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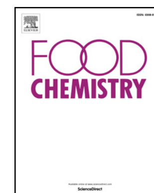

A novel process for phosphatidylserine production using a *Pichia pastoris* whole-cell biocatalyst with overexpression of phospholipase D from *Streptomyces halstedii* in a purely aqueous system

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ABSTRACT

This work was aimed to develop a novel phosphatidylserine (PS) production process for the food industry. The pld_{sh} gene, encoding phospholipase D from *Streptomyces halstedii* (PLD_{sh}) was cloned, and the codon optimized $pldm_{sh}$ gene was freely expressed by *Pichia pastoris* GS115 and successfully overexpressed on the cell surface of *P. pastoris* GS115 as displayed $PLDM_{sh}$ ($dPLDM_{sh}$) – a whole-cell biocatalyst for PS synthesis from phosphatidylcholine and L-serine. $dPLDM_{sh}$ was stable over a broad range of temperatures (20–60 °C) and pH values (4.0–8.0), indicating significant improvement in stability compared with its free counterpart expressed by *P. pastoris* GS115. Under the optimum conditions, the conversion yield of PS was 53%, and the relative yield remained above 40% after 4 repeated batch cycles of $dPLDM_{sh}$ catalysis in an aqueous system. Thus, $dPLDM_{sh}$ and the associated reaction system provided a novel strategy for efficient PS production for the food industry.

1. Introduction

Phosphatidylserine (PS), a kind of phospholipid component, has been extensively applied in the functional food industry (Vance & Steenbergen, 2005). As is known, supplemental PS in the diet has significant nutritional and biological functions, such as improving memory, preventing Alzheimer's dementia, relieving depression, increasing vigilance and attention, and decreasing stress (Claro, Patti, Abilio, Filho, & Silva, 2006; Hashioka et al., 2007; Hellhammer et al., 2004; Hirayama, Masuda, & Rabeler, 2006; Vaisman et al., 2008).

PS could be extracted from animal organs, soybeans, egg yolk, vegetable oils, and biomass. However, the low availability of such sources is likely to restrict its industrial-scale production. Additionally, PS obtained from animal organs, such as bovine brains, might be unsafe for human consumption due to the risk of infectious disease transmission (Chen, 2010).

PLD (EC. 3.1.4.4) can catalyze two types of reactions: hydrolysis of the phosphodiester bond of a phospholipid to produce phosphatidic acid (PA) and the corresponding alcohol and transphosphatidylation of phosphatidyl groups to various phosphatidyl alcohols (Uesugi & Hatanaka, 2009). An alternative method for preparing PS is the transphosphatidylation of phosphatidylcholine (PC) with L-serine by catalysis with phospholipase D (PLD), which has many advantages including mild reaction conditions, environmental friendliness, and easy scale-up to industrial production (Mao et al., 2017). Over the past decade, many PLDs have been identified in bacteria, fungi, plants, and mammals (Uesugi & Hatanaka, 2009). Of all the sources of PLDs, PLDs from *Streptomyces* have attracted considerable attention, they have been used as the primary catalysts for PS synthesis due to their higher transphosphatidylation activity, broader substrate specificity, and easier enzyme preparation than those from other sources (Hagishita, Nishikawa, & Hatanaka, 2000). To date, the production of PS by PLD-mediated transphosphatidylation of PC with L-serine has been reported by many groups. PLD, which has hydrolytic and transphosphatidylation activity, can synthesize PS from PC and L-serine and simultaneously hydrolyze PC and PS to PA in the aqueous phase, leading to greater consumption of substrate and low yield of product and hence a lower PS conversion ratio (Chen, Xu, Li, Hao, & Yan, 2013; Duan & Hu, 2012). In light of this, three strategies are proposed to synthesize PS: (i) construction of a biphasic system consisting of an organic solvent phase

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and an aqueous phase; (ii) solubilization of PC into mixed micelle using surfactant; (iii) use of calcium sulfate, silica, and diatomaceous earth as adsorbents of PC in a purely aqueous system (Pinsolle, Roy, Buré, Thienpont, & Cansell, 2013). Generally, PLD-catalyzed transphosphatidylation is performed in a biphasic system composed of a water-immiscible organic solvent phase containing the PC substrate and an aqueous phase containing the enzyme and L-serine to avoid the hydrolysis of PC and PS by PLD. Although many organic solvents, such as diethyl ether, ethyl acetate, *n*-hexane, 2-methyltetrahydrofuran, and γ-valerolactone, have been used to synthesize PS with a maximum PS yield of ~95% (Duan & Hu, 2013, 2012; Hirche, Koch, König, Wadewitz, & Ulbrich-Hofmann, 1997; Iwasaki et al., 2003), organic solvents containing toxic compounds may be inappropriate for food-processing and large-scale production. It was reported that PC could be solubilized into mixed micelle by surfactant molecules, resulting in an increased PS yield (Pinsolle et al., 2013). In an aqueous system containing the Triton X-100, sodium deoxycholate, and sodium cholate, the PS conversions of 94.7%, 57%, and 56% were obtained, respectively (Choojit, Bornscheuer, Upaichit, & H-Kittikun, 2016; Pinsolle et al., 2013). However, the use of surfactants led to the difficulty of the separation of the product, and most of the surfactant molecules may be undesirable for food production due to their toxicity (Li, Wang, Zhang, Zhao, & Niu, 2016). In addition, calcium sulfate, silica, and diatomaceous earth have been used as adsorbents of PC in the synthesis of PS in a purely aqueous system, making the process more complex.

So far, the low yield and complicated purification steps of *Streptomyces* PLDs have resulted in a high cost, and the unstable free enzymes are difficult to reuse, limiting the industrial application of PS production. The multiple reuses of the enzymes and improvement of their properties, such as stability, activity and selectivity, and high volume loading, could be achieved by the expensive immobilization technologies, but their preparation requires costly enzyme extraction and immobilization steps (Barbosa et al., 2013; Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013; Verma, Barrow, & Puri, 2013). To overcome these obstacles, the direct use of enzyme displayed on the surface of yeast cells as whole-cell biocatalysts has been developed, and this approach might provide a better method for reducing the cost of the biocatalyst because its preparation is simple and does not require further purification and immobilization steps (Yan, Zheng, & Li, 2014). In the past decade, many enzymes have been successfully displayed on the yeast cell surface as whole-cell biocatalysts, which appear to be the best candidates for use in an industrial bioconversion process (Jin et al., 2013). However, the PLD in an immobilized form is less commonly used than other enzymes (Song et al., 2012).

Thus it is imperative to develop a novel process for large-scale PS production for food industry. In a previous study, a strain of *Streptomyces halstedii* that had high PLD transphosphatidylation activity was isolated from soil samples. In the current work, *S. halstedii* PLD was freely expressed by *P. pastoris* GS115, and a free PLD (fPLD) was purified and characterized. Then, PLD was efficiently displayed on the cell surface of *P. pastoris* GS115 to produce a novel displayed PLD (dPLD) as a whole-cell biocatalyst, and the enzymatic properties of dPLD were investigated. In addition, the catalytic reaction process parameters and the practical biocatalytic behavior of dPLD in the synthesis of PS from PC and L-serine were further characterized in an aqueous system.

2. Materials and methods

2.1. Chemicals and enzymes

PC (≥99%, from soybean) and PS (≥97%, from soybean) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction endonucleases, T_4 DNA ligase, Pyrobest DNA Polymerase and genetic manipulation kits were all supplied by TaKaRa Bio (Dalian, China). Soybean lecithin (PC content ≥ 90%) was provided by Yuanye Bio (Shanghai, China). L-Serine was obtained from Solarbio Tech (Beijing, China). All other chemicals and reagents used were of analytical grade.

2.2. Plasmids, strains, and media

Plasmid pPIC9K (Invitrogen) was preserved in our laboratory. Plasmid pKFS was provided by Prof. Ying Lin (South China University of Technology), which was constructed by inserting the coding sequence of the flocculation functional domain (FFD) of lectin-like cell wall protein (Flo1p) with its own secretion signal sequence into plasmid pPIC9K as the yeast surface display vector. *E. coli* DH5α and *P. pastoris* GS115 were cultured in our laboratory for vector propagation and protein expression, respectively. The strain *S. halstedii* used in this study was obtained previously from soil samples and deposited in the Tianjin University of Science and Technology Culture Collection Center (TCCC 21102), Tianjin, China. Luria-Bertani (LB) medium, minimal dextrose (MD) medium, yeast extract peptone dextrose (YPD) medium, buffered minimal glycerol-complex (BMGY), and buffered minimal methanol-complex (BMMY) were prepared as previously described (Liu et al., 2016).

2.3. Construction of recombinant P. pastoris for free PLD_{sh} expression

Coding sequence of pld_{sh} without its signal peptide was analyzed by SignalP 4.1 Server and amplified by PCR using the forward primer 5′-GCGGAAGCGCCCACAC-3′ and reverse primer 5′-TCAGCCCTGGCA GAGGC-3′ with the genomic DNA of *S. halstedii* TCCC 21102 as template. The codon optimized pld_{sh} gene ($pldm_{sh}$) based on the codon bias of *P. pastoris* was synthesized by BGI Co. Ltd (Beijing, China) for high-level expression. Then, $pldm_{sh}$ gene was amplified using the forward primer 5′-CCGGAATTCGCTGAAGCTCCAACTCCA-3′ and reverse primer 5′-ATAAGAATGCGGCCGCTTA*GTGGTGGTGGTGGTGGTG*ACCCTGACA CAAACC-3′ containing *Eco*RI and *Not*I restriction sites (underlined) and 6-His tag for protein purification (italic). The resulting product was digested with *Eco*RI and *Not*I, and then inserted into the *Eco*RI-*Not*I-linearized plasmid pPIC9K. The resulting plasmid pPIC9K-$pldm_{sh}$ was transformed into *E. coli* DH5α competent cells. After analysis by digestion with restriction enzymes, pPIC9K-$pldm_{sh}$ was linearized by digestion with *Sal*I and transformed into *P. pastoris* GS115 by electroporation (0.2 cm cuvette, 1500 V, 200 Ω, and 25 μF). After incubation at 30 °C for 96 h, *P. pastoris* transformants were selected on MD plates, and then the transformants harboring multiple inserts were further screened from MD plates with 0.5–2.5 mg/mL of G418 gradient to express the free PLDMsh ($fPLDM_{sh}$).

2.4. Expression and purification of $fPLDM_{sh}$ from P. pastoris

The expression of $fPLDM_{sh}$ by recombinant *P. pastoris* harboring the plasmid pPIC9K-$pldm_{sh}$ was carried out as previously described (Liu et al., 2016). Methanol-induced *P. pastoris* harboring pPIC9K was used as the control. To purify the His-tagged $fPLDM_{sh}$, the culture supernatant was collected by centrifugation at 8000*g* at 4 °C for 20 min and applied to a Ni-NTA agarose resin column equilibrated with 20 mM Tris-HCl (pH 7.4) containing 20 mM imidazole and 500 mM NaCl. After removing unwanted protein with washing buffer, the target protein was finally eluted with 20 mM Tris-HCl (pH 7.4) containing 500 mM imidazole and 500 mM NaCl. The purity and apparent molecular mass of $fPLDM_{sh}$ were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using a 12% (w/v) separating gel. The protein concentration was measured with the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

2.5. Construction of recombinant P. pastoris for displayed PLD_{sh} expression

The pld_{sh} gene was obtained by PCR amplification with the forward

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