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An integrated process for enzymatic catalysis allowing product recovery and enzyme reuse by applying thermoreversible aqueous biphasic systems

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Abstract

Thermoreversible aqueous biphasic systems (ABS) composed of ammonium-based zwitterions (ZIs) and polymers are here disclosed to act as integrated bioreaction-separation processes. The biocatalytic reaction involving laccase occurs in homogeneous media, after which small changes in temperature induce the formation of two phases and the complete separation of the enzyme from the products in a single-step. These systems also allow the recover and reuse of the enzyme, along with the ZI-rich phase, contributing towards the development of sustainable biocatalytic processes.

In the past years, there has been an increased interest on the development of sustainable and efficient production processes, in which biocatalysis plays a pivotal role.1 Enzymes display a high activity, specificity and selectivity, minimizing the consumption of raw materials and the production of by-products when compared to their synthetic counterparts, thus contributing towards the development of sustainable catalytic processes.1, 2 Enzymes and biocatalysis have been used in several sectors, such as in soil bioremediation, nanobiotechnology, and biosensors, and by the textile, pulp and paper, cosmetic, food and pharmaceutical industries.3 Significant developments have been reported in the pharmaceutical industry with a decrease of the respective E-factor.4 Despite their advantages, the major challenges associated to enzyme-catalysed bioprocesses rely on the maintenance of the enzymes structural stability and activity, and on their reusability.5 Efforts have been made to overcome these issues, whereas one of the main strategies is the enzyme immobilization where the biocatalyst is adsorbed on, bound to, or encapsulated in a solid matrix.6, 7 Albeit this approach allows the reuse of the biocatalyst, immobilization may present some disadvantages, such as conformational changes of the enzymes, loss of activity, and their heterogeneity on the support.6, 8 Therefore, new approaches to recover

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biocatalysts from the reaction medium have been proposed, including for instance the use of membranes, centrifugation, liquid-liquid extraction,9 and immobilization of chemically modified enzymes.7, 10

Liquid-liquid systems appear as an interesting alternative in biocatalysis since they could allow the integration of the reaction and separation steps. Although liquid-liquid systems have been widely employed in the chemical industry for separation purposes due to their simplicity, low cost, and easy scale-up11, the commonly employed volatile organic solvents can be harmful to biologically active biomolecules. However, a milder environment to biomolecules can be afforded by water-rich systems, such as aqueous biphasic systems (ABS).12 ABS are composed of two immiscible aqueous-rich phases and can be formed by the mixture of two water soluble phase-forming components, such as two polymers, a polymer and a salt, or two salts, dissolved in aqueous media.13 Polymer-based ABS have shown to be biocompatible to deal with the separation of proteins, enzymes, viruses, cells, cell organelles, and other biological materials.14 Nevertheless, these systems are limited by a narrow polarity range preventing high extraction efficiencies and selectivities. This drawback may be overcome by ABS composed of ionic liquids (ILs).12, 15 Compared to the more conventional polymer-based systems, IL-based ABS display a lower viscosity, a faster phase separation, and higher extraction performance and selectivity.12

Previous studies on IL-based ABS have focused the determination of the respective phase diagrams and evaluation of their extraction efficiency for a wide range of compounds.12 Recently, there has been a significant interest on the development of dynamic and reversible IL-based ABS induced by the change of pH,16 temperature17 or by the addition of gases17, 18, that could ultimately lead to the development of integrated reaction-separation processes. However, only few works investigated the thermoreversible behaviour of IL-based ABS in separation processes. They have been investigated for the extraction of proteins (by temperature change),19 and in the production and separation of 5-hydroxymethylfurfural (HMF) from fructose (by pH change).20

In addition to the widely studied IL-based systems, novel ABS composed of water soluble ammonium-based zwitterions (ZIs) and inorganic salts were recently proposed,21 and their application in the selective separation of amino acids demonstrated. ZIs were suggested as substitutes of ILs, where the cation and the anion are covalently tethered, thus avoiding possible ions exchange between the ABS coexisting phases.22 However, the salts employed may lead to biocompatibility concerns when dealing with solutes sensitive to ionic strength, like proteins and enzymes.23 To minimize these issues, salts are here proposed to be replaced by polymers, such as polyethylene glycol (PEG), generally recognized as safe (GRAS) and used as additives in food products and drugs.10, 24, 25 Furthermore, ZI-based reversible ABS have not previously been considered as potential integrated reaction-separation platforms.

Based on the need of finding integrated reaction-separation processes for biocatalysis, in this work we investigated and designed novel ZI-polymer-based ABS with a thermal switchable behaviour, at temperatures amenable to deal with proteins. These systems were tested as integrated reaction-separation platforms, where a homogeneous catalytic reaction followed

by the enzyme separation from the product, by a change in temperature, is performed in one-step. To this end, laccase (EC 1.10.3.2) was used as the biocatalyst and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the subtract. Laccase was chosen since it has been attracting a large attention in the past years due to its ability to degrade a wide variety of substrates, being currently considered a promising biocatalyst in bio-based industries.6

Three water-soluble ammonium-based ZIs – *N,N,N*-tripentyl-3-sulfonyl-1-propaneammonium (N_{555}C3S), *N,N,N*-tripropyl-3-sulfonyl-1-propaneammonium N_{333}C3S), and *N,N,N*-trimethyl-3-sulfonyl-1-propaneammonium (N_{111}C3S), with different alkyl chain lengths, were synthesized and used in the preparation of ABS. Their molecular structures are depicted in Figure 1. Their synthesis was previously described13, while details on their purity are given in the ESI.

The ability of each ZI to form an ABS when mixed with PEGs of different molecular weight (1540, 2000, 4000 and 6000 $g \cdot mol^{-1}$) was firstly evaluated, and the respective ternary phase diagrams were determined at 25 °C. Further details on the experimental procedure, as well as on the detailed experimental weight fraction data are given in the ESI (Tables S1 to S5). Both N_{555}C3S and N_{111}C3S ZIs are able to form ABS at 25 °C with all polymers studied; however, N_{333}C3S, the ZI of intermediate alkyl chain length, does not undergo phase separation with any of the investigated polymers. A summary of the ZIs ability to form ZI-PEG-based ABS is provided in the ESI (Table S6). To the best of our knowledge, this irregular trend on the ABS formation with the increase of the alkyl side chain length of one of the phase-forming components was never observed, and is certainly a result of a change in the balance of competing interactions between the compounds present in the investigated ternary systems due to the increase of the ZI alkyl chains.26, 27

By plotting all the phase diagrams at 25 °C (*cf.* ESI, Figure S1), it is possible to appraise the influence of the length of the aliphatic moiety of the ZI, as well as of the molecular weight of the PEG on the solubility curves. The solubility curves indicate the minimum composition required to form an ABS, where mixtures with compositions above the curve are biphasic while those below are monophasic. The larger the biphasic region, the higher the ability of the ZI to induce liquid-liquid phase separation in polymer aqueous solutions. ZIs with shorter alkyl side chains are more prone to form ABS with polymers, while the ability of PEG to induce phase separation increases with its molecular weight, in agreement with trends previously observed for ABS composed of polymer/salt28 or polymer/IL.29 This is a consequence of the increasing hydrophobicity of PEGs with higher molecular weight.29

After the previous assessment on the ZI-PEG potential to form ABS at a common temperature (25 °C), these were then appraised at 35 and 45 °C, taking into account the operational temperature range of most enzymes and to avoid their activity loss. The optimum operation temperature of laccase is around 40 °C,30 although this temperature can vary with the pH and the laccase source, between 30 and 50 °C.31 Figure 2 depicts the phase diagrams obtained for ZI-PEG 6000-based ABS at several temperatures. The detailed experimental procedure used and the experimental data obtained for PEG 6000 and remaining polymers at different temperatures are provided in the ESI (Figure S2). It should be noted that N_{555}C3S does not form ABS with PEG 6000 at 45 °C. As previously observed

for ZI-salt-based ABS,21 ZI-polymer-based ABS also present two distinct behaviours with temperature, which depend on the ZIs alkyl chains length: (i) an increase of the immiscibility region with an increase in temperature for $N_{111}C3S$-based ABS; and (ii) a decrease of the biphasic region with a decrease in temperature for the $N_{555}C3S$-based systems. ABS composed of ZIs with smaller alkyl chains ($N_{111}C3S$) display a lower critical solution temperature (LCST)-type behaviour when mixed with polymers, following the same behaviour as polymer-salt ABS.32 Their formation seems thus to be dominated by a salting-out phenomenon, where the ZI acts as the salting-out species.29 On the other hand, the ABS formed with the most hydrophobic ZI investigated - $N_{555}C3S$ - present an upper critical solution temperature (USCT)-type behaviour, similar to those of polymer-polymer ABS.33 At higher temperatures, the interactions between the polymer and ZI are more favourable, enhancing thus their mutual solubility and reducing their ability to form ABS.34

The temperature-reversible behaviour of the studied systems is also depicted in Figure 2. To confirm this behaviour, a ternary mixture at the monophasic region was prepared at 25 °C for the system composed of $N_{111}C3S$, and at 45 °C for the system composed of $N_{555}C3S$. Then, the temperatures of these systems were changed to 45 °C and 25 °C for the systems composed of $N_{111}C3S$ and $N_{555}C3S$, respectively, to induce the formation of a biphasic system. Details on these mixtures compositions are described below, corresponding to the same mixtures at which biocatalysis was carried out. When the phase's separation occurs, a top polymer-rich phase and a bottom ZI-rich phase are formed. This proved thermoreversible behaviour was then applied to develop integrated reaction-separation biocatalytic processes. Herein, the homogeneous biocatalytic oxidation of ABTS by laccase was carried out in the monophasic region, followed by the separation of laccase from the oxidation product by promoting the phases' separation by temperature changes (increase or decrease according to the ZI employed in the ABS formation). The oxidation of ABTS occurred through the addition of laccase and ABTS to a monophasic mixture composed of ZI, PEG 6000 and water. The mixture was carefully mixed during 1 min, at 40 °C for the ZI $N_{111}C3S$ (20 wt% ZI + 20 wt% PEG 6000), and at 25 °C for the ZI $N_{555}C3S$ (39 wt% ZI + 13 wt% PEG 6000) – experimental details are given in the ESI.

For the two studied thermoreversible ABS, it was observed that laccase successfully catalysed the oxidation of ABTS (colourless) to a highly stable green-coloured $ABTS^{+}$ radical35 (Figure 3A). ABTS is a standard subtract used to appraise the laccase activity, for which the yield of reaction is considered as 100%. After that, a temperature change (from 25 to 40 ºC or *vice-versa*) was applied, inducing the separation of the system into two phases, while allowing the selective separation of the oxidized subtract and laccase, as shown in Figure 3. Further experimental details are given in the ESI.

Figure 3B shows the extraction efficiencies (*EE*%) of the studied systems for both laccase and oxidized ABTS, which correspond to the percentage recovery of each compound to a given phase. Remarkably, laccase and the reaction product partition to opposite phases, with extraction efficiencies higher than 81% achieved in a single-step. For the two studied ABS, laccase preferentially partitions to the ZI-rich phase and the reaction product to the PEG-rich phase, allowing thus the selective separation of the enzyme and product. A remarkable extraction efficiency of 100% was however obtained with the $N_{111}C3S$-based ABS, *i.e.*

laccase and the oxidized ABTS are completely separated for opposite phases with no losses or cross-contamination. It should be remarked that complete separations could also be achieved with other ZI-based ABS by a proper tailoring of the phases' hydrophobicity, either by changing the PEG molecular weight, ZI employed, or mixture composition.

In addition to the complete separation of the enzyme and the reaction product, it was further confirmed that laccase maintains its biocatalytic activity after the separation step (experimental details and results are given in the ESI). This maintenance in activity is of crucial relevance when envisaging the development of processes in which the enzyme can be recovered and reused. To prove this concept, the phase enriched in laccase (ZI-rich) was recovered and reused for the creation of a novel ternary system, as a new biocatalytic reaction medium. A summary of the enzyme recovery/reuse process is depicted in Figure 4A; experimental details are given in the ESI. Remarkably, it is possible to recover laccase and reuse it without losses in the catalytic activity, for at least 5 consecutive cycles (Figure 4B). This approach not only allows the recovery and reuse of the enzyme, but also allows the recovery and reuse of the ZI-rich phase required to form ABS, contributing towards the development of sustainable biocatalytic processes.

Two works using two-phase systems with laccase as a biocatalyst were previously reported.[36, 37] In the first work, it was shown the occurrence of enzymatic reactions (using laccase from *Polyporus versicolor*) in a two-phase system, yet composed of one organic solvent, such as hexane, toluene, tetrahydrofuran, acetone, among others, comprising the substrate and products, and an aqueous phase which contains the enzyme.[36] However, the enzymatic reaction occurs in heterogeneous media, *i.e.* at the interface, being thus essentially different from the approach here proposed. In addition to the use of hazardous organic solvents, the authors[36] were not able to characterize the compounds obtained in aqueous solution due to difficulties in obtaining them in sufficient amounts. Nicotra *et al.*[37] isolated and characterized four C-C and C-O dimers from the steroid hormone 17β-estradiol, formed by a catalytic reaction using laccase from *Trametes pubescens* in a biphasic system, formed by ethyl acetate and an aqueous buffered salt solution. Also in this approach, biocatalysis occurred at the interface. These heterogeneous reaction media usually require vigorous shaking and stirring to ensure the transfer of substrates and products between the phases, which can cause enzyme inactivation.[38] This may be responsible for the low yield obtained, ca. 27 %.[37] Elis *et al.*[9] reviewed others works on bioconversion in conventional (polymer-salt and polymer-polymer) ABS, using different enzymes or living cells, but in all these cases ABS were used as heterogeneous medium, *i.e.* with systems involving two-phases with the reaction occurring at the interface. Moreover, in these works, it was not possible to adequately separate the enzyme and the product for opposite phases. To overcome this problem, the use of ultrafiltration units has been proposed.[9] The thermoreversible systems here suggested thus overcome these limitations, allowing a homogeneous biocatalytic reaction, with all the advantages in terms of kinetics and yield, followed by a complete separation of the products and enzyme by a small temperature change.

In summary, temperature-driven reversible ABS composed of ZIs and polymers are efficient and sustainable integrated reaction-separation platforms in biocatalytic processes. Reaction and separation steps can be carried out sequentially by taking advantage of their temperature

switchable behaviour. The reaction step occurs at a homogeneous medium, followed by the enzyme and products separation in liquid-liquid systems promoted by small changes in temperature. This approach avoids the need of vigorous stirring to improve mass transfer, as typically carried out in heterogeneous reactions, thus contributing to the maintenance of the enzyme activity. Furthermore, the thermoreversible nature of ZI-PEG-ABS occurs at temperatures amenable for keeping the enzymes stability and activity (25-45 °C). These systems further allow the complete separation of the enzyme and the products in one-step, which are enriched in opposite phases, followed by the recovery and reuse of both the ZI-rich phase and enzyme. In addition to the example here discussed, reversible ZI-based ABS can be tailored to fit the requirements of other biocatalytic processes comprising value-added products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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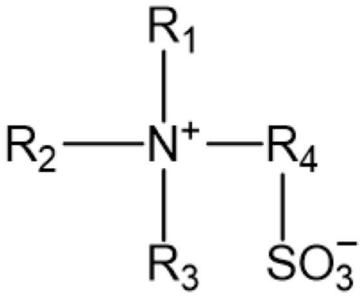
A



Acronym	R_1, R_2, R_3	R_4
$N_{111}C3S$	CH_3	$(CH_2)_3$
$N_{333}C3S$	$(CH_2)_3CH_3$	$(CH_2)_3$
$N_{555}C3S$	$(CH_2)_4CH_3$	$(CH_2)_3$

B

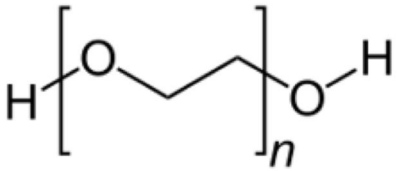


Figure 1.

(**A**) Chemical structures and acronyms of the ZIs used. (**B**) Chemical structure of PEG with the molecular formula H-(O-CH2-CH2)n-OH.

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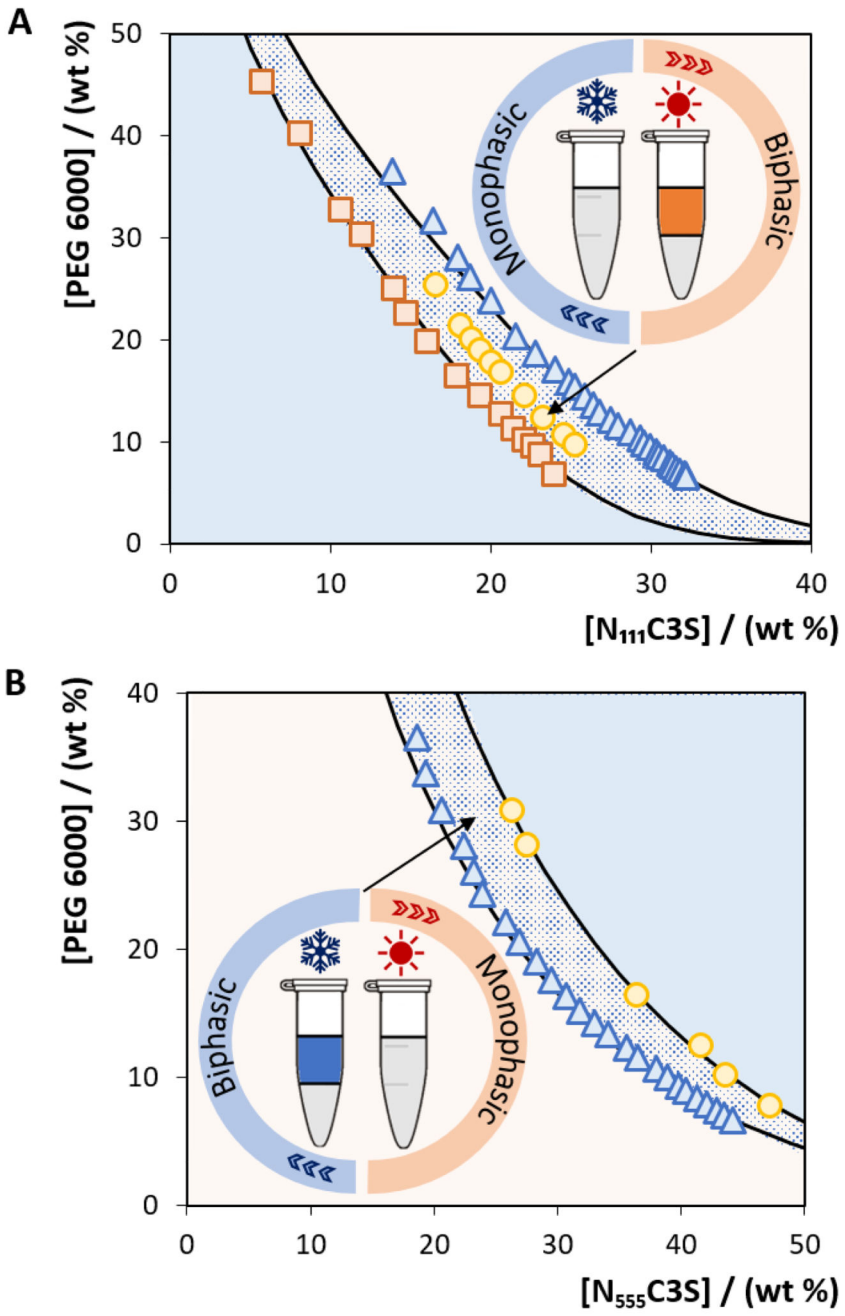

Figure 2.

Temperature effect in the phase diagrams of ternary systems composed of ZI + PEG 6000 + H_2O at 25 °C (▲), 35 °C (●) and 45 °C (■). (**A**) N_{111}C3S- and (**B**) N_{555}C3S-based ABS. There is no formation of ABS comprising PEG 6000 and N_{555}C3S at 45°C.

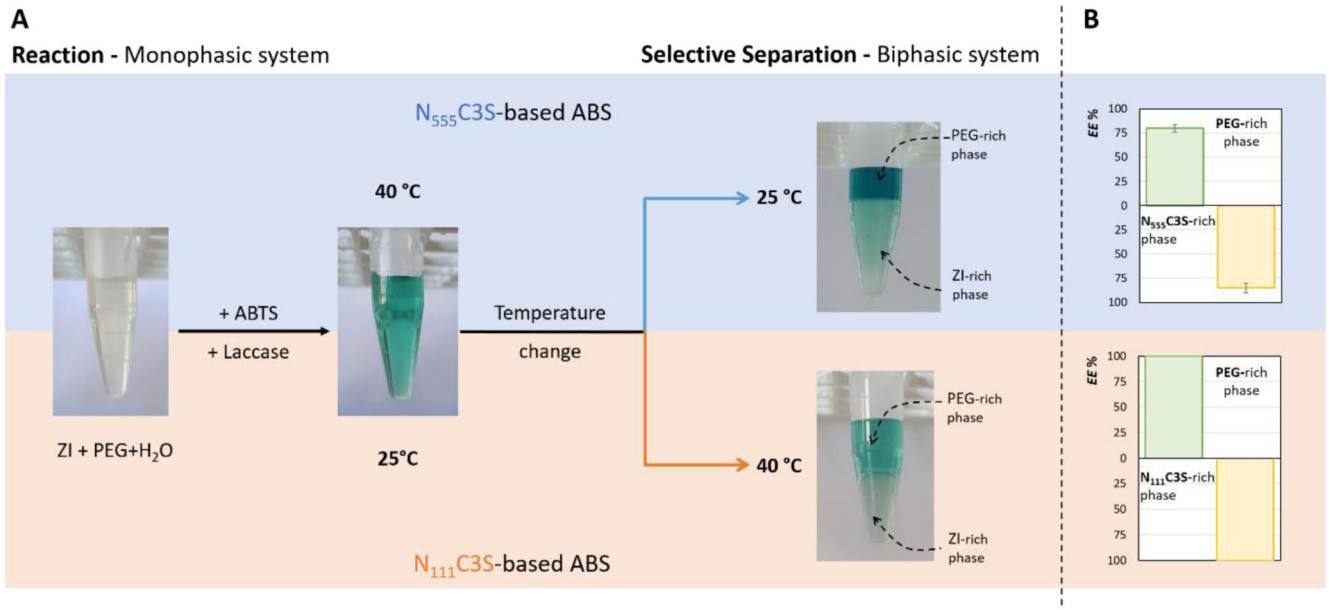

Figure 3.

(**A**) Oxidation of ABTS using laccase and their selective separation from the reaction product by changes in temperature. (**B**) Extraction efficiencies of laccase (*EE*%, orange bars) and extraction efficiencies of the green-colored $ABTS^{+}$ radical (*EE*%, green bars) in ABS formed by PEG 6000 and $N_{555}C3S$ or $N_{111}C3S$.

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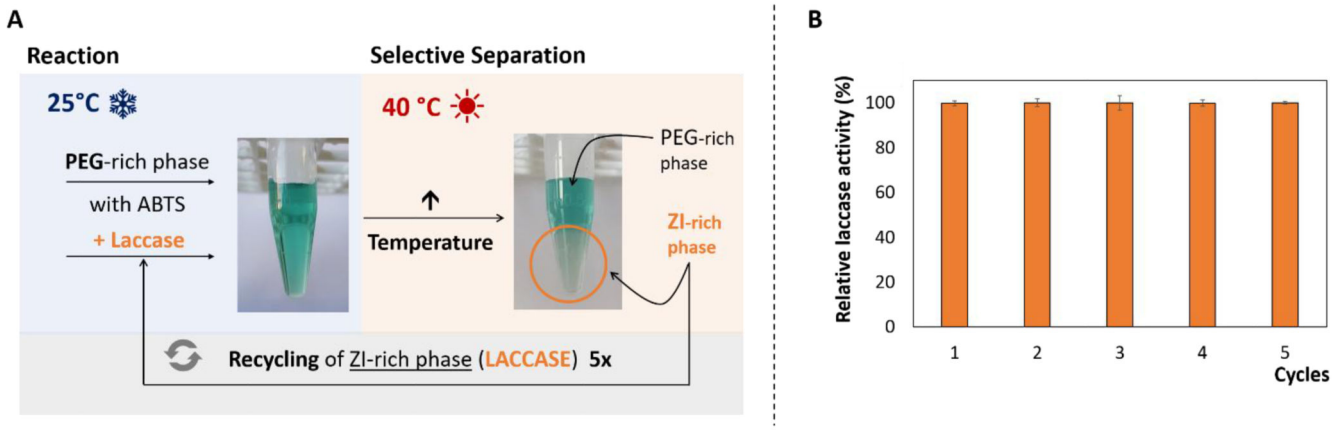

Figure 4.

(**A**) Flowchart of the integrated reaction-separation process developed, including the enzyme and ZI-rich phase recyclability. (**B**) Relative laccase activity in the ZI-rich phase in 5 cycles of oxidative reaction, comprising both the recovery and reuse of the enzyme and ZI-rich phase.

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