performance and selectivity of a *Thermomyceslanuginosus lipase* (TLL) for the concentration of clinically

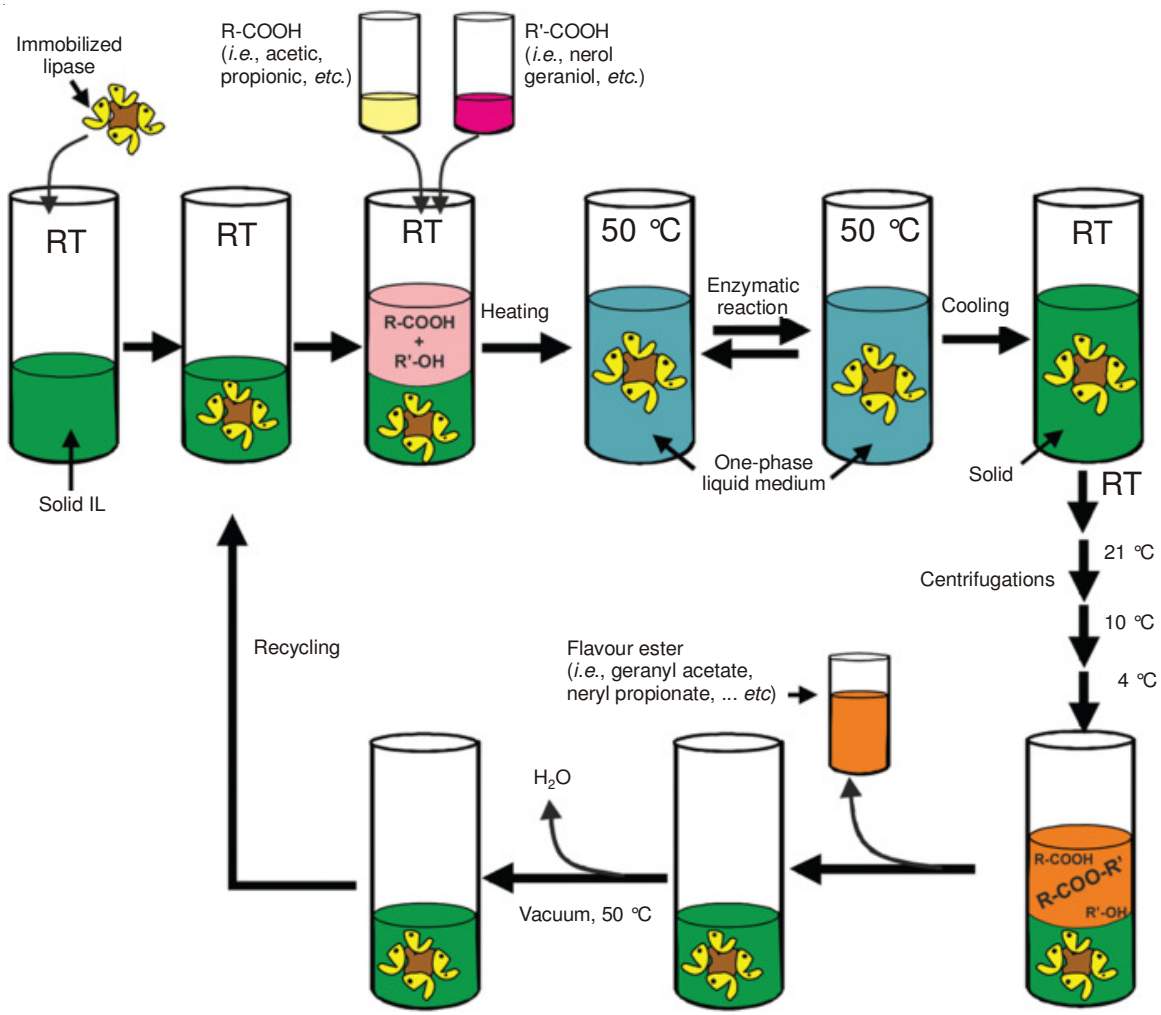

Fig. 3. Production of flavor esters bylipase-catalyze in switchable ionic liquid/ solid phases, and reusing the enzyme/ionic liquid system

important omega-3 fatty acids was increased when a protic ionic liquid (pIL), which was triethylammoniummesylate (TeaMs), was added[39]. TeaMs could influence both the secondary and tertiary structure of TLL, which led to the considerable catalytic activity and thermal stability of the enzyme. The hydrolytic activity of the lipase was enhanced from 64 to 80 % over a 36 h period and an increase in the thermal stability of TLL was also observed in the presence of TeaMs. The smooth kinetic resolution of 1-phenylethanol by *Candida antarctica* lipase B (CaLB) in PILs has been studied[40]. Good reaction rates and high enantioselectivities (92–>99 %) were observed during acylation of 1-phenylethanol with vinyl acetate at 40 °C.

Ionic liquids also possess many inconvenient characteristics, such as high cost, difficult purification steps and toxicity issues. Abbott and Davies[41] reported that imidazole-based ionic liquids with fluorinated anions suffer from high toxicity and commercial expense. Antibacterial activity, as well as cytotoxicity and toxicity towards multicellular organisms, has been determined across different studies[42]. The ecotoxicity of ionic liquids has also been studied with aquatic organisms (algae, crustacea, fish)[41] and terrestrial plants (soil toxicity)[43]. Therefore, careful designs for ionic liquid syntheses are required to reduce their toxicity and change their starting materials to use renewable resources[44]; these steps would makes this nonconventional medium attractive for performing large-scale enzymatic processes in future industrial applications.

Supercritical carbon dioxide ($scCO_2$): Supercritical fluids (SCFs) are a state of matter that exists at a pressure and temperature higher than the parent substance's critical point, but below the pressure required for condensation into a solid[17]. supercritical fluids have shared properties between liquids and gases because their densities are similar to liquids, but their viscosities are more gas-like. A particular advantage of using supercritical fluids during enzymatic catalysis is that these substances facilitate the easy separation of products or reactants from the reaction mixture. The unique nature of the supercritical fluids depends on both temperature and pressure. We can therefore fine-tune the reaction conditions and capitalize of the supercritical fluids exceptional abilities during extraction, reaction, fractionation and analytical processes[45,46]. Some parameters, such as the dielectric constant, partition coefficient or solubility, are pressure sensitive. Small changes in pressure and temperature near the critical point can alter the solubility of the substrates and produce high diffusivity and low viscosity in both substrates and products, which enables high rates of mass transfer in the reaction mixture. For enzymatic catalysis, the mass transport rates of the reactants to the active site of

the enzymes might be elevated because the supercritical fluids exhibit gas-like diffusivities and low viscosities[47].

As a green medium, supercritical carbon dioxide ($scCO_2$) is one of most suitable solvents for lipase catalysis used to produce flavors esters in the food industry. The lipase-biocatalyzed (Novozym 435) esterification of propionic acid with isopropyl alcohol, isobutyl alcohol and isoamyl alcohol in supercritical carbon dioxide[48] has been reported. The initial reaction rate decreases when the chain length of the primary alcohol increases. Additionally, the enzymatic esterification of propionic acid was faster with primary alcohols than with secondary alcohols. Furthermore, lactate esters were synthesized by esterifying *n*-butanol with lactic acid (LA) in a process catalyzed by immobilized lipase B from *Candida antarctica* (Novozyme 435) in supercritical carbon dioxide ($scCO_2$) with or without co-solvent[49]. The highest conversion (99 %) and corresponding maximum yield (11.2 gBL/(gE h)) were achieved after 26 h at 55 °C and 40 MPa in a $scCO_2$/*n*-hexane reaction medium. However, the higher acidity of the enzyme may be due to the pH change caused by the use of supercritical carbon dioxide and accumulation of lactic acid around the enzyme molecules. A potential cocoa butter analogue derived from camel hump fat has also been successfully prepared by enzymatic esterification using an immobilized lipase in supercritical carbon dioxide[50].

Because the separation of products or reactants from the reaction mixture is facile in supercritical carbon dioxide, this medium was usually used in combination with ionic liquids[51,52] and fluorous solvents[20,23] to form multiphase media. Heterogeneous catalysis in multiphase media possesses many advantages over homogeneous catalysis, such as catalyst recyclability and facile product separation. Heterogeneous catalysis offers also promising opportunities for the development of cleaner and more sustainable chemical processes (*e.g.*, the catalyst operates in one phase and the product is extracted in the second phase) without the use of volatile organic compounds in all catalytic systems. ionic liquids with low-toxicity and low vapor pressures are very useful tools that may take place of volatile organic compounds (VOCs) during extractions of liquid-liquid biphasic systems. The multiphase systems based on ionic liquids and supercritical carbon dioxide were first used as reaction media for lipase biocatalysis in 2002, which was the first operational approach during the development of truly green chemical processes in non-aqueous environments[51,52]. Using this approach, the mass transfer rate from the supercritical carbon dioxide phase to the ionic liquid phase containing the biocatalyst was enhanced with respect to the substrate and the extraction of the product(s) from the ionic liquid was simplified. Moreover, the product(s) obtained after the decompression of the supercritical fluid are uncontaminated by ionic liquid or any other organic solvent residues, while the CO_2 can still be recycled *via* re-compression. Bermejo's research group[53] has reported the effects of the enzyme concentration on the behaviour of supercritical carbon dioxide during the development of a lipase-promoted homogeneous enzymatic reaction in ionic liquid-CO_2 integrated media and the separation of the product (Fig. 4). The use of *Candida antarctica* lipase B (CaL B) as a catalyst for the hydrolysis of a triacet-based model reaction in a ionic liquid, which was 1-hydroxy-1-propyl-3-methyl imidazolium nitrate ($HOPMImNO_3$)-supercritical carbon dioxide. A large amount of enzyme was soluble in $HOPMImNO_3$ and remained active and stable without any observed change in the enzyme structure.

However, the use of supercritical carbon dioxide as a medium for lipase catalysis requires special equipment, making the process difficult and expensive for the preparation of low-value chemicals or products, such as biodiesel[54]. Currently, much of the basic research is focused on the activity of enzymes in supercritical fluids, especially super critical carbon dioxide. When attempting biocatalysis in a supercritical carbon dioxide medium, the major hurdles include the mitigation of the effect of carbamate formation on the enzymatic process and the stabilization of the pH fluctuations caused by carbonic acid formation that are observed during the reactions.

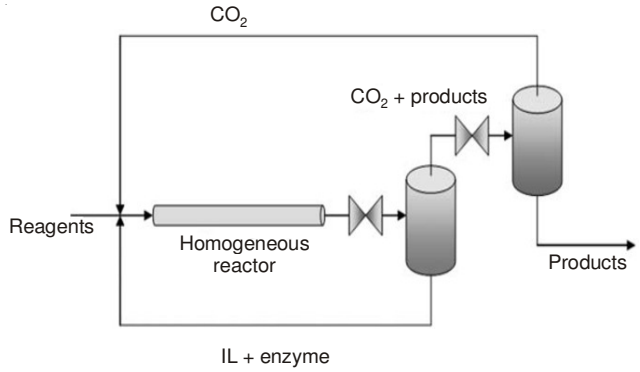

Fig. 4. Integrated reaction-separation process in a homogeneous IL-CO_2 medium

Conclusion

Because the preparation of novel media suitable for lipase catalysis has become more general and cost-effective, a number of interesting applications of lipase catalysis utilizing non-aqueous environments have become commercially attractive. These novel substances acted as reaction media in lipase catalysis, in addition to acting as extraction solvents for the facile separation of products from reaction mixtures. These behaviours facilitate the development of sustainable industrial bioprocesses.

However, certain disadvantages, such as unknown toxicological profiles and environmental persistence for long-chain perfluoroalkyl compounds, hinder the development and application of these novel substances in lipase biocatalysis. Novel ionic liquids, which are biodegradable and have little to no toxicity, are some of the most important substances used for the development of various green reactions and large-scale applications in chemical manufacturing. supercritical fluids such as supercritical CO_2, which have physical properties between the parameters of liquids and gases, provide an environment in which the products or reactants from a reaction mixture may be separated in the present of enzyme, which is undoubtedly beneficial for green synthetic processes. Currently, the use of supercritical carbon dioxide is comparatively difficult and expensive when industrially applied because it requires special equipment that is complicated to operate.

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