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Synthesis of butyl oleate catalyzed by cross-linked enzyme aggregates with magnetic nanoparticles in rotating magneto-micro-reactor

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ABSTRACT

In order to increase application of cross-linked enzyme aggregates (CLEAs) in industry production, a novel micro-reactor system that included a rotating magnetic field (RMF), a micro-reactor and CLEAs with magnetic nanoparticles (M-CLEAs) was designed to synthesize butyl oleate. Result showed that the presence of RMF significantly increased the yield of butyl oleate and the maximum increment was 23%. The yield of butyl oleate was impacted by the dosage and distribution of M-CLEAs in micro-reactor. M-CLEAs showed good reusability, since the morphology and the second structure of protein of M-CLEAs did not show evident change after 4 operative cycles. Although the three-dimensional fluorescence of M-CLEAs showed shift in fluorescence intensity and the maximum emission wavelengths, the yield of butyl oleate was not affected. This study provides a novel design that realized efficient, convenient and continuous application of CLEAs in biosynthesis, and M-CLEAs also show good promises in industry production.

1. Introduction

Cross-linked enzyme aggregates (CLEAs), a potential carrier-free immobilization design, have aroused extensive attention due to its high activity per unit volume, high stability against solvents, simple preparation process and economic advantages (Talekar et al., 2013; Cui et al., 2014; Nadar and Rathod, 2016). CLEAs of various enzymes, such as penicillin acylase (Illanes et al., 2007), acetylxylan esterase (Montorogarcía et al., 2010), *N*-acetylneuraminate aldolase (Inmaculada et al., 2014) and laccases (Matijošytė et al., 2010), have been prepared and used in biosynthesis. Even though CLEAs show promises, common problems for CLEAs are (1) their irregular shapes and sizes which may lead to slow diffusion of substrates into the CLEAs, (2) difficulty in recovering CLEAs since small and fragile CLEAs easily form clumps during centrifugation and filtration treatments, (3) adhering onto vesicle wall (Bidmanova et al., 2014; Wang et al., 2015; Sojitra et al., 2016), and (4) great internal mass-transfer limitations in CLEAs (Cui et al., 2016; Nadar and Rathod, 2016). These limited CLEAs application in industry production. Control on shapes and sizes of CLEAs has been concerned and made good progress (Talekar et al., 2012a; Rehman et al., 2016; Chen et al., 2017), however, viscosity and clumps of CLEAs is less concerned (Cui et al., 2014; Chaudhari and Singhal, 2017). In order to increase CLEAs application in industry production, it is necessary to solve these drawbacks of CLEAs.

Magnetic nanoparticles (MNPs) are a kind of nanophase materials which possess unique characteristics, such as high specific surface area, unique physicochemical properties, biocompatibility, biodegradability, low toxicity and low cost (Netto et al., 2013; Seenuvasan et al., 2014). MNPs offer facile separation by applying external magnetic, thus they appear as a cost-effective and biocompatible support for immobilization of industrial enzymes in mostly soluble form and in some cases with aggregated form (CLEAs) (Ansari and Husain, 2012; Zhang et al., 2015; Panwar et al., 2017). Therefore, MNPs have been used to improve enzyme performance and to achieve fast separation of enzyme and reaction system in enzyme immobilization (Johnson et al., 2011; Cao et al., 2012; Hosseinipour et al., 2015), including CLEAs (Talekar et al., 2012b; Cui et al., 2014; Nadar and Rathod, 2016). Presently, MNPs are used in CLEAs by two ways. One is that MNPs are used as carrier to immobilize CLEAs. Talekar et al. (2012b) immobilized α-amylase onto amino functionalized magnetite nanoparticles, and found that magnetic CLEAs exhibited excellent thermostability and storage stability, and the magnetic CLEAs retained 100% initial activity even after 6 cycles.

Abbreviations: CLEAs, cross-linked enzyme aggregates; RMF, rotating magnetic field; M-CLEAs, CLEAs with magnetic nanoparticles; MNPs, magnetic nanoparticles; HM-CSL-CLEAs, hybrid magnetic cross-linked lipase aggregates; SEM, scanning electron microscope; FTIR, Fourier transform infrared spectroscopy

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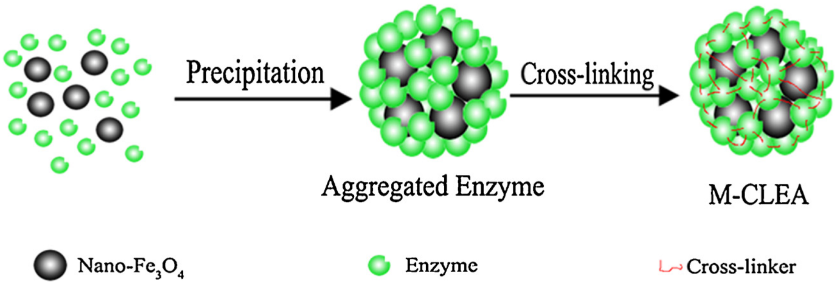

Fig. 1. Schematic diagram of M-CLEAs preparation.

Nadar and Rathod (2016) found that magnetic macromolecular glucoamylase CLEAs showed better thermostability and operational stability than traditional CLEAs. Jia et al. (2017) found that magnetic-cellulase-CLEAs exerted excellent activity, thermal stability, and pH stability. Tudorache et al. (2013) immobilized lipase CLEAs onto magnetic particles (1–17 μm) by glutaraldehyde. The other way is that MNPs are directly used as modifier to improve enzyme characteristics. Wang et al. (2017) used nano-Fe_3O_4 as additive to improve CLEAs activity and structure but no evident effects were observed. Cui et al. (2016) prepared the hybrid magnetic cross-linked lipase aggregates (HM-CSL-CLEAs) by coaggregation of lipase aggregates with non-functionalized magnetic nanoparticles and subsequent chemical cross-linking with glutaraldehyde, and found that HM-CSL-CLEAs exhibited higher thermostability, storage stability, and reusability than standard CLEAs. Martins et al. (2017) prepared CLEAs by chemical cross-linking with functionalized magnetite nanoparticles for glycompounds biosynthesis in microbioreactors. In these studies, magnetic CLEAs show stable reusability, better thermostability, good operational stability and storage stability. This strategy can overcome the drawbacks of CLEAs and might improve application potential of CLEAs in industry production (Su et al., 2018; Kopp et al., 2014; Cui and Jia, 2015). Up to now, however, most studies concern the preparation and characteristics of magnetic CLEAs, and their recovery is not really realized in consecutive reactions. This implies that magnetic CLEAs need further study.

Butyl oleate is widely used as diesel additive, polyvinyl chloride plastisizer, and water resisting agent in hydraulic fluids (Linko et al., 1995). Presently, butyl oleate is mainly produced by chemical synthesis, however, this process generally produces much acid wastewater and byproducts. Some studies have concerned biosynthesis of butyl oleate, unfortunately, production rate of butyl oleate is relative low. For example, Habulin and Knez's (1991) obtained the highest conversion rate of butyl oleate was 88.11% with the catalysis of immobilized *Mucor miehei* lipase after more than 60 h reaction. Ghamgui et al. (2004) obtained the conversion rate of 75% with the catalysis of immobilized *Rhizopus oryzae* lipase after 30 h reaction. Although Wang et al. (2010) obtained the conversion rate of 91% with the catalysis of immobilized *Rhizopus arrhizus* on hydrophobic polytetrafluoroethene membrane with nonwoven fabric after 2 h reaction, while it was flask-shaking experiment. Linko et al. (1992) obtained 98% yield of butyl oleate in 12 h with 0.3% of *Chromobacterium viscosum* lipase, but this result was from free enzyme. In similar studies on biosynthesis, Jiang et al. (2014) obtained 95.8% of ethyl oleate after 24 h reaction with *Pickering emulsion* immobilized on mesoporous organosilica. Gao et al. (2017) obtained 85.48% of butyl-levulinates by *Candida antarctica* immobilized on nanoflowers after 24 h reaction. These results indicate that higher yield of butyl oleate may be achieved by immobilized lipase.

The objective of this study was to biosynthesize butyl oleate by magnetic CLEAs (M-CLEAs). In order to achieve this goal, we designed a novel micro-reactor with rotating magnetic field, which achieve continuous, efficient and convenient application of M-CLEAs in the biosynthesis of butyl oleate. M-CLEAs before and after long-time reaction was observed through scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and three-dimensional fluorescence.

2. Material and methods

2.1. Materials

Crude lipase power of *Rhizopus arrhizus* (5000 U/g) was purchased from Beijing Tianfang Biological Corporation, China. Nano-Fe_3O_4 (50–100 nm) was purchased from Sigma-Aldrich (China). All solvents and chemicals were purchased from Beijing Chemical Reagents Company, China, and they are of analytical grade except for triolein, which is of chemical grade.

2.2. Preparation of crude lipase solution

Lipase solution (70 mg/mL) was prepared by adding 7.0 g crude lipase powder to 100 mL phosphate buffer (10 mM, pH 7.0). After 1 h vibration at 4 °C, the lipase solution was centrifuged at 6000 r/min. Supernatant solution was used for the preparation of M-CLEAs.

2.3. Preparation of M-CLEAs

M-CLEAs were prepared as literature reported (Cui et al., 2014). Nano-Fe_3O_4 (9 mg) was mixed with 1 mL of 70 mg/mL lipase solution, and homogenate at 4 °C for 30 s. Then 12 mL acetone was added with stirring at 4 °C for 30 min. After precipitation, glutaraldehyde was added into the suspension up to the final concentration of 2.075% (v/v) and vibrated (200 r/min) at 4 °C for 2 h. After cross-linking, M-CLEAs were separated by a magnet and washed repeatedly with phosphate buffer (10 mM, pH 7.0) until no lipase was detected in the supernatant, and then M-CLEAs were frozen drying for 24 h in a vacuum. Lastly, the M-CLEAs (2900 U/g) were stored at 4 °C. The general scheme of the preparation of M-CLEAs was illustrated in Fig. 1.

2.4. Measurement of kinetic parameters

The kinetic parameters (K_m and V_{max}) of CLEAs and M-CLEAs were determined in phosphate buffer (pH 7.0) by varying the substrate concentration (oleic acid concentrations are from 14 mM to 34 mM). The concentrations of CLEAs or M-CLEAs were kept constant. K_m and V_{max} were calculated by the Lineweaver-Burk equation using computed linear regression calculations.

2.5. Micro-reactor system

The novel micro-reactor system included a rotating magnetic field (RMF), a micro-reactor (2 cm i.d. and 20 cm length) and cross-linked lipase aggregates with magnetic nanoparticles (M-CLEAs). The micro-reactor had three sand core sieve plates, through which reaction solvents and product but not M-CLEAs could pass. Reaction fluids were continuously fed to the micro-reactor from the bottom entrance and passed though M-CLEAs and magnetic field. The magnetic field was produced by two magnets that were fixed on one side of the rectangular