Novel Magnetic Cross-Linked Lipase Aggregates for Improving the Resolution of (*R, S*)-2-octanol

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ABSTRACT Novel magnetic cross-linked lipase aggregates were fabricated by immobilizing the cross-linked lipase aggregates onto magnetic particles with a high number of -NH_2 terminal groups using *p*-benzoquinone as the cross-linking agent. At the optimal fabrication conditions, 100% of immobilization efficiency and 139% of activity recovery of the magnetic cross-linked lipase aggregates were achieved. The magnetic cross-linked lipase aggregates were able to efficiently resolve (*R, S*)-2-octanol, and retained 100% activity and 100% enantioselectivity after 10 cycles of reuse, whereas the cross-linked lipase aggregates only retained about 50% activity and 70% enantioselectivity due to insufficient cross-linking. These results provide a great potential for industrial applications of the magnetic cross-linked lipase aggregates. *Chirality 00:000–000, 2014.*

KEY WORDS: Yarrowia lipolytica lipase; magnetic nanoparticles; cross-linked enzyme aggregates; immobilization; (*R, S*)-2-octanol; enantioselectivity

INTRODUCTION

Lipases have exhibited capability in the preparation of optically active single enantiomers for synthesizing a variety of fine chemicals and pharmaceuticals.[1,2] Developing effective immobilization techniques to stabilize and reuse lipases is one of the key steps to make the enzymatic process economically viable.[1,3] Immobilization techniques can be divided into two types: carrier-bound and carrier-free. The disadvantage of carrier-bound enzymes is the dilution of catalytic activity due to the introduction of a large proportion of mass (~90-99% of the total mass), which results in lower volumetric and space-time yields and lower catalyst productivity. In addition, immobilization using a solid carrier is generally expensive and often requires the chemical modification of an inert matrix to allow for covalent coupling of the enzyme.[4] Carrier-free immobilization via cross-linking of the enzyme using bifunctional cross-linking agents is a promising strategy that overcomes the disadvantage of carrier-bound enzymes.[3] Due to the lack of a support, the enzyme activity is not diluted, and the specific activity of the resulting biocatalyst is increased.[5] Furthermore, an industrial enzymatic process using carrier-free immobilized enzymes can utilize smaller bioreactors than the enzymes immobilized on a solid support.[2] Among carrier-free immobilization technologies, cross-linked enzyme aggregates (CLEAs) technology is attractive for its low cost, simplicity, and efficiency.[6–9] The preparation of CLEAs consists of precipitating the enzyme and cross-linking the resulting physical aggregates with glutaraldehyde, a common cross-linking agent,[10,11] via the reaction with the reactive amino groups (mainly from Lys) on the enzyme surface. Therefore, immobilization using CLEAs technology is strongly dependent on the nature of the enzyme in terms of Lys content. Immobilization by CLEAs technology may not be highly efficient for electronegative enzymes with few surface reactive amino groups.[12]

In order to overcome the difficulty of utilizing enzymes with few surface reactive amino groups, several approaches for the preparation of CLEAs have been successfully developed. For enzymes with a low number of reactive amino groups on the external surface, the aggregation may be conducted in the presence of a polymer or macromolecules with many reactive amino groups.[13] However, Schiff bases resulting from the reaction of glutaraldehyde and amino groups are unstable under acidic conditions, and they have a tendency to break down and regenerate the aldehyde and amine.[14] This may result in the gradual release of the enzyme into the reaction medium due to inadequate cross-linking.[15] In addition, the CLEAs form clusters due to the separation of CLEAs from the reaction medium by centrifugation or filtration. This results in internal mass-transfer limitations, especially when enzymes act on macromolecular substrates.[13,16,17]

Magnetic cross-linked enzyme aggregates (MCLEAs), which were prepared by the addition of functionalized magnetite particles as an additive into enzyme solution, precipitation of enzyme to form aggregates, and then cross-linking of enzyme aggregates and particles, can circumvent the problem of clumping of CLEAs.[13] Because of the magnetic properties of the magnetite particles, the resulting MCLEAs can be easily controlled and separated from the reaction medium by the application of a permanent magnet or magnetic field, eliminating the need of filtration and centrifugation. However, MCLEAs prepared with glutaraldehyde still cannot solve the problem of the gradual release of the enzyme into the reaction medium.

In this work, in order to overcome the limitations of CLEAs technology, a novel strategy was developed to prepare MCLEAs by conducting the CLEAs onto magnetic particles with a high number of $-NH_2$ terminal groups using

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p-benzoquinone as the cross-linking agent. The prepared magnetic CLEAs were used as the biocatalyst to improve the resolution of (*R*, *S*)-2-octanol.

MATERIALS AND METHODS

Materials

Yarrowia lipolytica lipase (YLL) was kindly donated by Beijing CTA New Century Biotechnology (China). (*R*)-2-octanol, (*S*)-2-octanol, (*R*, *S*)-2-octanol, vinyl acetate, polyethylenimine (PEI), cationic polyacrylamide (CPAM), polymethylmethacrylate (PMMA), and *p*-benzoquine were purchased from Sigma-Aldrich (Shanghai, China). (*R*)-2-octanol acetate and (*S*)-2-octanol acetate were synthesized using the method previously reported.[18] Magnetic particles coated with PEI (Fe_3O_4-PEI, MP_1) and magnetic particles coated with CPAM (Fe_3O_4-CPAM, MP_2) were prepared according to our previous reports.[19,20] Fe_3O_4-PMMA with dendritic $-NH_2$ (MP_3) were prepared according to the method previously reported.[21] The other reagents were purchased from Beijing Chemical Reagents (Beijing, China).

Preparation of Magnetic Cross-Linked Lipase Aggregates

Magnetic cross-linked lipase aggregates (MCLLAs) preparation involves two steps: the precipitation of lipase and the subsequent cross-linking of the enzyme aggregates onto the surfaces of the magnetic particles. In a typical experimental procedure the magnetite particles (2.5 mg) were mixed with 5 mL of *Yarrowia lipolytica* lipase in buffer solution (5 mg protein/mL, 0.067 M phosphate buffer, pH 8.0) and shaken for 10 min at 20 °C. Then 20 mL of saturated ammonium sulfate solution was added with stirring at 4 °C for 40 min. After precipitation, *p*-benzoquinone was added to the suspension up to a final concentration of 5 mM and stirred for 20 h at 20 °C. After cross-linking, MCLLAs were separated using a magnet, washed three times with phosphate buffer, and immediately frozen and lyophilized. The cross-linked lipase aggregates (CLLAs) were prepared without the addition of magnetic particles as a control.

The immobilization efficiency (η) of the MCLLAs was calculated from the difference between the initial concentration of enzyme (C_0) and the concentration remaining after the preparation approach (C_1) using Eq. (1).[7]

$$\eta(\%) = \frac{C_0 - C_1}{C_0} \times 100\% \quad (1)$$

The activity recovery (λ) was estimated using Eq. (2).

$$\lambda(\%) = \frac{\text{Specific activity of MCLLAs}}{\text{Specific activity of the same amount of free lipase as that in the MCLLAs}} \times 100\% \quad (2)$$

The specific activity of the lipase was assayed using the *p*-nitrophenol method.[22]

Resolution of (R, S)-2-octanol Catalyzed by MCLLAs

The resolution of (*R*, *S*)-2-octanol catalyzed by MCLLAs was conducted as in our previous report.[23] The reaction solvent was the mixture of acetone and carbon tetrachloride, v/v =3:7. Reusability of the MCLLAs was studied in batch operations under the same conditions. After each batch reaction, the activity and enantioselectivity of the MCLLAs were measured and expressed as the residual activity and enantioselectivity by taking the activity and enantioselectivity of the first batch as 100%.

Analysis

The (*R*, *S*)-2-octanol and product were analyzed using gas chromatography (GC) using a previously reported method.[23] The extent of conversion (*c*) of (*R*, *S*)-2-octanol was determined by the decrease of (*R*, *S*)-2-octanol. The enantiomeric excess (*ee*) of the product was determined by the peak areas of the two isomers using Eq. (3). The enantioselectivity (*E*) was calculated using Eq. (4) according to a previously reported method.[24]

$$ee_p(\%) = \frac{R - S}{R + S} \times 100\% \quad (3)$$

where ee_p is the *ee* of the product, *R* is the concentration of the (*R*)-enantiomer, and *S* is the concentration of the (*S*)-enantiomer.

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \quad (4)$$

where *c* is the extent of the conversion of (*R*, *S*)-2-octanol.

RESULTS AND DISCUSSION

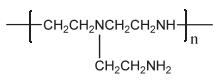
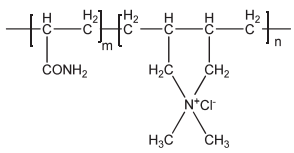
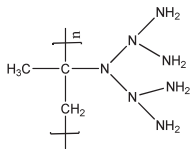
MCLEAs have emerged as a novel and versatile carrier-free immobilization technique, involving the precipitation of an enzyme from an aqueous solution and cross-linking the aggregates onto the surfaces of the magnetic particles using a bifunctional reagent.[25] Therefore, parameters that alter the enzyme precipitation and the aggregate cross-linking will affect the properties of the MCLEAs. The effects of various parameters during MCLEAs preparation on immobilization efficiency and activity recovery of MCLEAs were studied.

The nature of YLL and the type and quantity of magnetic particles

YLL, which belongs to the same gene family as *Thermomyces lanuginosus* lipase (TLL), is a lipase with multiple applications in the field of biotechnological processes.[26] However, due to its low Lys residue content, CLLAs prepared using YLL may not be highly effective due to inadequate cross-linking, which results in low mechanical stability or even the release of the enzyme during reactions.[15] In order to overcome this, three types of magnetite particles coated with large amino groups were added to the YLL solution to prepare MCLLAs via cross-linking of YLL aggregates and the amino functionalized magnetite particles. The results are shown in Table 1. All the magnetite particles tested improved the η and λ of the MCLLAs compared to the control (prepared without magnetite particles). The microparticle, MP_2, due to its low surface area, was coated with fewer amino groups, which resulted in a lower η compared to the nanoparticles MP_1 and MP_3.[27] MP_3, due to dendritic modifications, has a much larger number of amino groups (about 0.5 mmol/g, determined via ninhydrin colorimetry.[28]) on its surface than MP_1. Therefore, the addition of MP_3 into the YLL solution can lead to a high η due to sufficient cross-linking of YLL aggregates.

The ratio of the magnetic particles to the enzyme (protein) was an important factor in determining the performance of the MCLLAs.[7] The η and λ of the MCLLAs showed an upward trend for MP_3: YLL mass ratios in the range of 0.02–0.1 (Fig. 1). The increase of this ratio to values higher than 0.1

TABLE 1. MCLLAs prepared with different magnetic particles

	Magnetic particles			MCLLAs	
Entry	Type	Average diameter	Structure of covering layer	η (%)	γ (%)
1[a]	-	-	-	42 ± 3	85 ± 5
2	MP_1	12 nm		70 ± 2	91 ± 3
3	MP_2	58 μm		51 ± 3	87 ± 5
4	MP_3	200 nm		100 ± 2	120 ± 3

[a]Without the addition of magnetic particles.

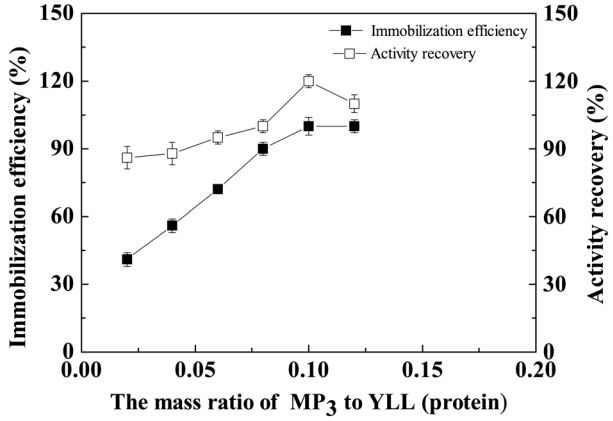


Fig. 1. Effect of the ratio of MP_3 to YLL on the immobilization efficiency and activity recovery of the MCLLAs.

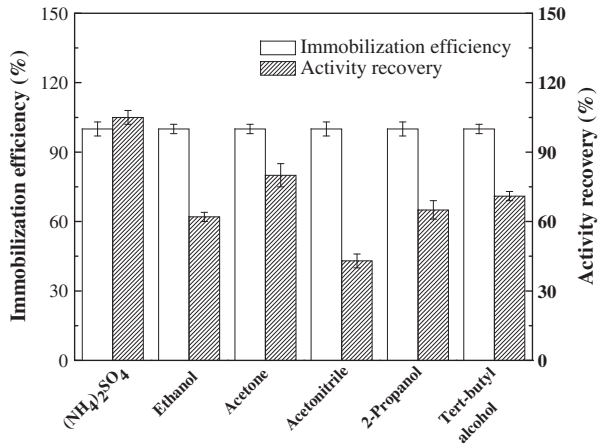


Fig. 2. Effect of the precipitation agent on the immobilization efficiency and activity recovery of the MCLLAs.

had no additional effect on η, but resulted in a decrease in λ (Fig. 1). This was most likely due to excessive cross-linking (due to excess MP_3), which resulted in larger particle sizes of the prepared MCLLAs and a loss of the ability of the inner YLLs to form complexes with substrates. Therefore, a ratio of MP_3 to YLL of 0.1 was used in further experiments.

Type and Quantity of the Precipitant

YLL was precipitated using different precipitation agents, such as $(NH_4)_2SO_4$, ethanol, acetone, acetonitrile, 2-propanol, and tert-butyl alcohol. The experimental data are presented in Figure 2. $(NH_4)_2SO_4$ was the most successful agent for the precipitation of YLL and led to high η and λ of the prepared MCLLAs. All the organic solvents used as precipitants affected the activity of YLL to a different degree. The optimal precipitant of YLL of $(NH_4)_2SO_4$ is in contrast to the optimal precipitant for the lipase from *Aspergillus niger*, which is dimethyl carbonate.[7] This is due to the different biochemical and structural properties of the enzyme which causes the optimal precipitant to vary from one enzyme to another.[25]

The amount of precipitant also affected the performance of the MCLLAs (Fig. 3). The λ in the YLL CLEAs increased with increasing $(NH_4)_2SO_4$ saturation of the enzyme solution during precipitation, which was in agreement with *Candida rugosa* lipase[29] and tyrosinase.[30] When the volume ratio of $(NH_4)_2SO_4$ solution to YLL solution increased to 4, "hyper activation" (greater than 100% λ), was observed due to the formation of more structured and fine-grained CLEAs.[31] A further increase in the ratio of the $(NH_4)_2SO_4$ solution to the YLL solution resulted in a decrease in λ due to the larger particle size of the MCLLAs.

Nature and Amount of the Cross-Linking Agent and Cross-Linking Time

p-Benzoquinone was used as the cross-linking agent because it reacts with the amino or hydroxyl groups of YLL

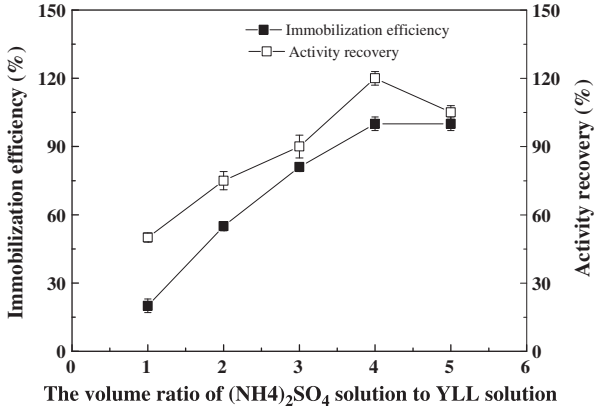


Fig. 3. Effect of the amount of $(NH_4)_2SO_4$ on the immobilization efficiency and activity recovery of the MCLLAs.

and the amino of MP_3 and forms stable MCLLAs due to the formation of C–O and C–N bonds tolerant to pH variation.[1] This circumvents the existing problems of glutaraldehyde, which cross-links the amino groups of enzyme aggregates via the formation of unsaturated bonds and Schiff bases, which are unstable.

An important parameter in the preparation of the MCLLAs was the amount of *p*-benzoquinone; this influenced the η and λ of the resulting MCLLAs (Fig. 4). The concentration of *p*-benzoquinone was clearly optimal at 4 mM. The η and λ of the MCLLAs prepared with increasing concentrations of *p*-benzoquinone increased to a maximum value and then λ decreased while η reached a plateau. This was because at lower *p*-benzoquinone concentrations, insufficient cross-linking occurred. On the one hand, portions of the YLL were not cross-linked and washed into the supernatant, resulting in a lower η. On the other hand, the unstable MCLLAs that resulted from the insufficient cross-linking released free YLL into the reaction medium, resulting in a lower λ. When the *p*-benzoquinone concentration was high, excessive cross-linking occurred, resulting in a loss of YLL's flexibility, which is crucial for enzyme activity. In addition, steric hindrance due to excessive cross-linking may have resulted in the rigidification of YLL and prevented the substrate from reaching the active site. Therefore, for an enzyme, there is an optimum cross-linking agent concentration at which sufficient cross-linking occurs while maintaining the enzyme's flexibility with high η and λ. However, the optimum concentration is strongly dependent on the functional groups (type and amount), which vary from one enzyme to another. This is confirmed by other studies.[1,7,13]

Cross-linking is a reaction; therefore, the time required to obtain the maximum λ is very important.[32] The MCLLAs of YLL were prepared using varying cross-linking times (4, 8, 12, 20, and 24 h). The results (presented in Fig. 5) showed that 8 h was required for complete cross-linking. And at the same time, the maximum λ, 139%, was achieved.

Resolution of (R, S)-2-octanol Catalyzed by the MCLLAs

The newly synthesized MCLLAs of YLL were used to catalyze the resolution of (*R*, *S*)-2-octanol. The enzymatic activity and enantioselectivity of the MCLLAs were 48.5 (±0.2) μmol/g/min and 850 (±5), respectively, which were higher than those (29.8 ± 0.3 μmol/g/min, 500 ± 2) of the CLLAs, and also higher than those of the magnetite-immobilized YLL and free YLL catalyzed under the same conditions. The *ee* of the product obtained by MCLLAs catalysis was 99.1%, which was higher than that (98.2%) of the product obtained by CLLAs catalysis.[23] The enhanced activity and enantioselectivity of MCLLAs may be partly due to the formation of the more structured and fine-grained CLEAs mentioned above and partly to the addition of the magnetic particles with a high number of $-NH_2$ terminal groups during the preparation of MCLLAs. Similar to BAS as additive (during the immobilization of formate dehydrogenase from *Candida boidinii* via CLEAs[33]), the magnetic particles may also act as a proteic feeder, preventing from excess cross-linking of important amino acid residues of YLL.

As with other immobilized enzymes, reusability is extremely important for potential industrial applications.[25] The reusability of the MCLLAs of YLL was studied in batch operations at optimum reaction conditions for a fixed reaction time. After each batch reaction, the enzyme activity and enantioselectivity in the MCLLAs were determined and expressed as the residual activity and enantioselectivity by setting the activity and enantioselectivity of the first batch as 100%. The results (Fig. 6) showed that almost no activity and enantioselectivity in YLL in MCLLAs were lost after 10 cycles of use, while they decreased continuously when CLEAs (prepared without MP_3) were tested over 5 cycles. These results suggested that the loss in YLL activity and enantioselectivity in CLEAs was due to the leaking of YLL

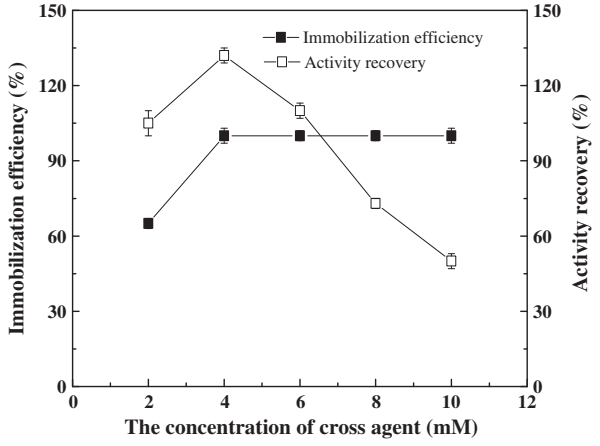


Fig. 4. Effect of the amount of cross-linking agent on the immobilization efficiency and activity recovery of the MCLLAs.

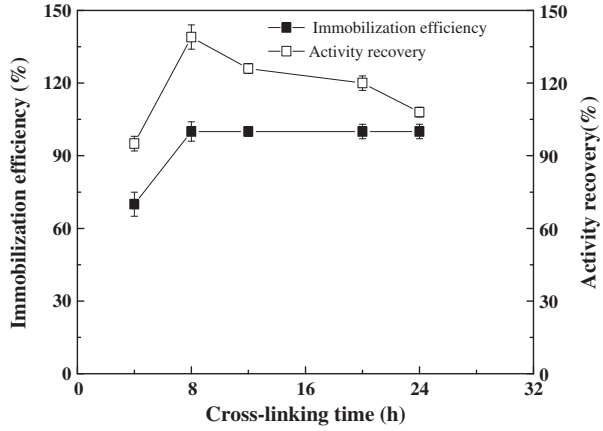


Fig. 5. Effect of cross-linking time on the immobilization efficiency and activity recovery of the MCLLAs.

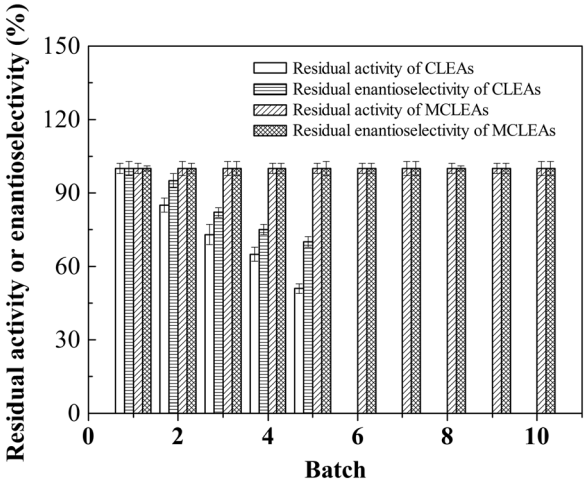


Fig. 6. The reusability of the MCLLAs used in resolution of (*R, S*) of 2-octanol.

into the reaction medium, because the number of functional groups of YLL was not sufficient to achieve efficient cross-linking of all the YLL molecules. Consequently, YLL could be released from the aggregates. Similar to other studies in which CLEA technology has been combined with other traditional immobilization methods to enhance the reusability of CLEAs (e.g., Hilal et al.[34] immobilized lipase CLEAs within microporous polymeric membranes; Wilson et al.[35] encapsulated penicillin G acylase CLEAs into very rigid lens-shaped polyvinyl alcohol hydrogel particles), we cross-linked YLL aggregates onto amino functionalized MP_3 to produce operationally stable MCLLAs due to adequate cross-linking.

CONCLUSION

Fabrication of MCLLAs using *p*-benzoquinone as the cross-linking agent in the presence of Fe_3O_4-PMMA with dendritic $-NH_2$ is an efficient strategy for YLL immobilization. The MCLLAs of YLL, which were synthesized under optimum conditions, were able to catalyze the efficient resolution of (*R, S*)-2-octanol, and demonstrated excellent reusability. These results suggest that the synthesized MCLLAs of YLL have potential for industrial applications in the preparation of optically active single enantiomers.

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