Journal of Biotechnology 271 (2018) 1–7

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Magnetic combined cross-linked enzyme aggregates (Combi-CLEAs) for cofactor regeneration in the synthesis of chiral alcohol

Erzheng Su[a,b,*], Meng Yang[a], Chenxi Ning[b], Xiaoqiang Ma[b,c], Senwen Deng[b]

[a] Enzyme and Fermentation Technology Laboratory, College of Light Industry and Food Engineering, Nanjing Forestry University, Nanjing 210037, China
[b] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China
[c] Department of Chemical & Biomolecular Engineering, National University of Singapore, 117585 Singapore

ARTICLE INFO

Keywords:
Chiral alcohol
Ketoreductase
Cofactor regeneration
Magnetic Fe_3O_4 nanoparticles
Magnetic Combi-CLEAs

ABSTRACT

Magnetic Fe_3O_4 nanoparticles were prepared and embedded into the Combi-CLEAs to produce the magnetic Combi-CLEAs in this work. The process for magnetic Combi-CLEAs preparation was optimized, and its properties were investigated. The optimum temperature, thermal stability and optimum pH of magnetic Combi-CLEAs were similar to those of Combi-CLEAs. The catalytic performance of magnetic Combi-CLEAs was tested with the biosynthesis of (*S*)-ethyl 4-chloro-3-hydroxybutyrate ((*S*)-CHBE). Magnetic Combi-CLEAs could tolerate higher substrate concentration in the biphasic system. The catalytic efficiency and long-term operational stability of magnetic Combi-CLEAs were obviously superior to those of Combi-CLEAs in both aqueous and biphasic systems. Embedding of magnetic Fe_3O_4 nanoparticles endowing rigidity contributed to these improvements. Furthermore, the preparation of magnetic Combi-CLEAs was easy, and its recovery during multiple batches of reactions could be fulfilled by magnetic field. Aforementioned advantages make the magnetic Combi-CLEAs hold obvious potential for industrial application.

1. Introduction

Cofactor NAD(P)H dependent ketoreductases have attracted increasing attention in the production of chiral alcohols for their high regio- and enantioselectivity (Patel, 2008; Pollard and Woodley, 2007). Efficient NAD(P)H regeneration turns out to be necessary in this process. Several strategies have been reported to realize NAD(P)H regeneration (van der Donk and Zhao, 2003; Liu and Wang, 2007). However, they all run with defects that restrict their application on a large scale.

Whole-cell systems were widely used to achieve NAD(P)H regeneration (Leipold et al., 2013; Kataoka et al., 1999; Kizaki et al., 2001). However, controllable expression of primary enzyme (responsible for primary chiral reaction) and auxiliary enzyme (responsible for regeneration of NAD(P)H that drives primary reaction) can hardly be achieved. In another way, co-immobilization of primary enzyme and auxiliary enzyme was realized to regenerate NAD(P)H, but restricted by complicated preparation process (Betancor et al., 2006; Vrtis et al., 2002). A novel strategy had recently been reported to prepare Combi-CLEAs comprising a ketoreductase (KRED 42) and a D-glucose dehydrogenase (GDH 1). Combi-CLEAs was employed for the first time in the production of valuable chiral alcohols to ensure the reuse of pyridine nucleotide cofactors. It had been demonstrated to be a robust regeneration system which could be prepared in a quick, simple and low-cost way. High substrate tolerance, reusability and long-term operational stability were confirmed in this system. Besides, a total turn-over number (moles of product formed/moles of cofactor presented in the reaction system, TTN) for NADH of 12,000 had been achieved with negligible amount of NADH as starter (Ning et al., 2014). However, owing to no carrier form, Combi-CLEAs had to face the problem of recovery. Centrifugation was absolutely needed, bringing limitation to its application.

Inexpensive magnetic nanoparticles may bring rigidity and magnetic property to Combi-CLEAs system with no complicated operation (Patel et al., 2016, 2017a; Kim et al., 2016). Thus, rapid collection of biocatalyst could be achieved with magnetic field (Patel et al., 2017b). In this work, an improved process based on the Combi-CLEAs system was reported. Magnetic nanoparticles were added during the cross-linking process and enveloped in the formed aggregates. The performance of free enzymes mixture, Combi-CLEAs and magnetic Combi-CLEAs were evaluated and compared for cofactor regeneration in this work.

* Corresponding author at: Enzyme and Fermentation Technology Laboratory, College of Light Industry and Food Engineering, Nanjing Forestry University, Nanjing, 210037, China.
E-mail address: ezhsu@njfu.edu.cn (E. Su).

https://doi.org/10.1016/j.jbiotec.2018.02.007
Received 19 September 2017; Received in revised form 19 January 2018; Accepted 12 February 2018
Available online 13 February 2018
0168-1656/

2. Materials and methods

2.1. Materials

Ketoreductase (KRED 42 with a specific activity of 27.4 U/mg, GenBank Access Number: AB213459.1) from *Pichia capsulate* and D-glucose dehydrogenase (GDH 1 with a specific activity of 5.4 U/mg, GenBank Access Number: KC426949.1) from *Bacillus subtilis* were both obtained from Shanghai Bairui Biotech. Co., Ltd (Shanghai, China), and numbered according to the product number. Ethyl 4-chloro-3-oxobutanoate (COBE) was purchased from Acros Organics (New Jersey, USA). (R)-ethyl 4-chloro-3-hydroxybutyrate ((*R*)-CHBE) and (S)-ethyl 4-chloro-3-hydroxybutyrate ((*S*)-CHBE) were purchased from Adamas Reagent (Shanghai, China). D-glucose was purchased from Lingfeng Chemical Reagent (Shanghai, China). NADH and NAD^+ were purchased from Solarbio Science & Technology (Beijing, China). All other chemicals used in this study were of analytical grade and commercially available.

2.2. Preparation of magnetic nanoparticles

The chemical co-precipitation method was chosen to prepare the magnetic nanoparticles because of its simplicity and convenience (Ranjbakhsh et al., 2012; Talekar et al., 2012). 1.25 g of ferrous chloride ($FeCl_2{\cdot}4H_2O$) and 3.40 g of ferric chloride ($FeCl_3{\cdot}6H_2O$) were dissolved in 100 mL deionized water at 60 °C with nitrogen gas flow removing oxygen. Then, 6 mL of 25% ammonium hydroxide (NH_4OH) was added and vigorously agitated for 40 min. The magnetite precipitate was collected using magnetic field and washed several times with the deionized water until a pH value of 7 was obtained. The powder was dried at 70 °C for 0.5 h. The obtained magnetic nanoparticles were characterized by field emission scanning electron microscope, infrared spectroscopy and X-ray diffraction.

2.3. Preparation of Combi-CLEAs and magnetic Combi-CLEAs

The Combi-CLEAs was prepared according to the optimized method developed in our previous work (Ning et al., 2014). The prechilled 1, 2-dimethoxyethane was added into the mixture containing same unit of crude ketoreductase (KRED 42) and D-glucose dehydrogenase (GDH 1) to a final concentration of 90% (v/v). After 20 min of precipitation at 4 °C, the glutaraldehyde was added to a final concentration of 0.2% (v/v) to start the cross-linking at 20 °C and 200 rpm for 1 h. Ten-fold volume of 0.1 M Tris-HCl buffer (pH 7.0) was then added to dilute the glutaraldehyde and end the cross-linking reaction. The mixture was centrifuged and the supernatant was discarded. The precipitates (i.e. Combi-CLEAs) were collected and washed with the 0.1 M Tris-HCl buffer (pH 7.0) for three times.

The procedure for magnetic Combi-CLEAs preparation was similar to that of Combi-CLEAs. The self-made magnetic nanoparticles were threw into the mixture containing same unit of crude KRED 42 and GDH 1 before the addition of 1, 2-dimethoxyethane. The magnetic Combi-CLEAs were collected by magnetic field. All other steps were the same as those of Combi-CLEAs. The magnetic nanoparticles amount, cross-linking temperature and time were further optimized to obtain the optimum procedure for magnetic Combi-CLEAs preparation.

2.4. Determination of enzyme activity

The GDH 1 activity was determined in assay mixture of 200 μL containing aliquot of enzyme solution, 0.2 mM NAD^+, 10 mM D-glucose, and 0.1 M Tris-HCl buffer (pH 7.0). A continuous assay using UV absorbance at 340 nm was employed to monitor the concentration of NADH during the reaction at 30 °C. One unit of GDH 1 was defined as the enzyme amount that catalyzed the production of 1 μmol of NADH per minute (Hilt et al., 1991).

The KRED 42 activity was determined in assay mixture of 200 μL containing aliquot of enzyme solution, 0.2 mM NADH, 6 mM COBE, and 0.1 M Tris-HCl buffer (pH 7.0). A continuous assay using UV absorbance at 340 nm was employed to monitor the concentration of NADH during the reaction at 30 °C. One unit of KRED 42 was defined as the enzyme amount that catalyzed the oxidation of 1 μmol of NADH per minute (Shimizu et al., 1990).

The activity of free enzymes mixture/Combi-CLEAs/magnetic Combi-CLEAs was determined in assay mixture of 500 μL containing 50 mM COBE, 0.1 mM NADH, 100 mM D-glucose, aliquot of biocatalyst, and 0.1 M Tris-HCl buffer (pH 7.0). The reaction was performed at 30 °C and 200 rpm. Samples were withdrawn and treated with double volume of ethyl acetate at room temperature for 20 min. The organic phase separated by centrifugation was dehydrated by using appropriate amount of anhydrous sodium sulfate, then determined by gas chromatography (GC) to measure the initial reaction rate. The specific activity of free enzymes mixture/Combi-CLEAs/magnetic Combi-CLEAs was defined as the μmol number of CHBE produced per minute and mg of biocatalyst at 30 °C and pH 7.0 (μmol min^{-1} mg^{-1}).

2.5. Characterization of magnetic Combi-CLEAs

Effect of temperature. The optimum temperature of free enzymes mixture/Combi-CLEAs/magnetic Combi-CLEAs was determined according to the standard method showed in Section 2.4 at temperature ranging from 15 °C to 65 °C. For thermostability analysis, free enzymes mixture/Combi-CLEAs/magnetic Combi-CLEAs was pre-incubated at 20 °C, 30 °C, 40 °C and 50 °C for 30 min to 120 min, and then placed at 30 °C for 15 min. The residual activity was determined at 30 °C according to the standard method showed in Section 2.4.

Effect of pH. The influence of pH on free enzymes mixture/magnetic Combi-CLEAs activity was determined by employing the following 0.1 M buffers: sodium citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 7.0–9.0) and glycine-NaOH buffer (pH 9.0–11.0).

2.6. Effect of COBE concentration on magnetic Combi-CLEAs activity

As for the effect of COBE concentration in aqueous system, the activity of free enzymes mixture/Combi-CLEAs/magnetic Combi-CLEAs was determined according to the standard method showed in Section 2.4 at respectively stable temperature with the COBE concentration ranging from 5 mM to 300 mM. As for the effect of COBE concentration in biphasic system, the activity of free enzymes mixture/Combi-CLEAs/magnetic Combi-CLEAs was determined in assay mixture consisted of 500 μL *n*-butyl acetate containing 25 mM–300 mM COBE and 500 μL Tris-HCl buffer (pH 7.0) containing 0.1 mM NADH and 100 mM D-glucose.

2.7. Reusability of magnetic Combi-CLEAs in aqueous system

The reusability of magnetic Combi-CLEAs in aqueous system was investigated in the reaction mixture of 500 μL containing 30 mM COBE, 0.1 mM NADH, 100 mM D-glucose, moderate amount of magnetic Combi-CLEAs, and 0.1 M Tris-HCl buffer (pH 7.0). The preliminary experiment showed that 30 mM COBE could be converted completely in 23 min. So, 23 min was chosen as the reaction time for each batch. After one batch, the magnetic Combi-CLEAs was recovered for next batch by magnetic field.

2.8. Catalytic performance of magnetic Combi-CLEAs in biphasic system

The catalytic performance of magnetic Combi-CLEAs in biphasic system was investigated by continuous batch reaction. The 20 mL batch reaction mixture was made of 10 mL *n*-butyl acetate containing 0.495 g COBE (3.0 mmoL) and 10 mL Tris-HCl buffer (pH 7.0) containing