Enzyme and Microbial Technology 75–76 (2015) 30–36

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Scaling-up the synthesis of myristate glucose ester catalyzed by a CALB-displaying *Pichia pastoris* whole-cell biocatalyst

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ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form 8 April 2015
Accepted 8 April 2015
Available online 17 April 2015

Keywords:
Candida antarctica lipase B
Yeast whole cell biocatalyst
1,2-O-Isopropylidene-α-D-glucofuranose
Myristate glucose ester
Scale synthesis

ABSTRACT

The novel whole-cell biocatalyst *Candida antarctica* lipase B displaying-*Pichia pastoris* (Pp-CALB) is characterized by its low preparation cost and could be an alternative to the commercial immobilized *Candida antarctica* lipase B (CALB). This study addresses the feasibility of using Pp-CALB in large scale glucose fatty acid esters production. 1,2-O-Isopropylidene-α-D-glucofuranose (IpGlc) was used as the acyl acceptor to overcome the low solubility of glucose in an organic solvent and to avoid the addition of toxic co-solvents. IpGlc significantly improved the Pp-CALB catalyzing esterification efficiency when using long chain fatty acids as the acyl donor. Under the preferred operating conditions (50 °C, 40 g/L molecular sieve dosage and 200 rpm mixing intensity), 60.5% of IpGlc converted to 6-O-myristate-1, 2-O-isopropylidene-α-D-glucofuranose (C14-IpGlc) after a 96-h reaction in a 2-L stirred reactor. In a 5-L pilot scale test, Pp-CALB also showed a similar substrate conversion rate of 55.4% and excellent operational stability. After C14-IpGlc was collected, 70% trifluoroacetic acid was adopted to hydrolyze C14-IpGlc to myristate glucose ester (C14-Glc) with a high yield of 95.3%. In conclusion, Pp-CALB is a powerful biocatalyst available for industrial synthesis, and this study describes an applicable and economical process for the large scale production of myristate glucose ester.

1. Introduction

Glucose fatty acid esters are a group of nonionic surfactants which are non-toxic, odorless, not irritating to skin, completely biodegradable and maintains a wide range of critical micelle concentrations (CMC) and hydrophilic-lipophilic balance (HLB) values. Because of different carbon chain lengths, glucose fatty acid esters have different HLB values. Glucose fatty acid esters with long carbon chains, such as myristic acid, palmitic acid, and stearic acid, are also good surfactants. These esters can be used in dispersed lubrication, emulsification, foaming, viscosity adjustment, preventing crystallization and antimicrobial applications; therefore, these chemicals have been widely used in food production, daily chemical, and pharmaceutical industries [1,2]. Synthesis of glucose fatty acid esters has received increasing attention because of their surfactant properties and environment-friendly advantages.

Compared to the chemical synthesis of glucose fatty acid esters, an enzymatic method is a superior option because of its mild reaction conditions, high catalytic efficiency, and inherent high selectivity of natural catalysts [3]. Additionally, enzymatic synthesis has an advantage in that the product may be accepted by customers as a 'natural product' [4]. Several commercial immobilized lipases, such as Novozym 435, Lipozyme RM IM and Lipozyme TL IM, have been used to catalyze the synthesis of glucose fatty acid esters [5,6]. However, the loss of activity and high cost of these enzymes, which results from complicated purification and immobilization steps in the lipase immobilization process, are considered the main hurdles in the industrialization of lipase-catalyzed glucose fatty acid esters production.

A lipase-displaying yeast cell operating as a whole-cell biocatalyst provides a more reasonable approach to lowering the cost of large-scale catalysis. Analogous to conventional enzymatic immobilization, the display of lipases can be considered as a type of

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http://dx.doi.org/10.1016/j.enzmictec.2015.04.002
0141-0229/

self-immobilization [7]. Exogenous lipases are firmly attached to the surface of yeast cells by connecting the lipases to a carrier protein anchored to the cell wall. Such lipase-displaying yeast cells can be produced during standard fermentation and used directly as biocatalysts without the additional steps of protein purification and immobilization. This process offers significant benefits in terms of lowering the cost of the biocatalysis process [8,9].

Although lipases have been widely used in the organic synthesis of products such as chiral compounds [10], flavor esters [11,12] and biodiesel [13–15], few examples have employed lipases to glucose fatty acid esters. The only study was our previous work that reported the synthesis of fructose mono laurate ester catalyzed by the *Candida antarctica* lipase B displaying-*Pichia pastoris* whole-cell catalyst (Pp-CALB) [16]. However, this work required improvement because of the low ester yield and the use of the toxic solvent DMSO, which leads to product purification difficulties and limits the applications in the food and pharmaceutical fields. Several strategies for the efficient production of glucose fatty acid esters could be used as references, including optimizing the types of lipase, optimizing the reaction medium, and using hydrophobic sugar derivatives [4].

This work focuses on the Pp-CALB catalyzed synthesis of glucose fatty acid esters in a large scale-reaction system. To overcome the low solubility of glucose (Glc) in organic solvents, 1,2-O-isopropylidene-α-D-glucofuranose (IpGlc, CAS NO. 18549-40-1) was used as the acyl acceptor. Acetone was used as the reaction medium to avoid the use of toxic co-solvents such as DMSO. DMSO is toxic to the operators. And it is also very difficult to separate from the product, which increasing the cost of the separation greatly. For acetone, the device should be seriously sealed and the condensate return system should be installed to ensure safety. Fatty acids with different carbon chain lengths were examined as the acyl donor. To explore the large-scale synthesis of glucose fatty acid esters, a 1-L scaled-up reaction system was performed in a 2-L stirred reactor. The temperature, dosage of molecular sieve and stirring speed were extensively analyzed. The reuse of Pp-CALB was also studied. The pilot scale synthesis of glucose fatty acid esters was then tested in a 2.5 L reaction system in a 5-L stirred reactor. Finally, to obtain pure glucose fatty acid esters, we also studied the hydrolysis of the 1,2-O-isopropylidene group in the product.

2. Methods

2.1. Materials, strains and media

All reagents, including IpGlc, were obtained commercially and were of analytical grade. The recombinant *Candida antarctica* lipase B displaying-*Pichia pastoris* strain GS115/CALB-GCW21-42 was constructed in this work, which used the Gcw21p and Gcw42p (NCBI accession No.XM_002491407, XM_002493171) cell wall proteins. The recombinant plasmid pKCALB-GCW21 was previously constructed by Jin et al. [15] in our laboratory. The pPICZαA plasmid was purchased from Invitrogen™. Zeocin resistance plates, Kanamycin resistance plates, tributyrin agar plates, minimal dextrose medium (MD), yeast extract peptone dextrose medium (YPD), buffered glycerol-complex medium (BMGY), and buffered methanol-complex medium (BMMY) were prepared for use as the yeast culture media [17].

2.2. Construction of the CALB-displaying P. pastoris GS115/CALB-GCW21-42

In this work, Gcw21p (NCBI accession No.XM_002491407) and Gcw42p (NCBI accession XM_002493171), which are glycosylphosphatidylinositol (GPI)-anchored cell wall proteins from *P. pastoris* GS115 [17], were used as anchors to display CALB on the *Pichia pastoris* cell surface. Both Gcw21p and Gcw42p displayed an excellent ability to display CALB (unpublished data).

The fusion gene CALB-GCW21 was amplified from the recombinant plasmid pKCALB-GCW21 using PCR using primers containing *Eco*RI and *Not*I sites. The amplified fragment and pPICZαA were both digested with *Eco*RI and *Not*I and then linked using a T4 ligase. The recombinant plasmid pZαA-CALB-GCW21 was transformed into the *P. pastoris* GS115 host strain. The transformant was selected through incubation on resistance plates containing Zeocin and on tributyrin agar plates supplemented with 0.5% tributyrin at 30 °C for 72 h. After gene sequencing verification, the high expression recombinant strain GS115/pZαA-CALB-GCW21 was screened by fermentation. The recombinant strain was the used as competent recipient cell to be transformed by pKCALB-GCW42.

The pKCALB-GCW42 was constructed as described in this paragraph. A gene fragment of the GPI anchored cell wall protein Gcw42p (without an internal N-terminal signal peptide) was amplified from the genomic DNA of *P. pastoris* GS115 via PCR using primers containing *Mlu*I and *Not*I sites. The amplified fragment was digested with *Mlu*I and *Not*I and subsequently inserted into the *Mlu*I and *Not*I sites of pKCALB-GCW21 to construct a recombinant expression plasmid pKCALB-GCW42. In this plasmid, the Gcw21p sequence was replaced by the gene fragment encoding Gcw42p.

The recombinant plasmid pK-CALB-GCW42 was then transformed into the host strain GS115/pZαA-CALB-GCW21. The transformant GS115/CALB-GCW21-42 was selected by incubating at 30 °C for 72 h on MD plates and Kanamycin resistance plates.

2.3. Preparation of the CALB-displaying P. pastoris whole-cell biocatalyst

The isolated transformant was precultured in BMGY medium at 30 °C. After 24 h, the culture was centrifuged at 6000 × *g* for 5 min and resuspended in BMMY medium containing 1% (v/v) methanol; the initial OD_{600} was controlled at 1. The culture was then cultured for 120 h at 30 °C. To maintain the inducted expression of the fusion protein, methanol was added to the culture every 24 h to maintain the 1% (v/v) concentration. *P. pastoris* GS115 transformed with pPIC9K and pPICZαA was used as a negative control. After the cells were harvested and washed several times, the cells were resuspended in 50 mM Tris-HCl buffer (pH 8.0), and 0.2 M trehalose (as a freeze-drying protectant) was added. This resuspension was followed by lyophilization for 24 h. This procedure produced the whole-cell biocatalyst (Pp-CALB) used in subsequent experiments.

The synthetic activity of Pp-CALB was measured by the ester synthesis method similar to that described by Sun and Xu [18]. In total, 0.05 g of whole-cell biocatalyst (or Novozym 435) was added to 10 mL of heptane containing 0.6 M hexanoic acid and 0.6 M ethanol and incubated at 50 °C for 30 min. The ethyl hexanoate present in the reaction mixture was assayed by gas chromatography. An Agilent 7890A gas chromatograph equipped with a hydrogen flame-ionization detector and an HP-5 silica capillary column (0.25 mm × 30 m, Agilent, Santa Clara, CA, USA) was used. The injector and detector temperatures were set at 250 °C. The carrier gas was nitrogen. The column temperature was held at 100 °C for 1 min, raised to 120 at 20 °C/min, then raised to 200 °C at 80 °C/min and maintained for 1 min.

One unit of transesterification activity was defined as the amount of whole cell catalyst producing 1 μmol of ethyl hexanoate per minute from hexanoic acid and ethanol at 50 °C. With this method, the synthetic activity of Pp-CALB was 1545 U/g, and that of Novozym 435 was 3200 U/g.

2.4. Pre-equilibration of Pp-CALB to control water activity

Lyophilized whole-cell biocatalysts were adjusted to the desired water activity by pre-equilibration with saturated salt solutions in separate sealed containers. The equilibration process was performed at 25 °C for 3 days. The salts used were LiBr (water activity, $\alpha_w = 0.06$), LiCl ($\alpha_w = 0.11$), $MgCl_2$ ($\alpha_w = 0.33$), $Mg(NO_3)_2$ ($\alpha_w = 0.53$), NaCl ($\alpha_w = 0.75$), and K_2SO_4 ($\alpha_w = 0.97$). The water activity of the equilibrated reagents was quantified through Karl Fischer titration on a 737 KF Coulometer (Metrohm, Herisau, Switzerland) [19].

2.5. Solubility of IpGlc and Glc in different organic solvents

IpGlc and Glc were over-added to glass vials containing 5 mL of organic solvents. The organic solvents include acetone, *tert*-butanol (*t*-butyl alcohol), *tert*-amyl alcohol (*t*-amyl alcohol), methyl ethyl ketone, acetonitrile, *n*-hexane and *tert*-butanol/acetone (1:3, v/v). The supersaturated solution was stirred at 50 °C and 200 rpm for 24 h. The solutions were then centrifuged at 10,000 rpm for 5 min. The solubility of the substrates was then quantified on an HPLC system (Waters model 2695) coupled with an refractive index detector (Waters model 2414) and an Aminex® HPX-87H Column (250 mm × 4 mm, BIO-RAD, USA) at 30 °C. The mobile phase was a mixture of methanol/water (90:10, by volume). The flow rate was 1 mL/min [20]. The concentrations of the substrates were determined from the peak area.

2.6. The synthesis of 6-O-lauric-1,2-O-isopropylidene-α-D-glucofuranose (C12-IpGlc) catalyzed by Pp-CALB in different organic solvents

In the synthesis of fructose mono laurate ester [15], 2-methyl-2-butanol/DMSO (20% v/v) was used as organic solvent as previously mentioned. However, DMSO was toxic and difficult to remove from the product because of its high boiling point and solubility. In the present work, we try to use other alternative solvents. In the synthesis of 6-O-lauric-1,2-O-isopropylidene-α-D-glucofuranose (C12-IpGlc), seven types of organic solvents (acetone, *t*-butyl alcohol, *t*-amyl alcohol, methyl ethyl ketone, acetonitrile, *n*-hexane and *t*-butyl alcohol/acetone (1:3, v/v)) were studied.

The reactions were performed at 50 °C in Erlenmeyer shake flasks (25 mL) containing a total reaction volume of 5 mL of 0.5 mmol IpGlc, 1.5 mmol lauric acid, 0.6 g 4 Å molecular sieve and 0.2 g Pp-CALB. The initial water activity was 0.11. The reaction vials were kept in reciprocating shaker maintained at 200 rpm. Aliquots