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Displaying *Candida antarctica* lipase B on the cell surface of *Aspergillus niger* as a potential food-grade whole-cell catalyst

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Abstract *Aspergillus niger* is a recognized workhorse used to produce food processing enzymes because of its extraordinarily high protein-producing capacity. We have developed a new cell surface display system *de novo* in *A. niger* using expression elements from generally recognized as safe certified microorganisms. *Candida antarctica* lipase B (CALB), a widely used hydrolase, was fused to an endogenous cell wall mannoprotein, CwpA, and functionally displayed on the cell surface. Localization of CALB was confirmed by enzymatic assay and immunofluorescence analysis using laser scanning confocal microscopy. After induction by maltose for 45 h, the hydrolytic activity and synthesis activity of *A. niger* mycelium-surface displayed CALB (AN-CALB) reached 400 and 240 U/g dry cell, respectively. AN-CALB was successfully used as a whole-cell catalyst for the enzymatic production of ethyl esters from a series of fatty acids of different chain lengths and ethanol. In a solvent-free system, AN-CALB showed great synthetic activity and afforded high substrate mole conversions, which amounted to 87 % for ethyl hexanoate after 2 h, 89 % for ethyl laurate after 2 h, and 84 % for ethyl stearate after 3 h. These results suggested that CwpA can act as an efficient anchoring motif for displaying enzyme on *A. niger*, and AN-CALB is a robust, green, and cost-effective alternative food-grade whole-cell catalyst to commercial lipase.

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Keywords Cell surface display · *Aspergillus niger* · Food processing enzyme · *Candida antarctica* lipase B · Solvent-free enzymatic synthesis

Introduction

There are growing demands for safe food ingredients that are isolated or synthesized by economic and green technologies in accord with the principles of green chemistry, and the use of naturally available substrates and food-grade catalysts is an essential part of producing process design [5, 31, 34]. When compared with chemical catalysts, enzymes are powerful catalysts for a number of reactions, as they provide higher selectivity, and the reaction conditions are often milder and environmentally friendlier. Enzymes produced from generally recognized as safe (GRAS)-certified microorganisms are preferred in the food processing industry [12]. In industrial applications, it is desirable that these products be obtained in solvent-free medium, because this approach avoids the costly separation of toxic and flammable organic solvents from the target product and enzyme in solvent-based methods [27, 33]. Therefore, developing food-grade enzymes with desirable capacity can provide significant benefits in terms of the safety properties of the obtained products and green features of the industrial processes.

Lipases, owing to their attractive characteristic of being efficient in catalyzing esterification reactions in non-aqueous phase, have been used in many chemical processing applications [10]. Among the lipases used in organic synthesis, *Candida antarctica* lipase B (CALB) has been found to be a particularly efficient and robust lipase with surprising specificity and activity [4]. Cell-surface displayed enzymes are tailor-made biocatalysts and appear to be an

attractive alternative to the expensive commercially available immobilized productions. In previous studies, synthesis of ethyl caproate [17], dibutyl adipate [40], polybutylene adipate [41], and enantiomeric ethyl lactate [18] catalyzed by CALB-displaying *Saccharomyces cerevisiae* whole cells had been described. A CALB-displaying *Pichia pastoris* whole-cell biocatalyst using the α-agglutinin anchor system was reported, which showed high synthetic activity for the production of esters from short-chain fatty acids [35]. In addition, Jin et al. [19] also investigated the synthesis of 12 short-chain flavor esters catalyzed by CALB-displaying *P. pastoris* whole cells in a 5-l batch stirred reactor. These results demonstrate that cell surface displayed enzymes may serve as robust biocatalysts with potential commercial applications for large-scale manufacturing of bulk chemicals, especially in the food, beverage, and cosmetic industries.

Aspergillus niger, a versatile platform microbe in biotechnology, is particularly suitable for food and drug production, and has been certified as a GRAS filamentous fungus by the Food and Drug Administration (USA) and the World Health Organization [25]. Enzymes produced by *A. niger* are likely to be accepted in the food processing industry [12]. Recently, *A. niger* has been used as a host for large-scale production of homologous and heterologous enzymes required in food industries. This organism has been proven to be beneficial because the production levels of secreted enzyme are extraordinarily high, and many specific *A. niger* production processes have been certified as GRAS [29, 32]. There are only a few reports describing cell surface display systems in filamentous fungi, and two cell surface display systems were developed in *Aspergillus oryzae* using different anchor proteins [3, 36]. Furthermore, several recombinant *A. oryzae* whole-cell biocatalysts expressing functional protein, 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 mycelia-bound activity, were also reported, and no anchor protein was used [1, 2, 16, 38, 39]. However, there have been no reports on cell surface display systems in *A. niger* and non-mutant commercially useful filamentous fungi without the introduction of additional mutations to the host to obtain auxotrophic phenotype.

In the present study, we have developed a novel cell surface display system in *A. niger* to expand the filamentous fungi cell surface display system for production applications. The methods described here provide an applicable guide for displaying enzymes on a wide range of filamentous fungi, especially some useful commercial fungi of which the genetics is little understood and no information is available about the complementation of the mutation transformation system. All the expression elements were from GRAS-certified food industrial strains, thus facilitating the use of the recombinant lipase as a food-grade whole-cell catalyst in the food processing industry. The synthetic capacity and practical properties of the *A. niger* mycelium-surface displayed CALB (AN-CALB) were further characterized in solvent-free system, and the results indicated that the system provides a potential biocatalyst for food processing industry applications.

Materials and methods

Strains, media, and materials

Escherichia coli TOP 10 (Life Technologies, Carlsbad, CA, USA) was used as a host for recombinant DNA manipulation and was grown in Luria–Bertani medium (1 % w/v tryptone, 0.5 % w/v yeast extract, 1 % w/v sodium chloride, pH 7.0) with ampicillin (50 μg/ml) added when necessary. *A. niger* SH-1 stored in our lab was used as a host for the construction of the cell surface display system [30]. Czapek–Dox (CD-NO_3) medium plates (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 $FeSO_4{\cdot}7H_2O$, pH 5.5) containing 2 % agar were used to grow the host *A. niger* SH-1. Modified Czapek–Dox (CD-Acea) medium plates (replacing 0.3 % $NaNO_3$ with 10 mM acetamide, and 2 % sucrose with 1 M sucrose, and 15 mM CsCl was added) were used to select the fungal transformants. The positive transformants were shaken in XAPY medium (4 % w/v xylose, 1.5 % w/v $(NH_4)_2SO_4$, 0.75 % w/v peptone and 1 % w/v yeast extract, pH 6.0). The fermentation broth was centrifuged at 6,000×*g* for 10 min, and the sediment was washed twice with normal saline and resuspended in an equal volume MAPY medium (0.5 % w/v maltose, 1.5 % w/v $(NH_4)_2SO_4$, 0.75 % w/v peptone and 1 % w/v yeast extract, pH 6.0) to induce the expression of the fusion protein, CALB-Flag-CwpA (CALB-C). A mouse anti-FLAG monoclonal antibody was obtained from Sigma (St. Louis, MO, USA). Alexa Fluor 488 conjugated goat antimouse IgG (H + L) was purchased from Molecular Probes (Eugene, OR, USA).

Construction of the cell surface display vector for *Aspergillus niger*

The *A. niger* cell surface display vector was constructed as follows. The selective marker *Aspergillus nidulans amd*S encoding fragment (**M16371**) was amplified from pAMDS (constructed in our lab) using a pair of *amdS* primers. The promoter sequence (Pgla) with the signal sequence and pro-sequence of the endogenous glucoamylase encoding gene was amplified from the genomic DNA of *A. niger*

Table 1 Primers used to amplify fragments for expression cassette construction used in this study

Names or genes	Primer sequences
amdS	5-GACCATGATTACGCCCTTAAGGGCCCTGAAGGTCGGATGTAC-3 5-TTGGAGTTCGGATCCGCGGCCGCTACTGATGTCTATTGGAAGAAAACT-3
Pgla	5-GGATCCGAACTCCAACCGGGGGGAGTAG-3 5-GCGCTTGGAAATCACATTTGCCAACCCT-3
TagdA	5-GTGATTTCCAAGCGCGTCGACGAGATGTACGTATGAAGGAAGCGTAACAGGATAG-3 5-AAAACGACGGCCAGTCACGTGGGCAGTAACCCATTCCCGGTTCTCTAGC-3
CALB	5-TTCCAAGCGCGTCGACGCCACTCCTTTGGTGAAGCG-3 5-CTTATCGTCGTCATCCTTGTAATCGGGGGTGACGATGCCGGAGCAGGT-3
CwpA	5-GATGACGACGATAAGAACCCCACCAAGGTTGCTCG-3 5-ACGCTTCCTTCATACGTATTAGACAGCGATGGCAATG-3
pUC19	5-ACTGGCCGTCGTTTTACAACGTCGT-3 5-GGCGTAATCATGGTCATAGCTGTTTC-3

The restriction enzyme sites are *underlined*

SH-1 using *Pgla* primers. The terminator region (**E12508**) of the alpha-glucosidase (TagdA) encoding gene of *A. oryzae* was amplified from pAGDA (constructed in our lab) using a pair of *TagdA* primers. These elements were then ligated sequentially with a fragment from the plasmid pUC19 (amplified with pUC19 primers) using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA, USA) in one step. The resulting plasmid was designated as pUAGA. The coding sequence for CALB was subcloned from the plasmid pICAS-CALB [17] using *CALB* primers. The coding sequence for the anchored protein CwpA was amplified from the genomic DNA of *A. niger* SH-1 using *CwpA* primers. These two fragments were digested with *Sal* I and *SnaB* I, and ligated with the longer fragment of pUAGA digested with *Sal* I and *SnaB* I to construct *A. niger* cell surface display vector pCALB-C in one step. The sequences of the PCR primers used are shown in Table 1.

Transformation and selection of the recombinants

The expression vector of pCALB-C was digested with *Afl* II and *PmaC* I, and the CALB-display expression cassette with the *amd*S selective marker was transformed into *A. niger* SH-1 by treating the protoplasts with polyethylene glycol according to the method described by Gomi et al. [14] and Tilburn et al. [42]. The transformants were selected on CD-Acea medium (without $NaNO_3$) containing 1 M sucrose as an osmotic stabilizer and 10 mM acetamide as the sole nitrogen source, and confirmed by mycelia PCR. Acetamide is a poor sole nitrogen source for *A. niger* SH-1 in the presence of sucrose and cesium chloride [22]. The *amd*S gene on cell surface display vector pCALB-C (Fig. 1) codes for an acetamidase enzyme which hydrolyses acetamide, enabling stable *amd*S$^+$ transformants to show vigorous growth on acetamide medium.

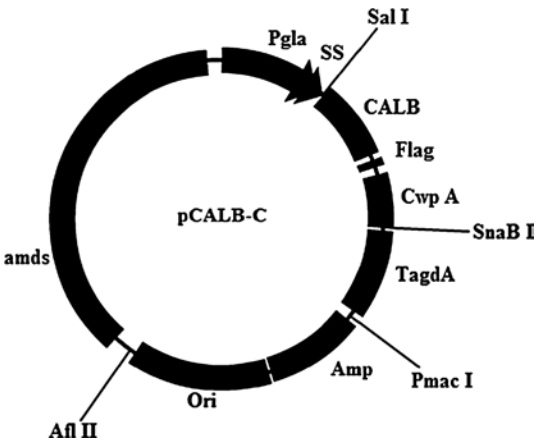

Fig. 1 Map of the plasmid pCALB-C for *A. niger* cell surface display system. The coding sequence for CALB was fused at the C-terminus with a Flag tag and an endogenous GPI-anchored protein CwpA to form the cell surface display cassette, under the control of the endogenous glucoamylase (Pgla) promoter, signal sequence, prosequence and the terminator of alpha-glucosidase (TagdA) encoding gene from *A. oryzae*. *A. nidulans* acetamidase-encoding gene (*amd*S) was used as the dominant selectable marker

Expression of cell surface displayed fused protein and lipase activity assay

The positive transformants of *A. niger* pCALB-C were incubated at 30 °C and 300 rpm for 2 days in a 500-ml flask containing 50 ml of liquid XAPY medium. The fermentation broth was centrifuged at 6,000×*g* for 10 min, and the sediment was washed and resuspended with equal volume of MAPY medium to induce fusion protein (CALB-C) expression. Furthermore, 0.5 % maltose (final concentration, w/v) was added to the culture every 12 h to maintain the induction of the fusion protein. The hydrolytic activity of CALB was determined spectrophotometrically using *p*-nitrophenyl butyrate (*p*NPB) as the substrate. A total of

25 mM *p*NPB was emulsified in 50 mM Tris–HCl buffer (pH 8.0) with 1 % Triton X-100. *A. niger* mycelium was collected by centrifugation at 6,000×*g* for 10 min at room temperature. The mycelium was washed twice with Tris–HCl buffer (pH 8.0), and the mycelium suspension was diluted when necessary. Subsequently, a 50-μl aliquot of the mycelium suspension and an equal volume of the substrate were mixed with 900 μl Tris–HCl buffer (pH 8.0) and allowed to react at 45 °C for 5 min. The reaction was stopped by centrifuging the reaction mixture at 16,000×*g* for 1 min at room temperature. Then, a 200-μl aliquot of the resulting supernatant was applied into a 96-well plate and the activity of CALB was assayed by measuring the absorbance of the liberated *p*-nitrophenol (*p*NP) at 405 nm using a kinetic microplate reader. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol *p*NP per minute. All the experiments were carried out at least in triplicate [35]. The synthetic activity was measured by the ethyl hexanoate synthesis method similar to that described by Jin et al. [19].

Immunofluorescence microscopy assay

The *A. niger* mycelium was immunostained and analyzed by laser scanning confocal microscopy according to the methods described by Kobori et al. [23] and Su et al. [35] with minor modifications. The induced mycelium was washed twice with ice-cold phosphate-buffered saline (PBS; pH 7.4) and resuspended in PBS supplemented with 10 mg/ml bovine serum albumin. An antibody against FLAG was used as the primary antibody at a dilution of 1:200 in a total volume of 0.2 ml. The mycelium was incubated with the antibody on a rotator at room temperature for 2 h. Subsequently, the mycelium was washed with PBS (pH 7.4), exposed to the secondary antibody, Alexa Fluor 488 goat antimouse IgG (H + L) diluted at a ratio of 1:200 in a total volume of 0.2 ml, and incubated for 1 h at room temperature. After washing three times, the mycelium was examined using a laser scanning confocal microscope (Carl Zeiss LSM 710, Oberkochen, Germany).

Preparation of the lipase-displaying whole-cell catalyst

The *A. niger* mycelium surface displaying CALB was prepared and used as a whole-cell biocatalyst as follows. The recombinant *A. niger* was induced with maltose for the expression of the fusion protein CALB-C as described above. The hypha was harvested and washed twice with 50 mM Tris–HCl buffer (pH 8.0), and then resuspended in 50 mM Tris–HCl buffer (pH 8.0) with 1 % trehalose as a freeze-drying protectant. The mycelium suspension was lyophilized for 24 h in a Christ Alpha 2–4 Freeze Dryer (Christ, Osterode, Germany). The lyophilized whole-cell biocatalyst water activity was adjusted by pre-equilibration with a saturated LiCl ($a_w = 0.11$) salt solution at 25 °C for 3 days to control the water content.

Characterization of the synthetic capacity of the *Aspergillus niger* mycelium-surface displayed CALB

In the solvent-free esterification system, only the reactants, fatty acids, and alcohol were involved, none conventional hydrophobic organic solvent (e.g., heptane, hexane et al.) was used in the ester synthesis reaction. A typical ester synthesis reaction was carried out in a 50-ml capped Erlenmeyer flask, and 50 mmol fatty acid and a defined amount of ethanol were added as reactants. All the reactants and whole-cell biocatalyst were pre-equilibrated separately with aqueous saturated LiCl solution ($a_w = 0.11$) in sealed containers at 25 °C for 3 days. The initial water content in the esterification reaction system was controlled by adding a certain amount of water to the esterification system at the start of the reaction. The reaction was initiated by the addition of lyophilized CALB-displaying *A. niger* mycelium to the reaction system and the mixture was incubated at an appropriate temperature and 200 rpm. Subsequently, samples of 100 μl were withdrawn periodically and mixed with 5 ml of ice-cold *n*-hexane. Ester synthesis was determined by measuring the amount of remaining fatty acid by titrating the withdrawn mixture with 25 mM potassium hydroxide-ethanol standard solution in the presence of phenolphthalein [20]. All the data were reported as average values of experiments performed at least in triplicate.

Results

Construction of the *Aspergillus niger* mycelium surface display system

To display CALB on the mycelium surface of *A. niger*, the sequence of the N-terminal portion of an endogenous glycosylphosphatidylinositol (GPI)-anchored protein CwpA was fused to the C-terminal sequence of CALB, and a FLAG tag was inserted between the sequences of CALB and CwpA for the immunoassay. This fused protein coding sequence was under the control of the endogenous strong gla promoter, signal sequence and pro-sequence (Fig. 1). The *A. nidulans* acetamidase encoding gene was used as the dominant selective marker that required no auxotrophic mutation in the host. The CALB-displaying cassette with the *amd*S selectable marker was transformed into *A. niger*, and the transformants were selected based on the phenotype that grew vigorously on CD-Acea medium with acetamide as the sole nitrogen source.

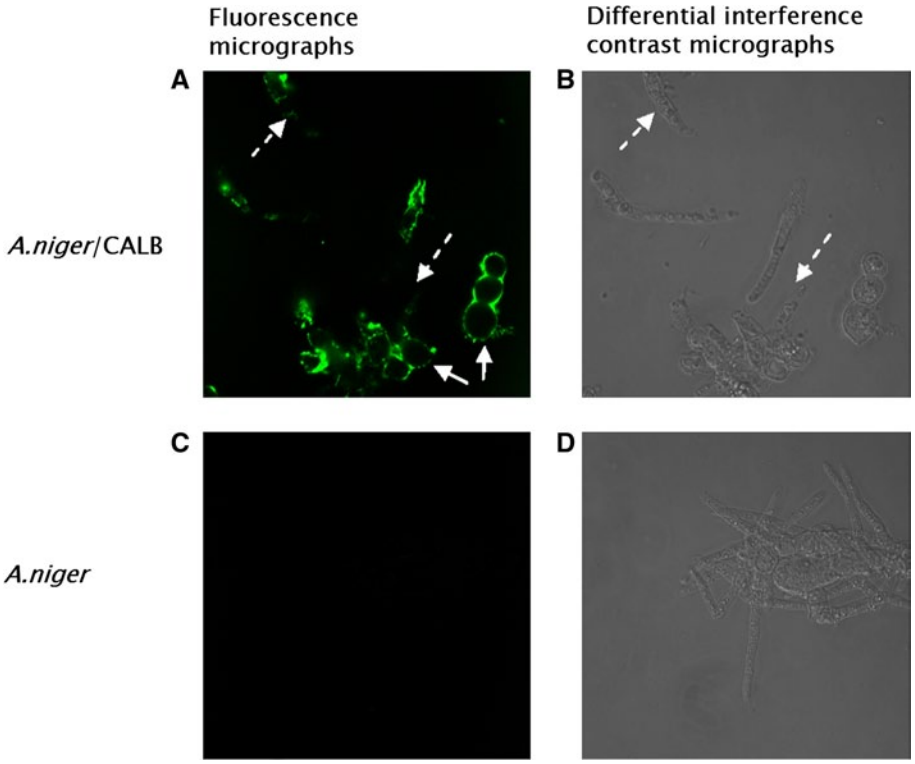

Fig. 2 Immunofluorescence microscopy assay of labeled *A. niger* mycelium by laser scanning confocal microscope. The mycelium of recombinant *A. niger* displaying CALB on the surface (**a**, **b**) and control host strain *A.niger* SH-1 (**c**, **d**) were detected after being labeled with mouse anti-FLAG monoclonal antibody and subsequently with goat anti-mouse IgG antibody conjugated with Alexa Fluor-488. Swelling hyphae with clear fluorescence and displaying fusion protein CALB-Flag-CwpA on the surface are indicated by *solid arrows*, lathy hyphae without fluorescence are indicated by *dotted arrows*

Confirmation of CALB display on the *Aspergillus niger* mycelium surface

To confirm whether CALB was displayed on the surface of *A. niger* mycelium, the hyphae were examined by immunofluorescence microscopy. Immunofluorescence labeling of the mycelium-surface displayed CALB-Flag-CwpA fusion protein with a mouse anti-FLAG monoclonal antibody as the primary antibody and Alexa Fluor 488 goat antimouse IgG as the secondary antibody was performed to verify the presence and localization of CALB on the cell surface. As shown in Fig. 2, a green fluorescence signal was clearly observed on the surface of *A. niger* mycelium anchoring CALB-Flag-CwpA (Fig. 2a, b), whereas no green fluorescence signal was observed on the control strain *A. niger* SH-1 mycelium (Fig. 2c, d). These results indicated that CALB was successfully displayed on the surface of *A. niger* mycelium.

Evaluation of lipase activity displayed on the *Aspergillus niger* mycelium surface

We evaluated the time course of lipase activity of the *A. niger* transformants using the host *A. niger* SH-1 as the control. In a 500-ml flask containing 50 ml of liquid medium, the *p*NPB hydrolytic activity of the *A. niger* whole-cell anchored CALB reached 400 U/g of dry cell and the ethyl hexanoate synthesis activity reached 240 U/g of dry cell, following induction by maltose for 45 h at 30 °C. In contrast, the hydrolytic activity and synthesis activity of *A. niger* SH-1 were <63 U/g of dry cell and almost zero, respectively. When controlled fed-batch cultivation was carried out in a 2-l bioreactor, the hydrolytic activity and synthesis activity on *A. niger* mycelium surface were 1,160 and 696 U/g of dry cell, respectively. There was no significant difference in the dry cell weight between the transformant strain and host strain when cultivated under the same conditions. As shown in Fig. 3a, when the mycelium was used to catalyze the hydrolysis of *p*NPB, the enzyme activity markedly increased with time, indicating that the fusion protein expression was induced by maltose and anchored on the *A. niger* mycelium surface. As expected, maltose induced high levels of cell surface displayed lipase, whereas xylose did not induce significant fusion protein expression. Similarly, Mackenzie et al. [26] and Withers et al. [46] also reported that there was no gla*A*-promoter-controlled gene expression during growth on xylose. Moreover, we found that xylose even repressed the induction effect of maltose (data not shown), which is consistent with the reports by Verdoes et al. [43] as well as Markus and Rinas [13]. Figure 3b demonstrates that the lipase hydrolytic activity was mainly observed on the mycelium surface of *A. niger* transformants anchoring CALB, whereas <23 % was detected in the culture supernatant. This observation of activity in the supernatant might be caused by the original secretory lipase from the host *A. niger* SH-1 (the activities

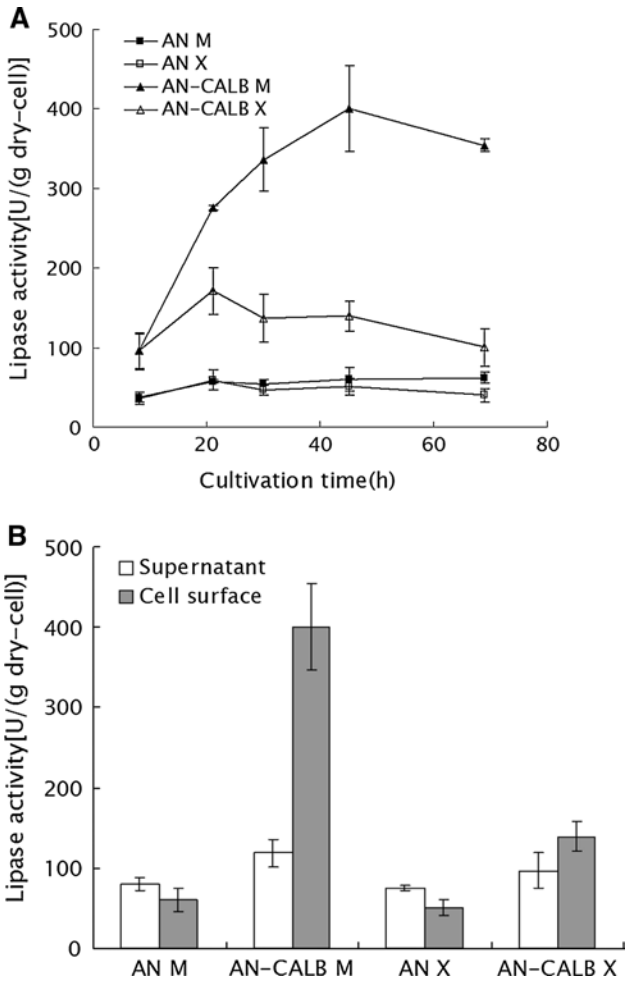

Fig. 3 **a** The time course of lipase hydrolytic activity displayed on *A. niger* mycelium surface. **b** The comparison of supernatant and cell surface lipase hydrolytic activity. AN M and AN X represent the control host strain *A. niger* SH-1 induced by maltose and xylose, respectively. AN-CALB M and AN-CALB X represent the recombinant *A. niger* displaying CALB induced by maltose and xylose, respectively

of the host and transformant shown in the supernatant fraction were nearly at the same level irrespective of whether being induced by maltose or xylose, 81, 118, 75, and 97 U/g of dry cell, respectively).

Catalytic performance of *Aspergillus niger* mycelium-surface displayed CALB in solvent-free esterification reaction system

Synthesis of ethyl esters from a series of fatty acids of different chain lengths and ethanol in solvent-free system was used to assess the catalytic activity of AN-CALB. Figure 4a shows the time courses of ethyl hexanoate (C6) production. AN-CALB exhibited notable reaction activity and gave a molar conversion rate of the substrate (hexanoic acid) of >87 % after 2 h reaction when 30 % water (mole percentage of fatty acid) was added at the start of the reaction. On the other hand, the mycelium of the control host strain *A. niger* SH-1 showed no catalytic activity, similar to that observed for the negative control system without enzyme. These results further supported that most of the induced CALB was successfully displayed on the surface of *A. niger* mycelium and retained its enzymatic activity.

When lauric acid (C12, Fig. 4b) and stearic acid (C18, Fig. 4c) were used as substrates, high product yield and esterification activity, which amounted to 89 % for ethyl laurate after 2 h and 84 % for ethyl stearate after 3 h, respectively, were observed. Furthermore, the initial water content in this solvent-free esterification reaction system was found to have a marked impact on the initial rates of AN-CALB. The reactants and enzyme were pre-equilibrated separately with saturated LiCl ($a_w = 0.11$) salt solution at 25 °C for 3 days, and the esterification activity of AN-CALB was found to increase significantly when 30 % water (mole percentage of fatty acid) was added at the beginning of the reaction. Table 2 shows the initial reaction rate of AN-CALB corresponding to fatty acid substrates of different chain lengths and various amount of water initially added to the reaction medium at the start of the reaction. When compared with 3 % water, 30 % water initially added led to 3.53-fold higher initial reaction rate for the synthesis of ethyl hexanoate, 6.89-fold higher initial reaction rate for the synthesis of ethyl laurate, and 9.58-fold higher initial reaction rate for the synthesis of ethyl stearate. In the range of 3–30 % water initially added, the esterification activity of AN-CALB increased along with the amount of water initially added; however, when 30–45 % water was initially added, there was almost no significant increase in the initial reaction rate of esterification (data not shown). With regard to the long-chain fatty acid substrate (stearic acid, C18), the positive effect of water activity on the reaction rate and conversion was much more obvious, which significantly shortened the time to reach maximum conversion from 9 to 3 h. When 3 % water was initially added to esterification system, the water formed during the course of esterification was also found to dramatically enhance the reaction rate.

Discussion

In the present study, we successfully established a novel cell surface display system in *A. niger* using an endogenous cell wall protein, CwpA, as the anchor protein. This system may be developed to provide a potential food-grade cell surface displayed lipase suitable for application in the food processing industry and could expand the filamentous fungi cell surface display system. CwpA is a GPI-anchored protein of *A. niger* that can be extracted from the cell wall by treatment with hydrofluoric acid. Damveld et al. [8] cloned

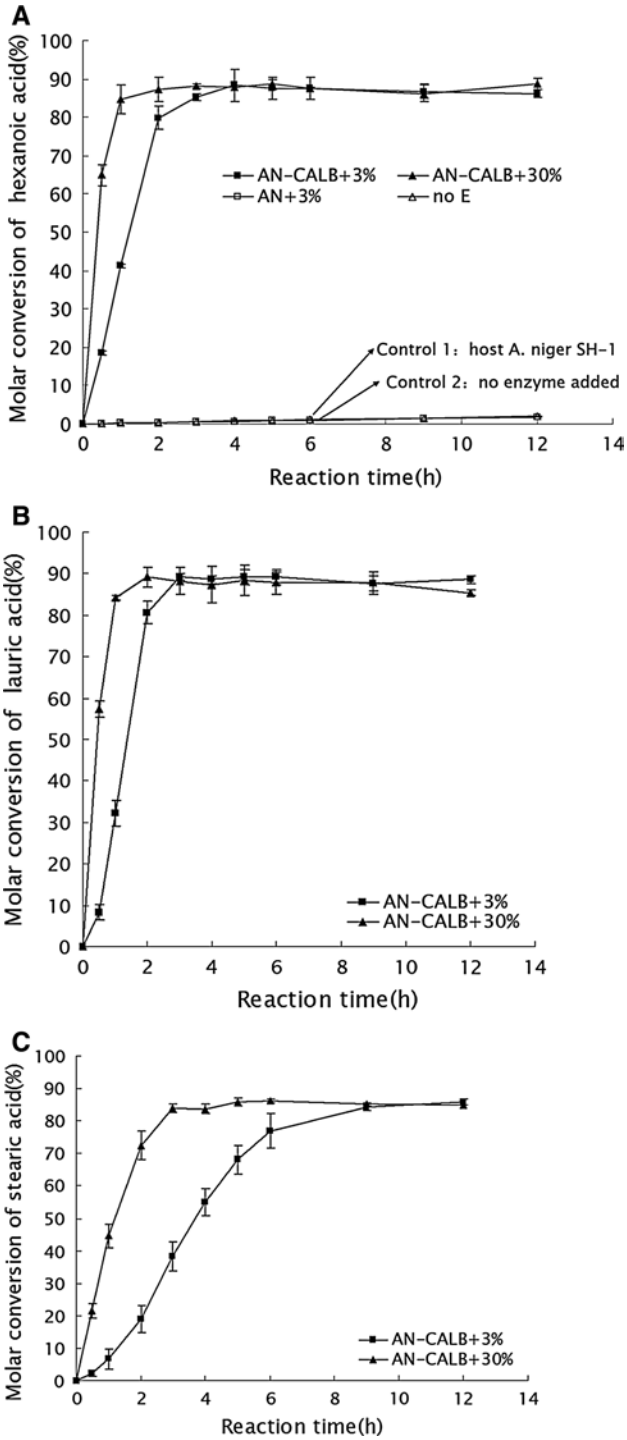

Fig. 4 **a** The time course of esterification reaction for the preparation of ethyl hexanoate in solvent-free system. The reaction mixture, consisting of 50 mmol hexanoic acid, 60 mmol ethanol, a defined amount of water, and 0.9 g lyophilized *A. niger* mycelium, was incubated at 50 °C and 200 rpm. **b** The time course of esterification reaction for the preparation of ethyl laurate in solvent-free system. The reaction mixture, consisting of 50 mmol lauric acid, 60 mmol ethanol, a defined amount of water, and 1.5 g lyophilized *A. niger* mycelium, was incubated at 60 °C and 200 rpm. **c** The time course of esterification reaction for the preparation of ethyl stearate in solvent-free system. The reaction mixture, consisting of 50 mmol stearic acid, 85 mmol ethanol, a defined amount of water, and 2.1 g lyophilized *A. niger* mycelium, was incubated at 60 °C and 200 rpm. The graph shows the percentage of molar conversion of the fatty acids substrate. AN-CALB+3 % represents the esterification reaction system with 3 % water initially added (mole percentage of fatty acid) promoted by the recombinant *A. niger* mycelium-surface displayed CALB, AN-CALB+30 % represents the esterification reaction system with 30 % water initially added promoted by the recombinant *A. niger* mycelium-surface displayed CALB, no E represents the negative control reaction system with 3 % water initially added and without enzyme, and AN+3 % represents the control esterification reaction system with 3 % water initially added promoted by the host strain *A. niger* SH-1 mycelium

the gene of CwpA and characterized this putative cell wall mannoprotein, but did not construct a cell surface display system. In the present study, immunofluorescence assay using laser scanning confocal microscopy revealed that the CALB-Flag-CwpA fusion protein was located on the surface of the recombinant *A. niger* mycelium. Clear fluorescence images indicated that the protein was localized at the swelled hyphal apices (mainly on the cell wall and septa), which is consistent with previous reports [15, 44]. A combination of the hydrolytic activity in an aqueous phase reaction and synthetic activity in a solvent-free organic phase reaction further supported that most of the induced fusion protein was anchored on the mycelium surface. Moreover, the present study also demonstrated that CwpA can be used as an anchoring motif for functionally displaying enzyme proteins on the cell surface of *A. niger*.

Transformation systems based on the complementation of auxotrophic mutations require host mutations that may lead to deleterious gene effects on important industrial strains. Furthermore, use of selectable markers conferring resistance to drugs for the development of transformation systems in filamentous fungi is not suitable for the purpose of food processing enzyme production [14]. To overcome these drawbacks, we selected *A. niger* with high protein-producing capacity as a host to construct the CALB-displaying whole-cell catalyst for applications in the food processing industry. All the expression elements were from GRAS certified food industrial strains, and *amd*S from *A. nidulans* was used as the dominant selectable marker, which has been certified as GRAS [29]. This novel cell surface display system does not leave hazardous genes in the host genome and involves minimal interference with the physiology of the host *A. niger*.

The host *A. niger* SH-1 grows poorly in the presence of acetamide as the sole nitrogen source, and we exploited this characteristic to develop a protoplast transformation system using the *amd*S gene as the dominant marker for selecting transformants on the basis of acetamide utilization. The transformants varied in their ability to grow on acetamide medium and showed different mycelium surface displayed lipase activities (data not shown). It is reported that strong

Table 2 The initial reaction rate of AN-CALB corresponding to different chain length fatty acid substrates and different amount of water initially added to the reaction medium

The amount of water initially added (mole percentage of fatty acid)	Initial rate (mmol h^{-1} g^{-1})		
	Ethyl hexanoate	Ethyl laurate	Ethyl stearate
3	20.47 ± 0.65	5.55 ± 1.25	1.07 ± 0.50
30	72.19 ± 3.08	38.23 ± 1.26	10.25 ± 1.03

growth of the positive transformants on acetamide medium requires high expression levels of the *amd*S gene, which might be result of multicopy integration of expression cassette or integration at a certain locus allowing efficient transcription [14, 28]. The difference in the number of active integrated copies and integration position on the chromosome may lead to the diversity of acetamidase activities, growth rate on acetamide, and cell surface displayed lipase expression of the positive transformants [21, 28].

In general, ester synthesis in non-aqueous phase requires a suitable mixture of reactants and solvent, however, in industrial food processing applications, solvent-free systems are preferable. Therefore, by using the enzymatic synthesis reaction of ethyl esters from a series of fatty acids of different chain lengths and ethanol in a solvent-free system as model, we evaluated the synthetic capacity of AN-CALB. It was demonstrated that AN-CALB could effectively catalyze ester synthesis in high substrate concentrations and attain high substrate mole conversions of around 85 % in a relatively short period. When compared with previous reports, in this study, AN-CALB showed good catalytic performance and synthesis efficiency. In our earlier study, using hexane and heptane as solvent, *P. pastoris* cell surface displayed CALB exhibited an ethyl hexanoate yield of 99 % in 2 h [19], whereas in the solvent-free system, the maximum conversion of hexanoic acid (C6) was similar to that presented by AN-CALB after 2 h. Water was found to have a profound effect on the lipase behavior, and as shown in Fig. 4, the synthetic activity of AN-CALB was dramatically improved when 30 % water (mole percentage of fatty acid) was initially added to the esterification system. Furthermore, as the fatty acid chain length increased, the water formed during the course of the esterification reaction with low initial water content showed a significant promotional effect on lipase activity. De Barros et al. [9] also reported that a lag time of 40 min was observed in the esterification of oleic acid with ethanol catalyzed by cutinase. A minimal amount of water is crucial for the enzyme to ensure its native conformation and to possess optimal activity [7, 11]. This has also been noted for the synthesis of ethyl palmitate in a solvent-free system promoted by commercial immobilized CALB, Novozyme 435, which afforded 97 % ethyl ester after 5 h [24]. In the ethyl palmitate synthesis system, addition of 5.3 M water at the start of the reaction was found to effectively increase the specific activity of CALB, and the initial water content was much higher than that used in the esterification reaction catalyzed by AN-CALB. Moreover, esterification of plant oil hydrolysate using commercial CALB, Novozyme 435, has been reported, which produced >90 % of methyl ester after 6 h without an organic solvent [45]. In another report, esterification of the hydrolysate from crude palm oil using Novozyme 435 was studied. In the presence of isooctane as solvent, the methyl ester content reached 98 % after 2 h [37]. Esterification of plant oil hydrolysate by *A. oryzae* whole-cell expressing CALB immobilized on biomass support particles has also been reported, which attained >90 % of methyl ester after 6 h in a solvent-free system [1]. Nevertheless, due to different reaction conditions, it is difficult to make a reasonable comparison of these studies. The results obtained in the present study indicated that AN-CALB may serve as a promising green and cost-effective alternative food-grade whole-cell catalyst to commercial lipase.

In summary, we have developed a new *A. niger* cell surface display system and demonstrated that AN-CALB could function as an excellent whole-cell catalyst, which can attain high substrate mole conversions within a relatively short period in solvent-free esterification reaction system. In addition, it was noted that CwpA can act as an anchoring motif for displaying enzyme proteins on the cell surface of *A. niger*, and the system described in this study can be used for cell surface display of other functional proteins in *A. niger*. Furthermore, based on the selectable marker, *amd*S from *A. nidulans*, our results could provide an applicable guide for displaying enzymes on a wide range of non-mutant commercially useful filamentous fungi such as *A. oryzae* [14], *A. niger* [21], *Penicillium chrysogenum* [6], and other filamentous fungi lack the ability to grow well on medium with acetamide as the sole nitrogen source.

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Conflict of interest The authors declare no competing financial interest.

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