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Microparticle-Based Strategy for Controlled Release of Substrate for the Biocatalytic Preparation of L-Homophenylalanine

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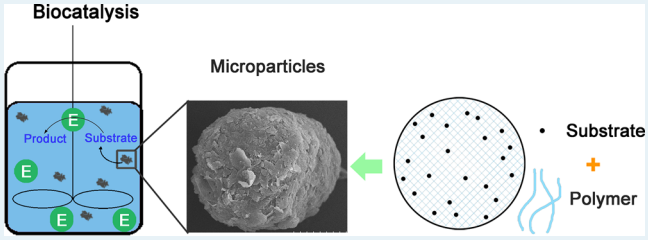
Supporting Information

ABSTRACT: Substrate inhibition is a universal challenge in biocatalytic process development. Herein, a controlled release of substrate from the microparticles was introduced and demonstrated to tackle this issue to increase the biocatalytic efficiency. Using phenylalanine dehydrogenase catalyzed production of L-homophenylalanine as a model reaction, and substrate-loaded microparticles were prepared and used as a reservoir to load a high amount of substrate and to control the release rate into the reaction media. Consequently, highly efficient biocatalysis could be sustainably achieved in the complex reaction system through constantly lowering the substrate concentration.



KEYWORDS: *biocatalysis, controlled release, microparticle, substrate inhibition, L-homophenylalanine*

Biocatalysis has been an important route in the production of enantiomerically pure compounds because of its inherent enantioselectivity, mild conditions, and potential to reduce total synthetic steps;[1,2] however, biocatalysts are usually inhibited to reduce activity or even inactivated by high substrate concentration.[3,4] Because substrate concentration is commonly a decisive factor to achieve high volumetric productivity and the scalability of an efficient process, substrate inhibition has become a universal issue for nearly all biocatalytic processes and their industrial usefulness.

To address this important issue, a variety of approaches have been explored, such as fed-batch addition of substrate,[5,6] development of aqueous−organic biphasic systems,[7] utilization of resins in the reaction system,[8,9] and use of ionic liquids as reaction media[10,11] and neat organic solvent.[12,13] Most of these approaches are designed to control substrate concentration in the reaction below the critical inhibition level, which could thus possibly diminish or reduce substrate inhibition and enable sustainable transformation.[14] However, these approaches have certain limitations to efficiently increasing the volumetric productivity. For example, batch-fed addition of substrate, the most commonly used manner, usually causes an unavoidable increase in the reaction volume and is less effective when substrate inhibition is very serious.[7,14] Organic solvents are associated with the issues on safety and volatility that are unsuitable for environmentally friendly processes.[14] In addition, the inclusion of resins in solid−liquid bioconversion usually requires extensive screening and testing, which may also hamper the stability of biocatalysts.[14] Thus, it is necessary to exploit a novel, green, and effective strategy for the substrate supply to robustly increase the efficiency of biocatalysis.

The concept of controlled release has been widely used in the pharmaceutical settings. For example, controlled release of drugs can sustainably give stable plasma drug concentration to increase efficacy, reduce toxicity, and improve patient compliance.[15,16] The controlled release systems are typically mixtures of a polymer and a drug so that the release behavior of the drug could be controlled or predesigned on the basis of diffusion, dissolution, osmosis, erosion, and other mechanisms.[17,18] Recently, micro- or nanoparticle-based drug delivery systems have attracted much attention owning to their small size, accurate control, and potential for easy preparation and design.[19] Therefore, we proposed that constantly controlling the released substrate concentration at a relatively low level by micro- or nanoparticle delivery systems could effectively avoid substrate inhibition and consequently increase the volumetric productivity for biocatalytic processes. Moreover, for the micro- or nanoparticles encapsulating organic compounds, particularly those with a predominant release mechanism of diffusion, the substrate release is typically driven or hindered by the conversion rate of the enzymes, serving as a demand-dependent delivery system.[14] This provides benefits to avoid substrate inhibition for complex bioconversions involving multiple enzymes and substrates. It is very difficult to decide adequate substrate concentration and the supply speed because of complicated and unpredictable kinetic behaviors during the reaction. In another aspect, the solid−liquid biphasic system could also prevent an undesirable increase in the volume from batch-fed processes for poorly water-soluble or highly toxic

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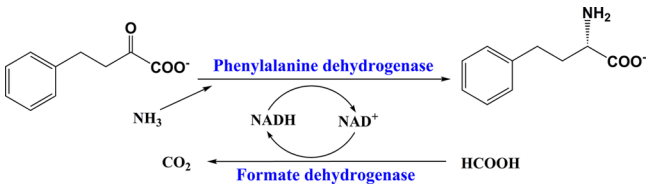
substrates. Therefore, controlled release of substrate could be an effective solution for the prevention of substrate inhibition and the increase of biocatalytic efficiency, regardless of the complexity of the reaction system.

To test our hypothesis, an appropriate reaction system and a polymer material used for the encapsulation of substrate are needed. First, the reductive amination of sodium 2-oxo-4-phenylbutanoic acid (OPBA-Na) to L-homophenylalanine (L-HPA) catalyzed by genetically modified phenylalanine dehydrogenase (mPDH) was chosen as a model reaction[20] (Scheme 1) on the basis of the following factors: (a) the conversion

Scheme 1. Reductive Amination of Sodium 2-Oxo-4-phenylbutanoic Acid (OPBA-Na) to L-Homophenylalanine (L-HPA) by Modified Phenylalanine Dehydrogenase (mPDH) from *Thermoactinomyces intermedius*[a]



[a]*Pichia pastoris* formate dehydrogenase (FDH) coexpressed with mPDH was coupled for the regeneration of NADH using HCOOH as a cosubstrate.

requires cofactor NADH, which is regenerated by coupling formate dehydrogenase (FDH) and its substrate formate; (b) the reaction contains multiple enzymes and substrates including mPDH, FDH, NADH, OPBA-Na, ammonia and formate; (c) at 25 °C and pH 8.0, the biocatalytic process seriously suffered from substrate inhibition, in which the initial velocity greatly declined when substrate concentration was above 150 mM, and the reaction was completely inhibited when OPBA-Na was over 250 mM (Supporting Information Table S1); (d) the reaction kinetics are unable to fit into any current models for accurate determination of the kinetic parameters; (e) our previous efforts on a batch-fed process resulted in a significant increase in the reaction volume, and the reaction was limited to only eight cycles;[20] and (f) product inhibition is not an issue because L-HPA precipitates out during the reaction and the byproduct is a gaseous CO_2.[21] Meanwhile, the use of nanoparticles as a vehicle for substrate supply was not appropriate because the amount of OPBA-Na loaded in the nanoparticles was too low to achieve meaningful productivity and recovery rate, and the release profiles of nanoparticles were unsatisfactory (data not shown). Consequently, microparticles were the ultimate choice for our study.

Next, a suitable polymer to prepare microparticles was screened by examining the encapsulation rate and the release profile of the substrate. As shown in Supporting Information Table S2, Eudragit RS100, an acrylic polymer with quaternary ammonium groups,[22,23] exhibited desirable characteristics for use as a material to load OPBA-Na compared to other polymers tested. To prepare OPBA-Na loading microparticles, the o/o emulsion solvent vaporization method was used to obtain the microparticles with irregular shapes and rough and condensed surface (Supporting Information Figure S1).

To maximize the loading efficiency and obtain a desired release profile, different ratios between the polymer and OPBA-Na were compared. As the percentage of the substrate increased, the particle yield slightly changed, accompanyied by a remarkable decrease in the particle size (Supporting Information Table S3), which is in accordance with previous reports.[23,24] The loading efficiency, calculated as the ratio of the substrate amount in the microparticles versus total particle amount, increased with the input of OPBA-Na. When the polymer/substrate ratio was 4:1, a loading efficiency of 17.48% was achieved to give the most significant sustainable release (Figure 1), which can deliver enough substrate into the reaction media.[25,26]

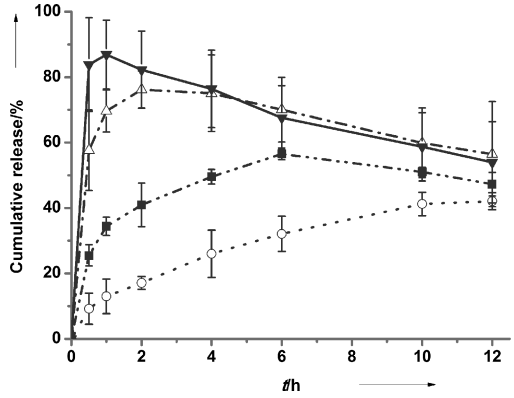


Figure 1. Release of OPBA-Na from the microparticles with different Eudragit RS100/substrate ratios. Data are presented as average values of three independent experiments, and error bars represent standard deviations. The release experiments were performed in 0.1 M potassium phosphate buffer (pH 8.0) containing 4 M NaCl at 25 °C, 250 rpm. ○ Ratio of 4:1; ■ ratio of 2:1; △ ratio of 1:1; ▼ ratio of 1:2.

As an ammonio methacrylate polymer, Eudragit RS100 is insoluble but pH-independently permeable in water, and compounds encapsulated in this material could be released by diffusion.[27] In this study, the release rate by a higher polymer/drug ratio was significantly slower than that by lower ones as a result of the possibility of an increased diffusion barrier (Figure 1). In particular, <60% of substrate was released in 6 h for ratios of 4:1 and 2:1, suggesting their great potential for controlled release of the substrate during the biocatalytic reaction. Meanwhile, the release profile revealed a biphasic behavior consisting of a retained phase in the beginning and a declined phase after the release reached maximum. Because OPBA-Na is stable in aqueous solution at pH 8 for 3 days (data not shown), substrate decomposition in the declined phase was not likely to occur. Meanwhile, adding NaCl into the reaction media could increase the amount of OPBA-Na to release (Supporting Information Table S4), indicating possible roles of charge interactions between OPBA and the polymer. On this basis, the release process should operate as a dynamic combination of diffusion and absorption. When most of the substrate encapsulated in the polymer diffused to the media, the adsorption played a predominant role to give a downward release profile. Similar adsorption was also observed for the product L-HPA and the enzymes. Therefore, NaCl at optimal 4 M concentration was added in the release and reaction media in the following experiments to disrupt the adsorption while maintaining enzyme activity (Supporting Information Table S4). Although NaCl is gentle to most enzymes, some enzymes may have limited tolerance to such a high concentration. Fortunately, in this case, a high concentration of NaCl did not appear to have a negative impact on either enzyme.

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Subsequently, we employed the microparticles with polymer/substrate ratios of 2:1 and 4:1 to take part in the model reaction at a substrate concentration of 250 mM. Theoretically, on the basis of Figure 1, substrate inhibition could be avoided when the substrate concentration was controlled lower than the critical inhibition concentration of 150 mM. Surprisingly, the bioconversion was only <30% after 12 h, in which the actual substrate concentration was >200 mM to cause substrate inhibition (data not shown). This accelerated release in the reaction was most likely from the shifted reaction equilibrium,[14] or the dissociation of the substrate from the particles in the presence of complicated reaction components.

Given that the particle size could greatly affect the release rate, it is desirable to increase the particle size to achieve controlled slower release. Hence, sieves were used to screen particles for larger size and slower release (Supporting Information Figure S2).[23,25] After comparison, the microparticles with size over 250 μm were proven to be suitable, with which 20% substrate was released in 12 h (Figure 2a) while loading efficiency remained constant (17.48%). Then these particles were used to supply 250 mM OPBA-Na (1.43 g microparticles) for the reductive amination. This is a concentration that is impossible for conventional batch reaction by the same enzymes and cofactors (see Supporting Information and Table S1). This time, 85% of OPBA-Na was released out and successively transformed to L-HPA in 12 h (Figure 2b). This was a remarkable increase compared with 3% conversion by conventional batch reaction with severe substrate inhibition. Through the process, the microparticles swelled a little but remained intact. After collecting and drying, the microparticles were intact and well-separated with shapes and sizes that were similar to those at the beginning. However, white precipitates of L-HPA were observed on the surface of the microparticles. Thus, another 2 M NaCl was added into the resulting solution for further dissociation of L-HPA. Meanwhile, the pH was adjusted to 11 for dissolving L-HPA, and the remaining microparticles were removed via filtration. The product was finally recovered from the filtrate by adjusting the pH back to neutral to obtain a recovery yield of 68.8%. This slightly lower yield than previous batch-fed processes with the same enzymes could be due to the strong binding of L-HPA to the particles. Then, the pH value for dissolving L-HPA was increased to 14 to further dissolve strongly bound product, and the final yield was increased to 72.4%, higher, than previous batch-fed processes.[20] This continuous transformation for 12 h suggested that both mPDH and FDH maintained stable catalytic activity in the presence of the microparticles and NaCl. In addition, the substrate concentration was constantly controlled under the critical inhibition concentration (150 mM). Collectively, the initial reaction velocity increased 18-fold, suggesting that Eudragit RS100 microparticles could avoid substrate inhibition and increase overall catalytic efficiency.

Finally, to demonstrate the feasibility of the present strategy, a further increase in the substrate concentration and reaction scale was performed. In a 50 mL reaction system, OPBA-Na in the microparticles was increased to 500 mM (100 g/L, 28.7 g microparticles). After 33 h, the conversion was 80% (Figure 2c). Over the course of the reaction, the substrate sustainably released from the microparticles and was immediately transformed to L-HPA with >99% ee. In particular, the substrate concentration was well controlled under the inhibition level throughout the reaction. Total 400 mM (71.6 g/L) L-HPA was produced, and the space–time productivity reached 51.7 g L^{-1}

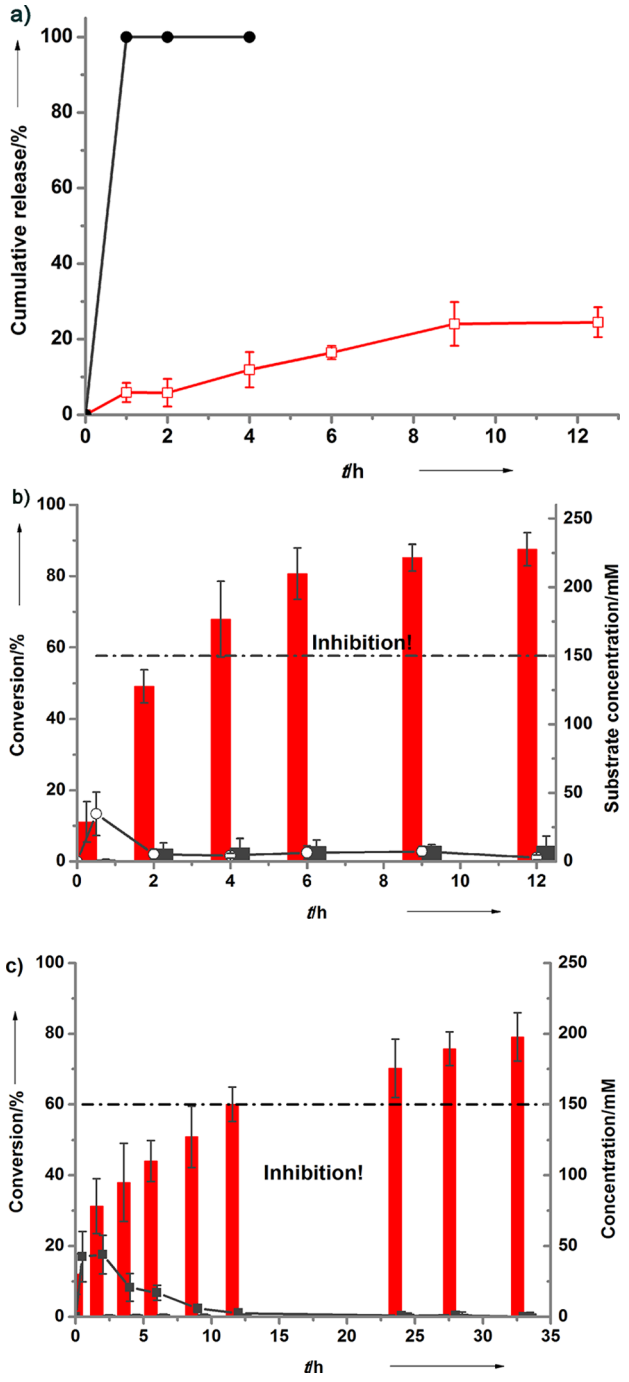

Figure 2. Reductive amination of OPBA-Na with the microparticles. A polymer/substrate ratio of 4:1 and particle size >250 μm were used. Data are presented as average values of three independent experiments, and error bars are standard deviations. (a) Release profile of OPBA-Na from the microparticles. ●, Dissolution behavior of OPBA-Na; □, Eudragit RS100 microparticles with particle size >250 μm. (b) Time course of reductive amination of OPBA-Na using the microparticles to load 250 mM substrate in 5 mL system. Red bar, percentage of conversion with the microparticles; black bar, percentage of conversion of conventional batch substrate addition; ○, actual substrate concentration; dashed line, critical inhibition concentration. (c) Time course of reductive amination of OPBA-Na using the microparticles to load 500 mM substrate in the 50 mL system, and finally, 400 mM L-HPA was formed. Red bar, percentage of conversion with the microparticles; black bar, percentage of conversion of conventional batch substrate addition (less than 1%). ■, actual substrate concentration; dashed line, critical inhibition concentration.

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day^{-1}. To the best of our knowledge, this is equal to the highest substrate concentration ever reported for this biocatalytic reaction.[20,28] Moreover, the final reaction volume was increased less than 2-fold over the beginning, significantly lower than that of the batch-fed reaction (an over 5-fold increase), showing the particular advantages for substrates with low aqueous solubility in a very limited operational room, which is quite common for the preparation of a large number of pharmaceuticals or intermediates.[14] Therefore, by applying a microparticle-assisted substrate supply, the substrate inhibition in biocatalysis could be completely eliminated to robustly enhance the biocatalytic efficiency. In addition, the potential reuse of the polymer and the advancement in developing less expensive materials for more efficient encapsulation can further facilitate the implementation of the present strategy. Although the present study was intended only to deal with the issues associated with substrate inhibition in biocatalytic processes, an effective solution to simultaneously address both substrate and product inhibitions would be more desirable and beneficial because these inhibitions typically coexist to affect the catalytic efficiency in most biocatalytic reactions.

In summary, we have demonstrated the concept of a microparticle-based strategy to control substrate release for efficient biocatalysis. Using a Eudragit RS100 microparticle system, a high substrate concentration was achieved for mPDH-catalyzed reductive amination of OPBA-Na. Because the microparticle encapsulation technique can be applied to various types of chemicals for controlled release, the present study provides a general solution for efficient, environmentally friendly biocatalytic processes for the preparation of valuable chemicals.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details concerning materials, preparation, and characterization of microparticles and experimental procedures of the biocatalytic reactions. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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