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Biocatalytic behavior of a new *Aspergillus niger* whole-cell biocatalyst with high operational stability during the synthesis of green biosolvent isopropyl esters

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ABSTRACT

Using microbial cell-surface displayed enzymes to produce chemicals is a promising green chemistry procedure, but few studies evaluated filamentous fungi cell-surface display systems. Here, a novel *Aspergillus niger* whole-cell biocatalyst with excellent operational stability was constructed using a heterologous anchor protein, and the details about its practical catalytic characteristics during the synthesis of green biosolvent isopropyl esters (IPEs) were investigated. *Candida antarctica* lipase B (CALB) was chosen as a model enzyme, fused to a *Saccharomyces cerevisiae* glycosylphosphatidylinositol protein, SED1, and displayed on the *A. niger* surface, the hydrolytic activity of the *A. niger* mycelium-surface displayed CALB reached 440.30 U/g dry cell. In solvent-free esterifications of medium-long chain fatty acids and isopropanol, the *A. niger*/CALB-Flag-SED1 whole-cell biocatalyst showed great organic substrate tolerance, high thermostability, no significant lag time in a dry initial condition and no synthetic activity decrease after five batch reactions at 65 °C. The highest yields of isopropyl laurate, isopropyl myristate, and isopropyl palmitate, 79.21, 81.62, and 81.41%, respectively, were obtained after a 6-h reaction. Acetone washing helped to remove the excessive water accumulating during esterification and benefitted the operational stability. The relative acid conversions of the three IPEs in batch 5 were 99.79, 99.10, and 96.83%, respectively, of those in batch 1. The *A. niger*/CALB-Flag-SED1 whole-cell biocatalyst is a promising alternative to commercial immobilized products, and SED1 can serve as an efficient anchor module to display industrial enzymes in an *A. niger* expression system.

1. Introduction

Fatty acid isopropyl esters (IPEs) have been in increasing demand because of their numerous applications in the cosmetic, pharmaceutical, food, and other industries [1–4]. By virtue of its skin penetration enhancement function for actives and excellent spreading properties, isopropyl myristate is used as a co-solvent in transdermal pharmaceutical preparations and as a substitute for natural oils in cosmetics [5–9]. Nowadays, new synthesis methods satisfying the concept of green chemistry are being developed to produce esters in large quantities and at low cost [1]. To avoid the drawbacks of chemical methods, such as high temperatures, undesirable by-products, and objectionable flavors [1], enzymatic catalysis is considered to be a green chemistry procedure to manufacture IPEs for personal care use. Additionally, a solvent-free reaction system can lower costs, enhance yields, and increase safety [1]. Process economics, particularly the cost of enzymes or biocatalysts needs to be comparable with current chemical manufacturing practices. At the moment, enzymes and biocatalysts used in esterification are more expensive than chemical catalysts [10–14]. Hence, it is attractive to develop new enzymes or biocatalysts and characterize the practical synthetic properties during the manufacturing processes.

Microbial cell-surface display techniques allow for the efficient expression of functional proteins on the cell surface through linkage with a genetically fused anchor protein. Recombinant strains that display active industrial enzymes on their cell surface are considered to be promising whole-cell biocatalysts in numerous applications [15]. These tailor-made biocatalysts appear to be attractive alternatives to expensive, commercially available immobilized products. Because of their attractive characteristics, lipases (EC 3.1.1.3) have been used as pillar biocatalysts in many

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large-scale commercial applications, especially in the food and chemical industries [11,13]. *Candida antarctica* lipase B (CALB), a well-known and widely used industrial biocatalyst, is a particularly efficient and robust lipase with surprising specificity and activity [16]. In previous studies, CALB was chosen as a model enzyme to develop and test several microbial cell-surface display systems, including *Escherichia coli* [17], *Saccharomyces cerevisiae* [18,19], and *Pichia pastoris* [14,20,21] systems. Furthermore, several recombinant *Aspergillus oryzae* whole-cell biocatalysts expressing functional CALB [22–24], taking advantage of the phenomenon that cell immobilization on biomass support particles strongly prevents the active secretion of enzyme into the culture medium and results in high mycelium-bound enzymatic activity, were also reported, and no anchor protein was used [25].

In our previous study, CALB was functionally displayed on the mycelium-surface of *Aspergillus niger* using an endogenous cell wall mannoprotein, CwpA, and showed great synthetic activity for the enzymatic production of ethyl esters from a series of fatty acids of different chain lengths in solvent-free system [26], but it still need to improve the *A. niger* cell surface display system and develop new *A. niger* cell surface display system to enrich the filamentous fungi cell surface display system. The use of different anchor proteins can significantly influence the activity of displayed enzymes [27,28]. SED1, a major stress-induced structural glycosylphosphatidylinositol (GPI) protein in *S. cerevisiae*, has been reported to be an efficient anchoring domain for displaying functional enzymes in *S. cerevisiae* [29] and *P. pastoris* [30]. Though the major components of fungal cell walls are glycoproteins, glucan (mainly 1,3-β-glucan) and chitin (1,4-β-N-acetylglucosamine) polymers, there are still considerable differences in the cell wall compositions and contents between filamentous fungi and yeasts [31]. For example, α-glucans are not present in the cell wall of *S. cerevisiae*, whereas they are important cell wall polymers in *A. niger* [32]. During the production of homologous and heterologous proteins controlled by the *A. niger* glucoamylase (glaA) promoter, fed-batch submerged cultivation with carbon substrate limitation to preventing catabolite repression is a commonly used strategy, which can also cause stress to *A. niger* and lead to cell wall remodeling and complex constituent variation of the cell wall polymers [33]. The feasibility of using the heterologous GPI protein SED1 as an anchor module in filamentous fungi needs to be confirmed. Furthermore, the details about the practical catalytic properties of the *A. niger* cell surface displayed lipase, especially the operational stability, have not yet been reported.

In this study, CALB was still chosen as a model enzyme to test the new *A. niger* cell surface display system using the GPI anchoring region derived from *S. cerevisiae* SED1. The gene cassette for the cell-surface display of CALB was integrated into the *A. niger* genome, and the lipase activity of the *A. niger* whole-cell biocatalyst was evaluated. Additionally, the catalytic reaction process parameters and the practical biocatalytic behavior of the *A. niger* mycelium-surface displayed lipase in synthesis of green biosolvent IPEs were further characterized in a solvent-free system. The results presented here indicate that the SED1 anchoring region from *S. cerevisiae* can serve as a highly efficient anchor module to display industrial enzymes in an *A. niger* expression system and provide a potential promising alternative to commercially available immobilized products.

2. Materials and methods

2.1. Strains and vector

The *A. niger* SH-1 strain, stored in our laboratory [34], was used as the host for the construction of the cell surface display system. *E. coli* TOP 10 (Life Technologies, Carlsbad, CA, USA) was used as the host for recombinant DNA manipulation. Plasmid pUAGA developed in our previous study [26] was used to construct the *A. niger* cell-surface display vector.

2.2. Media

Luria-Bertani (LB) medium containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, at a pH 7.0, was used for cultivation of *E. coli*. Fifty micrograms of ampicillin per milliliter were added to LB medium for growing the *E. coli* transformants. Czapek–Dox (CD-NO_3) medium plates containing 2% (w/v) sucrose, 0.3% (w/v) $NaNO_3$, 0.2% (w/v) KCl, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 0.001% (w/v) $FeSO_4{\cdot}7H_2O$, 2% (w/v) agar, at a pH 5.5, were used for cultivation of *A. niger* SH-1. Modified Czapek–Dox (CD-Acea) medium plates containing 1 M sucrose, 10 mM acetamide, 15 mM CsCl, 0.2% (w/v) KCl, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 0.001% (w/v) $FeSO_4{\cdot}7H_2O$, 2% (w/v) agar, at a pH 5.5, were used to select *A. niger* transformants. XAPY medium containing 4% (w/v) xylose, 1.5% (w/v) $(NH_4)_2SO_4$, 0.75% (w/v) peptone and 1% (w/v) yeast extract, at a pH 6.0, was used to grow positive *A. niger* recombinants. MAPY medium containing 0.5% (w/v) maltose, 1.5% (w/v) $(NH_4)_2SO_4$, 0.75% (w/v) peptone and 1% (w/v) yeast extract, at a pH 6.0, was used to express the fusion protein CALB-Flag-SED1.

2.3. Chemicals and reagents

Mouse anti-Flag monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 488 conjugated goat antimouse IgG (H + L) was purchased from Molecular Probes (Eugene, OR, USA). Isopropanol (high-performance liquid chromatography grade), lauric acid (98%), myristic acid (98%), and palmitic acid (98%) were purchased from Sigma-Aldrich. All other chemicals were also obtained commercially and were of analytical grade.

2.4. Construction of the Aspergillus niger cell surface display plasmid pCALB-SED1

The sequence encoding mature CALB was subcloned from the plasmid pICAS-celAL-CALB [19] using *CALB* primers (5′-***TTCCAAGCGCGTCGAC***GCCACTCCTTTGGTGAAGCG-3′, 5′-CTTATCGTCGTCATCCTTGTAATCGGGGGTGACGATGCCGGAGCAGGT-3′, underlined sequence indicates the sequence encoding the Flag peptide tag). The sequence encoding the anchoring region of SED1 (**X66838**) was amplified from the *S. cerevisiae* genomic DNA using *SED1* primers (5′-***GATGACGACGATAAG***CAATTTTCCAACAGTACAT-3′, 5′-***ACGCTTCCTTCATACGTA***TTATAAGAATAACATAGC-3′). The ***bold italic*** sequences in *CALB* primers and *SED1* primers indicate the overlap sequences for the In-Fusion cloning reaction. These two fragments were ligated with the longer fragment from pUAGA [26] digested with *Sal* I and *Sna*B I to construct the *A. niger* cell-surface display vector pCALB-SED1 using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA, USA) in one step. The cloning procedure is illustrated in Fig. 1. All ligations of DNA fragments were confirmed by DNA sequencing.

2.5. Transformation and selection of the Aspergillus niger recombinants

Acetamide is a poor sole nitrogen source for the host strain *A. niger* SH-1 in the presence of sucrose and caesium chloride [35]. The positive transformants can hydrolyze acetamide and grow on a medium containing acetamide as the sole nitrogen source since the *amdS* gene from the cell surface display vector pCALB-SED1 (Fig. 1) coding for an acetamidase was integrated into their genome. The