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Highly efficient synthesis of ethyl hexanoate catalyzed by CALB-displaying *Saccharomyces cerevisiae* whole-cells in non-aqueous phase

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ABSTRACT

A recombinant *Saccharomyces cerevisiae* displaying *Candida antarctica* lipase B (CALB) on the cell surface was constructed and used as a whole-cell biocatalyst to catalyze the esterification of hexanoic acid and ethanol for the preparation of ethyl hexanoate, a fragrance compound of liquor. Various reaction parameters affecting the esterification catalyzed by CALB-displaying *S. cerevisiae* whole-cells were investigated. The optimal reaction conditions were reaction temperature 40 °C, hexanoic acid concentration 0.2 M, the ratio of hexanoic acid to ethanol 1:1.25 and the amount of cells 60 g/l (17 U/g-dry cell). Molecular sieves (3 Å) were added to the reaction medium as the water absorbent. The yield reached 98.2% after reaction for 12 h under the optimal conditions. The CALB-displaying *S. cerevisiae* whole-cell biocatalyst exhibited quite a good operational stability in the esterification, and more than 95% of its original activity was retained after 10 batches reaction. Thus, CALB-displaying *S. cerevisiae* whole-cell biocatalyst is promising for esters synthesis in non-aqueous phase.

1. Introduction

Ethyl hexanoate is a typical fragrance compound of Chinese liquor and Japanese sake with an annual demand of more than 2000 tons [1,2]. Presently, the synthesis of ethyl hexanoate is mainly based on chemical methods, but its application is somewhat hampered by the environmental concern of the process as well as the increasing demand for natural flavor compounds [2–4]. On the contrary, enzymatic synthesis seems to be a promising alternative due to the mild reaction conditions and being environmentally friendly. More importantly, esters from enzyme-mediated reactions can be considered natural [2,5,6]. To date, few studies have been made of the enzymatic synthesis of ethyl hexanoate except that Abbas *et al.* described the esterification of ethanol and hexanoic acid catalyzed by lipase from *Mucor* sp. immobilized on Amberlite IRC 50 and Xu *et al.* reported the same reaction with whole-cell lipase from *Rhizopus chinesis* CCTCC M201021 [1,5].

Lipases, which have proved to be efficient catalysts for catalyzing the esterification reactions in non-aqueous phase, have been widely applied in preparing various flavor and fragrance esters, such as methyl caproate, ethyl caproate, butyl caproate, allyl caproate, ethyl butyrate, butyl butyrate, isovaleryl butyrate, geranyl butyrate, geranyl acetates, ethyl valerate, hexyl acetate and butyl acetate [1–7]. Among them, lipase from *C. antarctica* B (CALB) exhibited high substrate specificity and catalytic efficiency in the esterification [8–11]. However, little has been known about the catalytic performance of CALB in the production of ethyl hexanoate. Although CALB is promising for the synthesis of esters, the high cost of commercial immobilized CALB limits its industrial application [9,12]. Compared with immobilized enzymes, enzyme-displaying yeast whole-cell biocatalyst seems to be an alternative due to its simplicity, high enzymatic activity and cost-effective as well [9,10,13,14]. Recently, lipase-displaying yeast whole-cell biocatalyst has attracted more and more attentions since it can be used for the synthesis of useful product, such as biodiesel fuel [15].

Recently, Kato *et al.* [9] described the construction of CALB-displaying yeast using α-agglutinin anchor system, and the whole-cell biocatalyst showed a preference for short chain fatty acids. Tanino *et al.* [10] also established CALB-displaying yeast with Flo1p as the anchor, which can catalyze the esterification of adipic acid and *n*-butanol. To get a deeper insight into the catalytic performance of CALB-displaying yeast whole-cell biocatalyst, various kinds of reactions, including synthesis of ethyl hexanoate, should be explored.

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In this work, a CALB-displaying *S. cerevisiae* whole-cell biocatalyst, which was constructed in our laboratory, was applied in the synthesis of ethyl hexanoate. The effects of some major influential factors, such as reaction temperature, substrate concentration, the molar ratio of acid to ethanol and amount of cells addition on the reaction were investigated. Furthermore, the operational stability of CALB-displaying *S. cerevisiae* whole-cell biocatalyst was also examined in order to explore its potential in production of ethyl hexanoate.

2. Materials and methods

2.1. Materials, strains and media

Ethyl haxanoate and *n*-butyl acetate (used as standard) were purchased from Pure Chemical Analysis Co. (Bornew, Belgium). *n*-Heptane was of HPLC grade and purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were also obtained commercially and of analytical grade. Restriction enzymes were purchased from Takara Lt. Co. (Kyoto, Japan).

C. antarctica LF058, *S. cerevisiae* MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) and yeast display vector pICAS were kept in our laboratory [16]. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA), as the host strain for the recombinant DNA manipulation, was grown in LB medium [1% (w/v) tryptone (Difco, Detroit, MI, USA), 0.5% (w/v) yeast extract (Difco), 0.5% (w/v) sodium chloride] containing 50 μg/ml ampicillin at 37 °C for 16 h. *S. cerevisiae* was cultured in SD medium [0.67% (w/v) yeast nitrogen base without amino acids (YNB, Difco) and 2% (w/v) glucose] containing 2% (w/v) casamino acids (Difco) and supplemented with appropriate amino acids when necessary (SDC medium) at 30 °C for 48 h.

2.2. Construction of expression plasmid pICAS–celAL–CALB

To improve the enzyme activity displayed on the yeast surface, an artificially synthesized celAL linker, based on *Neocallimastix patriciarum* Cellulase A (celA) with a FLAG peptide at the C-terminus, was introduced between the CALB and α-agglutinin. This extremely Asn-rich linker is unlikely to be heavily glycosylated, as there are only a few Ser residues and one potential N-glycosylation site present [17]. Restricted enzyme sites, including Bgl II and Xho I at the N-terminus and Sal I at the C-terminus, were also introduced into the linker.

The synthetic linker digested with Bgl II and Sal I, was inserted between the Bgl II and Xho I (an isocaudarner of Sal I) sites of pICAS, thus resulted in the expression vector pICAS–celAL (Fig. 1a). The gene of *CALB* encoding mature CALB with a Pro region was amplified from chromosomal DNA of *C. antarctica* LF058 by polymerase chain reaction (PCR) with the following primers depending on the sequence registered in GenBank (Z30645) [18].

Sense primer is 5′-CTATCA*AGATCT*CTGCCACTCCTTTGGTGAAGC-GTC-3′, antisense primer is 5′-TACATA*CTCGAG*ATAGGGGTGACGAT-GCCGGAG-3′. The amplified fragment was digested with Bgl II and Xho I and ligated into the expression vector pICAS–celAL. The recombinant plasmid was named as pICAS–celAL–CALB (Fig. 1b). The insertion was confirmed by DNA sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Preparation of CALB-displaying whole-cell biocatalyst

The plasmid pICAS–celAL–CALB constructed above was transformed into *S. cerevisiae* MT8-1 cells using Yeast Maker® Kit (Clontech BD, Mountain View, CA, USA) according to the protocol specified by the supplier. Transformants were spread and selected with the activities of lipases displayed on the cell surface on the plate-assay medium. The medium for the plate-assay was prepared as followings: autoclaved SD medium, 1% (w/v) gall powder as an emulsifier and 2% (w/v) agar were mixed 50 μg/ml ampicillin with 0.2% (v/v) tributyrin. The activities of the lipases were examined by the halo formed around the colony. The transformants selected were cultured in 10 ml SDC medium at 30 °C for 48 h, and then the activities of the lipases were assayed by spectrophotometric method using *p*-nitrophenyl butyrate as a substrate. The transformant with the highest hydrolytic activity was cultivated in SDC medium at 30 °C for 96 h. After cultivation, the cells were collected, washed with distilled water twice, and then lyophilized with Christ Alpha 2-4 Freeze Dryer (Christ, Osterode, Germany) for 24 h as the whole-cell biocatalyst to be used. According to the procedure described above, an approximate 10 g lyophilized *S. cerevisiae* whole-cell biocatalyst can be obtained in 1 l of fermentation culture.

2.4. Assay of the hydrolytic activity of whole-cell biocatalyst

The hydrolytic activity of whole-cell biocatalyst was determined spectrophotometrically as described by Kato *et al.* [9]. *p*-Nitrophenyl butyrate, as the substrate, was emulsified in 50 mM potassium phosphate buffer (pH 7.5) containing 0.5% Triton X-100, followed by adjusting its concentration to 2.5 mM. Lyophilized whole-cell biocatalyst (2 g) was suspended in 50 ml potassium phosphate buffer. Then 500 μl aliquot of the cells and an equal volume of the substrate were mixed and incubated at 30 °C for 5 min. The reaction mixture was centrifuged at 10,000 × *g*, 4 °C for 10 min. A 200 μl aliquot of the resulting supernatant was measured at 405 nm with spectrophotometer (Tecan Sunrise, Männedorf, Switzerland). A control reaction was performed with the same procedure without whole-cell biocatalyst [19].

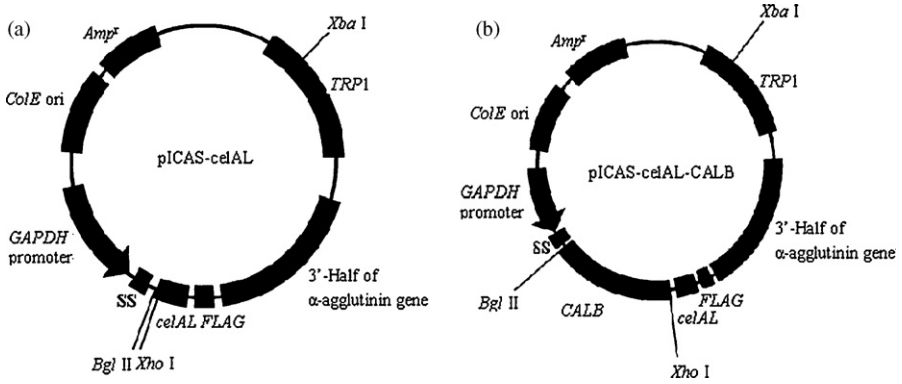

Fig. 1. Schematic overview of the yeast cell surface display plasmid pICAS–celAL (a) and pICAS–celAL–CALB (b). SS, secretion signal sequence of the glucoamylase gene.

2.5. Esterification reaction

All the reaction media were dehydrated by gentle shaking with 3 Å molecular sieves overnight before being used. In a typical experiment, 5 ml of *n*-heptane containing 1 mmol hexanoic acid and a predetermined quantity of ethanol was added to a 25-ml Erlenmeyer shaking flask capped with a septum. The reaction was initiated by addition of 0.2 g lyophilized whole-cell biocatalyst to the reaction system at 200 rpm and an appropriate temperature for 24 h. Samples of 50 μl were periodically withdrawn and centrifuged (10,000 × *g*, 10 min), and 20 μl upper layer was mixed with 1 ml *n*-heptane containing *n*-butyl acetate for GC analysis. Details about the ratio of hexanoic acid to ethanol and temperature were specified for each case.

In the reactions the whole-cell biocatalysts were reused, the relative activity of the biocatalyst was determined by the ratio of the yield of each batch to the yield of the first batch and the relative activity of the first batch was regarded as 100%.

2.6. GC analysis

The yield of ethyl hexanoate in the reaction mixture was assayed with a Agilent 7890A gas chromatograph equipped with a hydrogen flame-ionization detector and a DB-FFAP silica capillary column (0.25 mm × 30 m, Agilent, Santa Clara, CA, USA). The column temperature was held at 50 °C for 1 min, then upgraded to 60, 68, 95 °C and 200 °C at 10, 40, 20 and 40 °C/min, respectively, and then kept for 2 min. Injector and detector temperatures were both set at 250 °C. The carrier gas was nitrogen.

The initial velocity (V_0) and the yield of the reaction were calculated from the GC data.

The average error for this determination was less than 0.7%. All reported data were averages of experiments performed at least in duplicate.

3. Results and discussion

3.1. Preparation of yeast cells displaying CALB for whole-cell biocatalyst

A CALB-displaying *S. cerevisiae* was constructed before investigating its catalytic performance. Mature CALB with a Pro region from *C. antarctica* LF058 was initially displayed on the yeast cell surface by directly fusing with α-agglutinin. Unfortunately, the resulting lipase showed very low activity (data not shown). So the celA linker was introduced between the CALB and α-agglutinin, which had been successfully used to connect CALB and cellulose-binding domain without interfering with lipase activity [11]. The expression plasmid, pICAS–celAL–CALB, was constructed according to the method described in Section 2.2 and transformed into *S. cerevisiae* MT8-1. The recombinant *S. cerevisiae* displaying the lipase on the cell surface was cultivated and lyophilized as a whole-cell biocatalyst to be used for the esterification of hexanoic acid and ethanol. The hydrolytic activity of the lyophilized whole-cell biocatalyst was determined as 17 U/g-dry cell, which was similar to that of *S. cerevisiae* displaying CALB CBS6678 using the Flo1p anchor system [10].

3.2. Effect of substrate concentration on the esterification of hexanoic acid and ethanol

It is well known that the substrate concentration is an important parameter affecting enzyme activity and potential application [1,20]. Thereby, the effect of varying concentrations of hexanoic acid in the range of 0.05–1.4 M on the initial velocity and yield of esterification was investigated. As shown in Fig. 2, the initial velocity increased with the concentration of hexanoic acid up to 0.2 M, beyond which further rise in the hexanoic acid concentrations resulted in a sharp drop in the initial velocity. It is indicated that there exists substrate inhibition in the esterification, which is consistent with previous reports [6,7,20]. Notably, the yield could still reach as high as 96.5% after 24 h even when the hexanoic acid concentration was 1.4 M. It is suggested that the enzyme displayed on the cell surface has a good tolerance to hexanoic acid. This maybe result from that the cell, which the lipase was bound to, had the ability to provide suitable microenvironment when enzyme existed in solvent system and inhibit the accumulation of polar substrate from damaging microaqueous layer [1,21]. Here, considering the residuary activity of whole-cell biocatalyst after each batch and the whole productivity of per mass unit of hexanoic acid, the optimal concentration of hexanoic acid was regarded as 0.2 M.

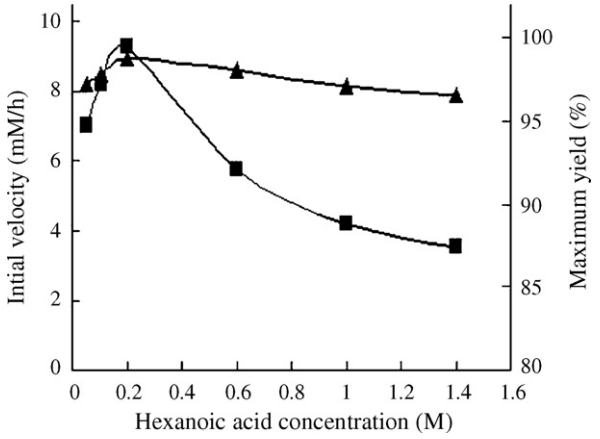

Fig. 2. Effect of the substrate concentration on the esterification. The reactions were carried out in 5 ml of *n*-heptane containing varying concentrations of hexanoic acid ranging from 0.05 to 1.4 M with molar ratio of hexanoic acid to ethanol of 1:1.25, and 0.2 g lyophilized whole-cell biocatalyst at 200 rpm and 40 °C. Molecular sieves (3 Å) were added to the reaction system to remove the water produced in the reaction. Symbols: (■) initial velocity; (▲) maximum yield.

3.3. Effect of molar ratio of hexanoic acid to ethanol on the esterification

Thermodynamically, a high substrate concentration may push the reaction towards the product formation and speed up the reaction. In the case of esterification reactions of acids and alcohols, when acid concentration is fixed, the esterification catalyzed by whole-cell is greatly affected by the molar ratio of acid to alcohol [1,4]. As illustrated in Fig. 3, a clear decline in the initial velocity was observed when the molar ratio of hexanoic acid to ethanol was beyond 1:1. There are several possible reasons for this. One is that the enzyme was partly inactivated by the excessive ethanol in the reaction system [1]. On the other hand, the substrates such as methyl and ethyl alcohols, which are too small, could not release enough energy to change lipase conformation to the desired catalytically active form [5,22,23].

However, the maximum yield of the reaction increased with the molar ratio of hexanoic acid to ethanol up to 1:1.25, beyond which the maximum yield showed no appreciable improvement with further increase in the molar ratio of hexanoic acid to ethanol. It is obvious that a little excessive amount of ethanol was necessary for the high yield, which was in good accordance with the reported results [1]. Thereafter, the molar ratio of hexanoic acid to ethanol was set at 1:1.25 in the subsequent experiments.

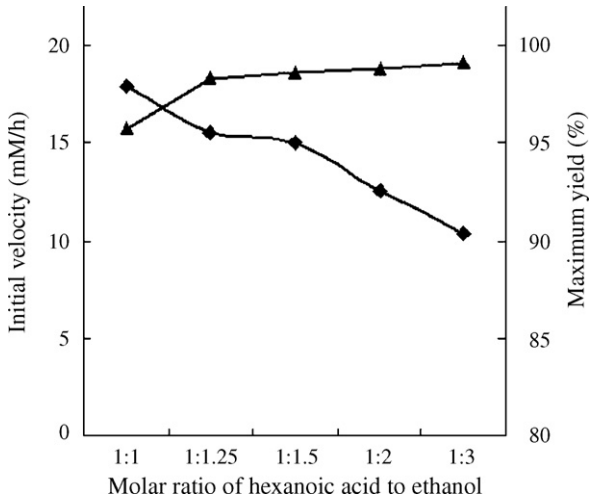

Fig. 3. Effect of the molar ratio of hexanoic acid to ethanol on the esterification. The reactions were carried out in 5 ml of *n*-heptane containing 1 mmol hexanoic acid, varying amount of ethanol and 0.2 g lyophilized whole-cell biocatalyst at 200 rpm and 40 °C. Molecular sieves (3 Å) were added to the reaction system to remove the water produced in the reaction. Symbols: (■) initial velocity; (▲) maximum yield.

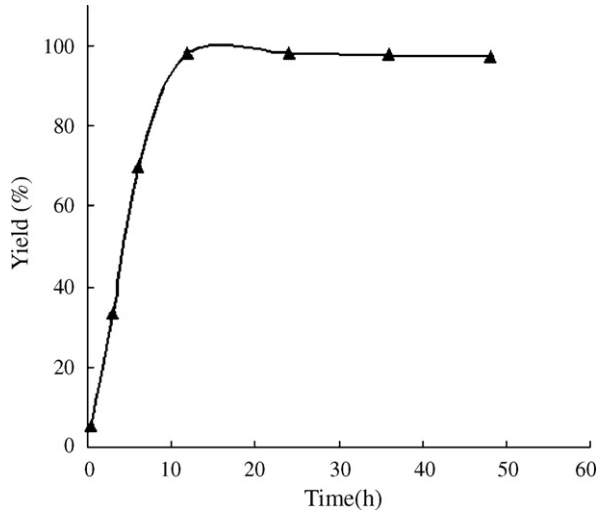

Fig. 5. Time course of whole-cell biocatalyst-mediated esterification. Reaction conditions: 5 ml of *n*-heptane containing 1 mmol hexanoic acid, 1.25 mmol ethanol, 0.3 g lyophilized whole-cell biocatalyst at 200 rpm and 40 °C. Molecular sieves (3 Å) were added to the reaction system to remove the water produced in the reaction.

3.4. Effect of temperature on the esterification

Temperature has great effect on the activity, selectivity and stability of a biocatalyst and the thermodynamic equilibrium of a reaction as well. As shown in Fig. 4, within the range from 25 to 60 °C, higher temperature resulted in higher initial velocity since higher temperature accelerates molecular diffusion. However, the maximum yield of the reaction increased with the increase of temperature from 25 to 40 °C and reached the maximum of 98.2% at 40 °C. Further rise in temperature beyond 40 °C could slightly pull down the reaction yield. This is due to the partial inactivation of the enzyme in organic solvent at high temperature for a long time because CALB protein undergoes partial unfolding by heat-induced destruction of non-covalent interactions [24]. Thus, appropriate temperature for this reaction was set at 40 °C. It is worth noting that a high yield of 96.0% was obtained even at 60 °C, indicating that CALB displayed on the yeast surface showed good thermostability, which was in good agreement with reports by Kato *et al.* [9] and Tanino *et al.* [10].

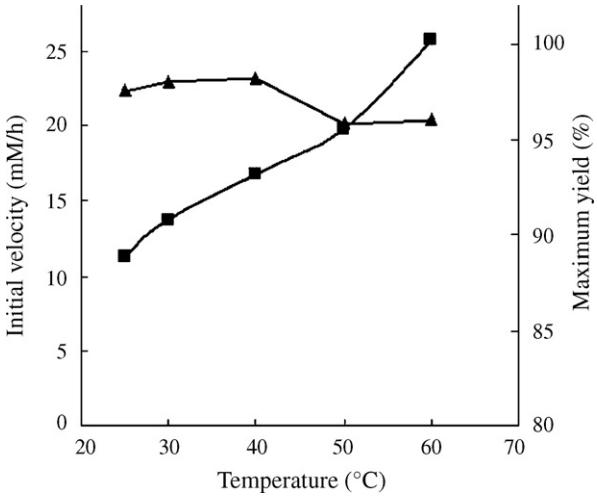

Fig. 4. Effect of temperature on the esterification. The reactions were carried out in 5 ml of *n*-heptane containing 1 mmol hexanoic acid, 1.25 mmol ethanol and 0.2 g lyophilized whole-cell biocatalyst at 200 rpm and different temperature. Molecular sieves (3 Å) were added to the reaction system to remove the water produced in the reaction. Symbols: (■) initial velocity; (▲) maximum yield.

3.5. Effect of amount of whole-cell biocatalyst and time course of the esterification

Lyophilized whole-cell biocatalyst was added into esterification reaction system with different concentration levels from 10 to 80 g/l at a substrate concentration of 0.2 M. The yield underwent a dramatic rise when the concentration of whole-cell biocatalyst changed from 10 to 60 g/l after 12 h, and then no significant change in the yield was observed with the cell concentration more than 60 g/l. The possible reason is that lots of active sites of the internal enzyme molecules cannot be exposed to the substrates and bulk cells particles limit mass transport [1]. Moreover, lower activity of the inner fraction of the biocatalyst may decrease the catalytic efficiency of per mass unit of the whole-cell biocatalyst, which was also found in the esterification catalyzed by other lipases [25–27]. So, the optimal amount of cells was 60 g/l in the reaction mixture.

The time course of whole-cell biocatalyst-mediated esterification of hexanoic acid with ethanol under the optimal conditions was depicted in Fig. 5. The reaction proceeded at a fairly high rate in 30 min, followed by a substantial deceleration. This could be accounted for by the decreasing hexanoic acid concentration and the acceleration of the hydrolysis of the product ethyl hexanoate. Additionally, the partial inactivation of the enzyme by ethanol as well as hexanoic acid contributed to the deceleration of the reaction [5,22,23]. After around 12 h, the yield reached the maximum and kept constant of more than 98.1% in the time of 12∼24 h and then slightly went down owing to the hydrolysis of ethyl hexanoate. The similar result was also obtained by enzymatic synthesis of ethyl hexanoate catalyzed by immobilized lipase from *M.* sp. [5]. The results here suggested that CALB-displaying *S. cerevisiae* is a promising biocatalyst, which can be a good alternative to immobilized enzyme in the synthesis of flavor esters [9].

3.6. Operational stability of whole-cell biocatalyst

To further examine the potential of whole-cell biocatalyst for ethyl hexanoate production, its operational stability was investigated. The whole-cell biocatalyst was recovered by centrifugation after each batch reaction (one batch for 12 h), followed by wash-

ing with *n*-heptane to remove any substrate or product absorbed onto the whole-cell biocatalyst and then introduced into the fresh reactants. The relative activity of the whole-cell biocatalyst still remained above 95% after being reused for 10 batches. The possible reason is that washing the whole-cell biocatalysts with highly volatile solvent, such as *n*-heptane, can drain out the yeast cells and avoid yeast cells clumping, which limits mass transport and decreases the exposure of the active sites to substrates [7]. In addition, the whole-cell biocatalyst also exhibited good storage stability without significant decrease in activity after storing for 4 months at room temperature. These results further prove that the whole-cell biocatalyst is promising for the production of ethyl hexanoate on industrial scale.

4. Conclusions

CALB fusing with celA linker was displayed on the cell surface of *S. cerevisiae* with α-agglutinin as an anchor protein. And the CALB-displaying *S. cerevisiae* was successfully employed as a whole-cell biocatalyst to catalyze the esterification of hexanoic acid and ethanol to produce ethyl hexanoate in *n*-heptane. Under the optimal conditions the yield was as high as 98.2% after reaction for 12 h. Furthermore, the whole-cell biocatalyst exhibited good operational stability as well as storage stability, thus promising in industrial production.

Acknowledgements

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