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Research Article

High-level soluble expression of phospholipase D from *Streptomyces chromofuscus* in *Escherichia coli* by combinatorial optimization

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ABSTRACT

Background: Phospholipase D (PLD) is used as the biocatalyst for phosphatidylserine (PS) production. In general, PLD was expressed in insoluble form in *Escherichia coli*. High-level soluble expression of PLD with high activity in *E. coli* is very important for industrial production of PLD.
Results: *Streptomyces chromofuscus* PLD coding gene was codon-optimized, cloned without signal peptide, and expressed in *E. coli*. The optimal recombinant *E. coli* pET-28a+PLD/BL21(DE3) was constructed with pET-28a without His-tag. The highest PLD activity reached 104.28 ± 2.67 U/mL in a 250-mL shake flask after systematical optimization. The highest PLD activity elevated to 122.94 ± 1.49 U/mL by feeding lactose and inducing at 20°C after scaling up to a 5.0-L fermenter. Substituting the mixed carbon source with 1.0 % (w/v) of cheap dextrin and adding a feeding medium could still attain a PLD activity of 105.81 ± 2.72 U/mL in a 5.0-L fermenter. Fish peptone from the waste of fish processing and dextrin from the starch are both very cheap, which were found to benefit the soluble PLD expression.
Conclusions: After combinatorial optimization, the high-level soluble expression of PLD was fulfilled in *E. coli*. The high PLD activity along with cheap medium obtained at the fermenter level can completely meet the requirements of industrial production of PLD.
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1. Introduction

Phosphatidylserine (PS) is an important ingredient of the cell membrane. It plays role in cell signaling and blood coagulation. PS can also regulate the activities of different enzymes and be used as the activating agent for protein kinase C [1,2]. In clinical practice, PS has been proved to prevent Alzheimer's disease [3,4]. Besides, PS can be taken as a nutritional supplement for particular crowd such as depression patients [5]. Therefore, it has been extensively applied in functional food and pharmaceutical industries [6].

PS can be found in different biological tissues, including bovine brain, soybean, and egg yolk. Chemical extraction from biological tissues is the used method for preparing PS at present [7]. However, transmitting bovine spongiform encephalopathy may be a risk [8]. In addition, the cost of extracting PS from biological tissues is very high because the PS content in tissues is usually very low [9]. In this situation, the enzymatic method for PS production may be a wise choice. Phospholipase D (EC 3.1.4.4, PLD) is an enzyme that can catalyze the transphosphatidylation reaction between the polar head group of phosphatidylcholine (PC) and L-serine to produce PS [10,11]. As PC is easily acquired from soybean, the most important problem is the preparation of highly active PLD.

PLD occurs ubiquitously in various organisms including bacteria [12], yeast [13], plants [14], and mammals [15]. The consensus motif of the PLD superfamily is H(X)K(X)4D, which is buried into a relaxedly conserved region that is generally appeared in two separate copies in PLD [16]. These two conserved HKD regions are considered to be active centers of PLD, which catalyze two reactions: hydrolysis reaction and transphosphatidylation reaction [17]. In general, the eukaryotic PLDs show much higher hydrolytic activity than transphosphatidylation activity [18]. However, microbial PLDs have higher transphosphatidylation activity than the eukary-

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otic ones [19,20]. Therefore, the PLDs from the microbial sources are appropriate biocatalysts for synthesizing the phospholipid derivatives [21]. Especially, the PLDs from *Streptomyces* sp. hold very high transphosphatidylation activity and are thus used as the biocatalysts for industrial PS production [22].

The PLD activity is extremely low in wild *Streptomyces* sp. [23]. Heterologous recombinant expression of *Streptomyces* sp. PLD had been attempted. *Streptomyces racemochromogenes* PLD was cloned into a self-constructed plasmid pES103 and heterologously expressed in *Streptomyces lividans*. The PLD activity (near to 30 U/mL) expressed by recombinant *S. lividans* was approximately elevated by 90-fold compared with the original strain [24]. PLD from *Streptoverticillium cinnamoneum* was cloned into a self-constructed plasmid pUC702 and expressed into *S. lividans* heterologously. After optimizing the initial glucose concentration and feeding the carbon/nitrogen sources, the maximum PLD activity attained about 55 U/mL after culturing for 60 h [25]. However, cultivation of recombinant *S. lividans* for PLD production required expensive thiostrepton and long fermentation time [24,25]. PLDs from *Streptomyces* PMF, *Streptomyces antibioticus,* and *Streptomyces* sp. were also cloned and tried to express in *Escherichia coli* [26,27,28]. Unfortunately, most of the PLD was expressed in insoluble form in *E. coli.* High-level soluble expression of PLD with high activity in *E. coli* is an urgent task for industrial production of PLD.

In order to satisfy the requirement of PLD application, in this work, the coding gene of PLD from *Streptomyces chromofuscus* was codon-optimized and cloned into *E. coli*. The influences of pET vector type, culture medium type, inducer, and inducing temperature on this PLD expression were firstly studied. Next, the effects of carbon/nitrogen source and phosphate on the PLD expression were investigated at great length. In addition, optimization of inducing mode and feeding medium in a 5-L fermenter were implemented to further raise the PLD yield. After the above systematic optimization, high-level expression of PLD with high activity was finally fulfilled in *E. coli*.

2. Materials and methods

2.1. Plasmids, strains, culture media, and media component

The coding gene of PLD from *S. chromofuscus* was codon-optimized and synthesized by Generay Biotech (Shanghai) Co., Ltd. (China). pUC 57 vector (Generay) was employed for subcloning and DNA sequencing. The Sec pathway is known for the translocation of polypeptides across the cytoplasmic membrane into the periplasm of *E. coli.* The PelB signal sequence was used for the secretion of extracellular protein by the Sec pathway. So pET-25b and pET-20b without lac operator were chosen to enhance extracellular production of PLD in the periplasm. The pET-28a and pET-32a with Trx·Tag were chosen to express PLD in the cytoplasm. Hence five plasmids: pET-20b (+), pET-25b (+), pET-28a (+), pET-32a (+), and pET-28a (+) without histidine tag were employed for construction of PLD expression plasmids. *E. coli* strain BL21 (DE3) was adopted as the host for PLD expression. Luria-Bertani (abbreviated as LB, 10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone), Super Broth (abbreviated as SB, 15 g/L NaCl, 15 g/L yeast extract, 30 g/L tryptone), Terrific Broth (abbreviated as TB, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 5 g/L glycerol, 12 g/L tryptone, 24 g/L yeast extract) and YTGN broth (abbreviated as TY, 5 g/L Na_2HPO_4, 8 g/L glycerol, 10 g/L yeast extract, 20 g/L tryptone) were used. The yeast extract and tryptone were bought from Oxoid Co, Ltd. (UK). Other carbon and nitrogen sources were industrial grade with the lowest price in the Chinese market.

2.2. Protein concentration, PLD activity, and SDS-PAGE analyses

Bradford method was used to determine the protein concentration by taking the bovine serum albumin as a standard [29]. SDS-PAGE analysis was performed using 12 % (w/v) polyacrylamide gel.

PLD activity was measured referring to the previous work with minor modification [30]. The reaction mixture containing 0.1 % (w/v) PC, 0.02 % (v/v) Triton X-100, 0.111 % (w/v) $CaCl_2$, and 50 μL of PLD sample was diluted to 100 μL with 0.1 M pH 8.0 Tris-HCl buffer. After incubating at 37°C for 5 min, 20 μL of 0.1 M EDTA solution prepared with 1.0 M pH 8.0 Tris-HCl buffer was used to stop the reaction and cooled at room temperature for 5 min. To the reaction mixture, 10 μL of 1.0 M pH 8.0 Tris-HCl buffer containing 21 mM phenol and 0.59 mM 4-aminoantipyrine, and 10 μL of 1.0 M pH 8.0 Tris-HCl buffer containing 12.5 U/mL of choline oxidase and 10 U/mL of horseradish peroxidase were added. The mixture was incubated at 37°C for 2 h and its absorbance at 505 nm was determined. A calibration curve was developed based on a series of standard choline chloride solutions. Under the above determination conditions, the amount of enzyme that can produce 1 μmoL choline per minute was defined as one unit (U) of PLD hydrolysis activity.

2.3. Construction of recombinant plasmids, PLD expression, and cell disruption

Three primers F1-Forward 5′-CATGCCATGGGCACCACCGGTACTCC-3′ (*Nco* I site), F2-Forward 5′-TATCATATGGCACCACCGGTACTCC-3′ (*Nde* I site), and R-Reverse 5′-CCC AAGCTTATTCAGGATCGTAGGTACGTT-3′ (*Hind* III site) were used to clone the codon-optimized PLD without signal peptide. The pUC 57 with PLD gene was utilized as the PCR template. The obtained PLD gene and expression plasmids were digested with the *Hind* III and *Nco* I or *Hind* III and *Nde* I restriction enzymes (Takara, Nanjing) simultaneously, and ligated to construct the PLD recombinant plasmids: pET-20b+PLD, pET-25b+PLD, pET-28a+hPLD (His-tag retained), pET-28a +PLD (His-tag removed), and pET-32a+PLD (Fig. 1). Then, they were transformed into the *E. coli* BL21 (DE3), respectively.

A single colony of the recombinant *E. coli* strain was transplanted to 10 mL LB media in a 50-mL shake flask containing specific antibiotics, and shaken at 200 rpm and 37°C overnight as seed. 1.0 % (v/v) seed was added into 50 mL fermentation medium in a 250-mL shake flask and cultivated at 200 rpm and 37°C for 2.5–3.0 h up to the OD_{600} achieved 0.8~1.0. 2.5 g/L lactose or 0.1 mM IPTG was added to induce PLD expression for 36 h at 25°C. Collection of the cells was carried out by centrifuging at 8000 rpm for 10 min. The cells were resuspended with 0.1 M pH 8.0 Tris-HCl buffer, then disrupted with the ultrasonic disruptor in ice bath at 300 W for 10 min (one cycle: working 3 s and intervals 5 s). The disrupted cell solution was centrifuged at 8000 rpm for 10 min, and the supernatant was collected and sampled for PLD activity, protein concentration, and SDS-PAGE analyses.

2.4. Different recombinant E. coli strains screening

Five PLD recombinant strains: pET-20b+PLD/BL21(DE3), pET-25b+PLD/BL21(DE3), pET-28a+hPLD/BL21(DE3), pET-28a+PLD/BL21(DE3), and pET-32a+PLD/BL21(DE3) were induced in 50 mL TB medium in a 250-mL shake flask at 25°C for 36 h and shaken at 200 rpm, to investigate their effects on cell growth, PLD expression, and activity.

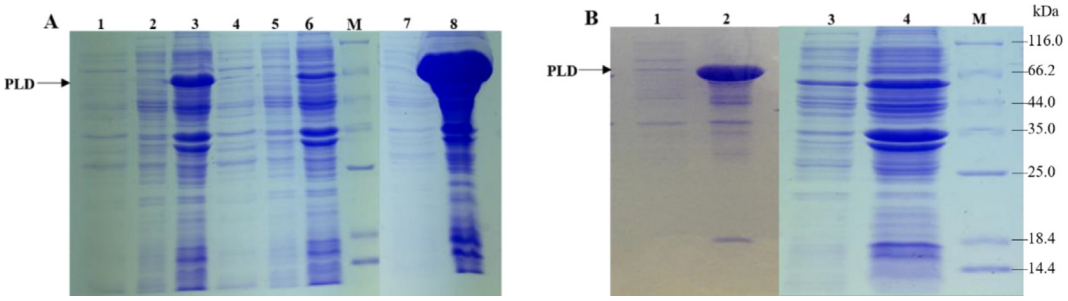

Fig. 1. The SDS-PAGE expression profiles of different recombinant *E. coli* strains. (A) Lane 1–Lane 3, fermentation supernatant, supernatant and precipitate after inducing and disrupting of pET-20b+PLD/BL21(DE3), respectively; Lane 4–Lane 6, fermentation supernatant, supernatant and precipitate after inducing and disrupting of pET-25b+PLD/BL21(DE3), respectively; Lane 7–Lane 8, supernatant and precipitate after inducing and disrupting of pET-32a+PLD/BL21(DE3), respectively. (B) Lane 1–Lane 2, the supernatant and precipitate after inducing and disrupting of pET-28a+hPLD/BL21(DE3), respectively; Lane 3–Lane 4, the supernatant and precipitate after inducing and disrupting of pET-28a+PLD/BL21(DE3), respectively. Ten microlitres of samples of the fermentation supernatant after inducing, 10 μL of samples of the supernatants and 5 μL of precipitates after inducing and disrupting. Recombinant *E. coli* strains containing different plasmids were induced in TB medium with 0.1 mM IPTG at 25°C for 36 h. M: molecular mass markers.

2.5. Culture media selection, inducer and inducing temperature optimization

The recombinant *E. coli* strain selected in Section 2.4 was cultured in LB, TB, SB, and TY media to investigate the effect of medium type on PLD expression. IPTG at the concentrations of 0.05~1.0 mM or lactose at the concentrations of 0.5~10.0 g/L was employed as the inducer to induce PLD expression. Temperatures at 20°C, 25°C, 28°C, 32°C, and 37°C were set to investigate the influence on the PLD expression.

2.6. Carbon source optimization

Firstly, the original carbon source in the starting fermentation medium was replaced with 1.0 % (w/v) of glucose, sucrose, sorbitol, glycerol, starch, and dextrin. After obtaining the better "fast" carbon sources and "slow" carbon sources, mixtures of "fast" and "slow" carbon sources at the ratio of 1:1 (w/w) were further investigated. Then, concentration gradients (0.5~2.0 %, w/v) of the best mixture of "fast" and "slow" carbon sources were investigated. Finally, the ratio of "fast" and "slow" carbon source was set at 1:9, 3:7, 5:5, 7:3, and 9:1 (w/w) to study their influences on the PLD expression and the growth of recombinant strain. "Fast" carbon sources were those containing compounds that could be used directly by microorganisms. In contrast, "slow" carbon sources could only be used after decomposition.

2.7. Optimization of nitrogen source and phosphate

Some domestic cheap nitrogen sources such as soybean meal, ox bone peptone, fish peptone, peanut powder, and corn steep liquor at the concentration of 1.2 % (w/v) were used to substitute the Oxoid tryptone in TB medium. After selecting a cheap alternative carbon source, its concentration was set at 0.6~2.0 % (w/v) to investigate the effects on the PLD expression and the growth of recombinant strain. Furthermore, Angel yeast extract at the concentrations of 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, and 4.4 % (w/v) were used to replace the Oxoid yeast extract to further low the cost of medium. Total phosphate concentration gradients (0~149 mM) were also investigated to further improve the production of PLD.

2.8. Cultivation in a 5-L fermenter

Firstly, based on the above optimization, the cultivation was directly scaled up to 5-L fermenter containing 3.0 L of medium from 250-mL shake flask loading 50 mL medium. The medium and conditions used in the fermenter were the same as those in the shake flask. Then, in order to increase the PLD yield in the fermenter, the inducing temperature and mode were modified to balance the PLD expression and the growth of the recombinant strain. The carbon source (mixed sorbitol (0.7 %, w/v) and starch (0.3 %, w/v)) in the optimized medium was also replaced with 1 % (w/v) of dextrin to further reduce the cost of medium used in scale-up cultivation.

3. Results and discussion

3.1. Cloning and expression of the S. chromofuscus PLD gene in E. coli

The PLD gene from *S. chromofuscus* was codon-optimized, synthesized, and ligated to pUC 57. Taking pUC 57 as the PCR template, the full-length PLD gene without signal peptide was successfully cloned using specific primers F1-Forward and R-Reverse. The PLD gene of *S. chromofuscus* has an open reading frame of 1539 bp in length, which encodes a polypeptide of 510 amino acids with a deduced molecular weight of 55 kDa.

The signal peptide sequence of PLD gene could influence the processing of the active PLD in *E. coli* [31,32,33]. Deletion of the signal peptide sequence might benefit the soluble expression of PLD in *E. coli*. Therefore, five PLD expression plasmids without signal peptide, pET-20b+PLD, pET-25b+PLD, pET-28a+hPLD (His-tag retained), pET-28a+PLD (His-tag removed), and pET-32a+PLD, were constructed and imported into *E. coli* BL21 (DE3) (Fig. S1). The PLD expression in BL21 (DE3) was certified by SDS-PAGE (Fig. 1). It could be found that all recombinant plasmids could express PLD in BL21 (DE3). However, the soluble expression of different plasmids was obviously different (Fig. 1A and B). The pET-20b and pET-25b with pelB leader sequence could hardly express the PLD in soluble form (Fig. 1A, lanes 2 and 5). The pET-28a+hPLD expressed obviously more PLD into inclusion body than into soluble proportion (Fig. 1B, lanes 1 and 2). The pET-32a+PLD chiefly expressed PLD in the insoluble form as shown in Fig. 1A (lane 8). The pET-28a+PLD could express more PLD in soluble form than all the other four plasmids (Fig. 1B, lane 3). The N-terminal of the PLD linking with the Trx-tag (pET-32a), pelB leader sequence (pET-20b, pET-25b), or His-tag (pET-28a) might influence the expressed PLD folding into the soluble form, and thus forming insoluble inclusion body.

The activity and specific activity of PLD were also determined and compared between the five recombinant strains. The PLD activity and specific activity expressed by pET-28a+PLD/BL21 (DE3) reached 26.22 ± 0.84 U/mL and 2.06 ± 0.13 U/mg, respectively, which were the highest among the five recombinant strains

(Table 1). The PLD activities expressed by pET-20b+PLD/BL21(DE3) and pET-25b+PLD/BL21(DE3) were very low, which were hardly detected. The PLD activities expressed by pET-28a+hPLD/BL21 (DE3) and pET-32a+PLD/BL21(DE3) were lower, because more insoluble inclusion bodies were produced (Fig. 1). Therefore, the pET-28a+PLD/BL21(DE3) was selected as the recombinant strain for PLD production.

3.2. Effect of the basic medium on the PLD expression

Nutrient-rich medium may be favorable to the cell growth and the enzyme expression in recombinant *E. coli* [34]. Therefore, SB, LB, TY, and TB media were taken as basic media to culture the recombinant *E. coli* pET-28a+PLD/BL21(DE3) and investigate the PLD expression. As shown in Fig. 2, it could be found that the total expression and soluble expression of PLD were both higher in TB medium (Fig. 2A, Lane 7 and 8). The LB, SB, and TY media could not effectively enhance the total PLD expression and improve the soluble expression of PLD (Fig. 2A, Lane 1–6). Besides, when the pET-28a+PLD/BL21 (DE3) was cultivated in TB, the OD_{600} attained was higher than those obtained in LB, SB, and TY. The highest OD_{600} reached 12.82 ± 0.273, and high OD_{600} accompanied by the highest PLD activity of 26.22 ± 0.84 and specific activity of 2.06 ± 0.13 (Fig. 2B). The four basic media contained different kinds of components. LB and SB media had fewer kinds of nutrients among them, resulting in low PLD expression levels. Nutrient-rich TY and TB media contained different kinds of phosphate. KH_2PO_4 and K_2HPO_4 in TB medium might be more beneficial to PLD expression. In addition, the suitable ratio of carbon to nitrogen in TB medium could be also important for cell growth and PLD expression in recombinant *E. coli*. Therefore, TB was selected as the basic medium for next optimization.

3.3. Effect of the inducer type, concentration, and inducing temperature on the PLD expression

IPTG can show toxicity toward human beings, and its price is high. Lactose can be used to replace the IPTG for induction of recombinant protein expression [35]. In addition, if the majority of inclusion bodies could transform into the active soluble form by inducing with lactose, the PLD activity would be significantly improved. IPTG (0.05~1.0, mM) or lactose (0.5~10.0, g/L) was used to induce the recombinant PLD expression. As shown in Fig. 3, high IPTG concentration was unfavorable to the growth of pET-28a +PLD/BL21 (DE3), which resulted in the cell density OD_{600} decreased gradually along with the increase of IPTG concentration. The PLD activity and specific activity raised first and descended later along with the increase of IPTG concentration. The highest PLD activity and specific activity reached 32.96 ± 0.94 U/mL and 2.42 ± 0.23 U/mg at 0.5 mM IPTG, respectively (Fig. 3A). Unlike IPTG, high concentration of lactose could facilitate the growth of pET-28a+PLD/BL21 (DE3). At 2.5 g/L of lactose, the highest PLD activity and specific activity of recombinant *E. coli* strain attained 35.28 ± 1.04 U/mL and 3.08 ± 0.05 U/mg, which were elevated to 1.07-fold and 1.27-fold in comparison with 0.5 mM IPTG induction, and the OD_{600} (13.9 ± 0.46) was 1.37-fold higher than that of 0.5 mM IPTG induction (Fig. 3). Thus, lactose could be used as inducer for PLD expression in pET-28a+PLD/BL21 (DE3). When the lactose concentration exceeded 2.5 g/L, the PLD activity produced by pET-28a+PLD/BL21 (DE3) would decrease, but the OD_{600} still increased (Fig. 3B). Therefore, an appropriate concentration of lactose was applicable as the inducer for PLD expression in *E. coli*. About 2.5 g/L of lactose was the optimal inducer concentration.

Induction should also be carried out at a neither too high nor too low temperature. The pET-28a+PLD/BL21(DE3) was induced at 37°C, 32°C, 28°C, 25°C, and 20°C. As shown in Fig. 4, high or low temperature was harmful to the PLD expression. Although high temperature could speed up the PLD synthesis, it would lead to more PLD expression in insoluble form (Fig. 4B, lane 8–10). At a temperature more than 30°C, the insoluble PLD produced largely, and the soluble PLD was so few that the bands were hardly visible (Fig. 4B, lane 3–5). Low temperature could reduce the formation of insoluble inclusion bodies. A better balance between soluble PLD expression and cell growth could be attained at 25°C, which generated the highest PLD activity (35.28 ± 1.04 U/ml) and specific activity (Fig. 4A).

3.4. Effect of carbon source

Carbon source is one of the six elements of the culture medium, which can provide the carbon for biosynthesis of protein and the energy for cell growth. Optimization of carbon source can often improve the expression of recombinant protein and/or cell growth, which may enhance the expression level of recombinant protein in *E. coli*. For this purpose, the carbon source effect on the PLD expression of pET-28a+PLD/BL21 (DE3) was investigated with TB medium as the basic medium. About 1.0 % (w/v) of six carbon sources was used to replace the 0.5 % (w/v) glycerol in TB (Table 2). 1.0 % (w/v) of glycerol was the most unfavorable carbon source, which resulted in the lowest PLD activity. All other carbon sources were favorable to PLD expression because they could obviously improve the PLD yield. The best carbon source was sorbitol which led to the highest PLD activity of 61.09 ± 1.25 U/mL after 36 h induction. The glucose was second only to sorbitol as a carbon source. Sucrose, dextrin, and starch showed similar results.

Among the five better carbon sources, glucose and sorbitol are "fast" utilization carbon sources, and dextrin and starch are "slow" utilization carbon sources with very low price. Therefore, in order to balance the metabolism of "fast" and "slow" carbon sources and reduce the cost of carbon sources, combinations of carbon sources were next investigated. About 1.0 % (w/v) of mixed "glucose and dextrin," "glucose and starch," "sorbitol and dextrin," or "sorbitol and starch" at the weight ratio of 1:1 was used as the carbon source of TB medium. High PLD activity and specific activity were produced by the mixed "glucose and starch" and mixed "sorbitol and starch," which attained to 69.92 ± 1.02 U/mL, 4.12 ± 0.06 U/mg, 83.36 ± 0.80 U/mL, and 4.17 ± 0.16 U/mg at 36 h, respectively.

The concentration gradients (0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 % w/v) of mixed "glucose and starch (1:1, w/w)" or mixed "sorbitol and starch (1:1, w/w)" in the TB were further studied. As shown

Table 1
Effect of expression plasmid on the growth of recombinant *E. coli* strain and expression of PLD.

Plasmid	Host	OD_{600}	Enzyme activity (U/mL)	Specific activity (U/mg)
pET-20b+PLD	BL21(DE3)	16.10 ± 0.251	0.78 ± 0.02	0.26 ± 0.05
pET-22b+PLD	BL21(DE3)	15.84 ± 0.41	1.62 ± 0.07	0.73 ± 0.04
pET-32a+PLD	BL21(DE3)	25.20 ± 0.35	2.23 ± 0.41	0.64 ± 0.06
pET-28a+hPLD	BL21(DE3)	23.62 ± 0.75	15.86 ± 0.54	1.86 ± 0.75
pET-28a+PLD	BL21(DE3)	10.21 ± 0.17	26.22 ± 0.84	2.06 ± 0.13

Five recombinant *E. coli* strains were induced with 0.1 mM IPTG in TB medium at 25°C for 36 h.

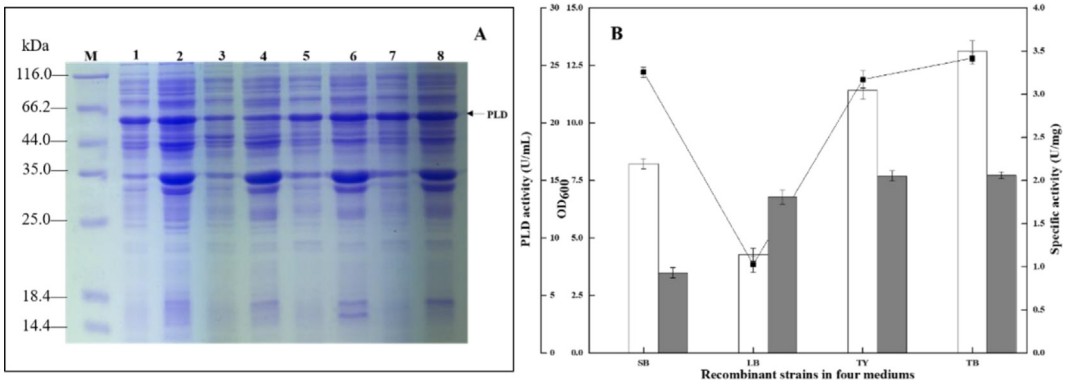

Fig. 2. Effect of different basic media on the PLD expression. (A) Lane 1–Lane 8, the supernatants and precipitates after inducing and disrupting of pET-28a+PLD/BL21(DE3) in SB, LB, TY, and TB, respectively. 10 μL samples of the supernatants (Lanes 1, 3, 5, and 7) and 5 μL samples of the precipitates (Lanes 2, 4, 6, and 8) after inducing and disrupting. M: molecular mass markers. (B) PLD activity (white area), cell density OD_{600} (■) and specific activity (light gray area). SB, LB, TY, and TB represented the recombinant *E. coli* strain pET-28a+PLD/BL21 (DE3) induced with 0.1 mM IPTG in SB, LB, TY, and TB media at 25°C for 36 h.

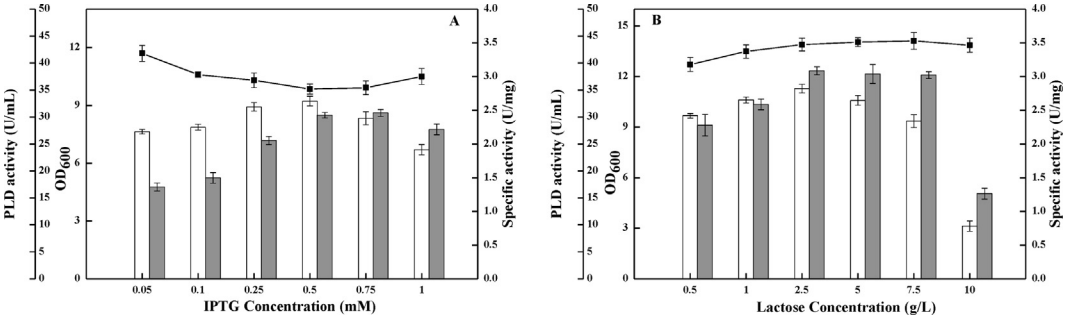

Fig. 3. Effect of varied concentration of IPTG or lactose on the PLD expression. PLD activity (white area), cell density OD_{600} (■), and specific activity (light gray area). (A) pET-28a+PLD/BL21 (DE3) induced with IPTG (0.05~1.0 mM), and (B) pET-28a+PLD/BL21(DE3) induced with lactose (0.5~10.0 g/L) at 25°C for 36 h.

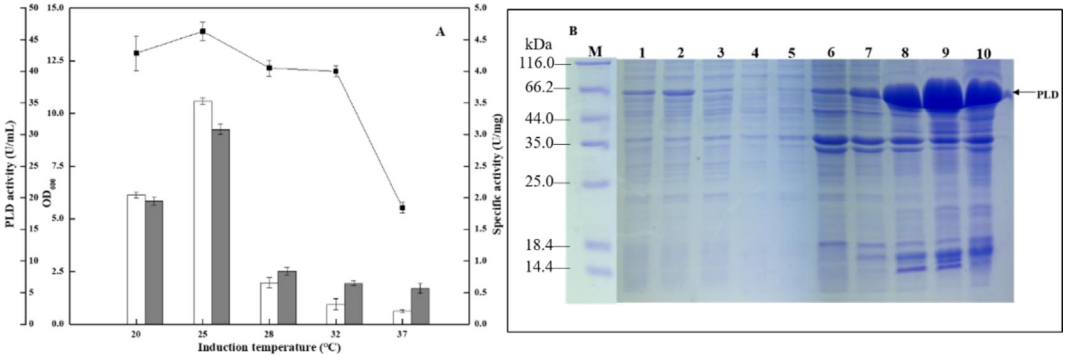

Fig. 4. Effect of induction temperature on PLD expression. (A) pET-28a+PLD/BL21(DE3) induced with 2.5 g/L of lactose at 20°C, 25°C, 28°C, 32°C, and 37°C for 36 h. PLD activity (white area), cell density OD_{600} (■), and specific activity (light gray area). (B) SDS-PAGE analysis of pET-28a+PLD/BL21(DE3) induced at various temperatures. Ten microliters of samples of the supernatants (Lane 1–5) and 5 μL of samples of precipitates (Lane 6–10) obtained after inducing with 2.5 g/L of lactose at various temperatures for 36 h.

in Fig. 5, the highest PLD activity attained to 83.97 ± 1.56 U/mL by 0.75 % (w/v) of mixed "glucose and starch (1:1, w/w)" (Fig. 5A), and 83.36 ± 0.8 U/mL by 1.0 % (w/v) of mixed "sorbitol and starch (1:1, w/w)" at 36 h (Fig. 5B).

Finally, the weight ratio between starch and glucose at 0.75 % (w/v) or between sorbitol and starch at 1.0 % (w/v) was investigated at 1:9, 3:7, 5:5, 7:3, and 9:1 (Fig. 5). All of the OD_{600}, PLD activity, and specific activity raised first and then declined. As far as 0.75 % (w/v) of mixed "glucose and starch" was concerned, the maximal PLD activity and specific activity attained to 83.97 ± 1.56 U/mL and 4.17 ± 0.18 U/mg at the weight ratio of 5:5 between glucose and starch at 36 h (Fig. 5C). As for 1.0 % (w/v) of mixed "sorbitol and starch," the highest PLD activity and specific activity attained to 94.57 ± 1.93 U/mL and 5.16 ± 0.12 U/mg at the weight ratio of 7:3 between sorbitol and starch at 36 h (Fig. 5D). The highest PLD activity and specific activity obtained by 1.0 % (w/v) of mixed "sorbitol and starch (7:3, w/w)" were 1.13-fold and 1.23-fold higher than those attained by 0.75 % (w/v) of mixed "glucose and starch (5:5, w/w)," respectively. Therefore, 1.0 % (w/v) of mixed "sorbitol and starch" at the weight ratio of 7:3 was selected as the optimal carbon source.

3.5. Effect of nitrogen source, Angel yeast extract, and phosphate

Nitrogen source is another important component of the culture medium, which can provide the nitrogen for biosynthesis of protein. Oxoid tryptone is more expensive than other nitrogen sources. Most of the cost of TB medium comes from the cost of

Table 2
Effect of carbon source on the recombinant *E. coli* growth and PLD expression.

Induction time (h)	24 h			36 h		
Carbon sources (1.0%, w/v)	OD_{600}	Enzyme activity (U/mL)	Specific activity (U/mg)	OD_{600}	Enzyme activity (U/mL)	Specific activity (U/mg)
Glucose	11.46 ± 0.62	41.75 ± 1.52	2.95 ± 0.08	13.17 ± 0.90	46.05 ± 1.54	3.09 ± 0.05
Glycerol	11.97 ± 0.12	3.50 ± 0.02	0.33 ± 0.04	12.16 ± 0.25	5.07 ± 0.02	0.51 ± 0.02
Sucrose	9.79 ± 0.24	21.33 ± 0.42	1.4 ± 0.05	8.73 ± 0.54	25.70 ± 0.72	2.29 ± 0.15
Sorbitol	12.32 ± 0.65	52.70 ± 0.96	3.39 ± 0.17	14.84 ± 0.47	61.09 ± 1.25	3.78 ± 0.08
Dextrin	11.75 ± 0.34	29.90 ± 0.57	2.38 ± 0.05	12.67 ± 0.33	34.63 ± 0.88	3.88 ± 0.04
Starch	11.31 ± 0.75	19.27 ± 0.46	1.68 ± 0.07	12.23 ± 0.39	27.76 ± 0.63	1.83 ± 0.03
Glucose + Dextrin (1:1, w/w)	12.80 ± 0.52	34.89 ± 0.38	3.00 ± 0.04	15.77 ± 0.81	36.89 ± 0.72	2.53 ± 0.07
Glucose + Starch (1:1, w/w)	11.70 ± 0.82	46.89 ± 0.47	3.90 ± 0.07	14.44 ± 0.37	69.92 ± 1.02	4.12 ± 0.06
Sorbitol + Dextrin (1:1, w/w)	13.40 ± 0.35	15.63 ± 0.81	1.39 ± 0.03	17.53 ± 0.66	34.18 ± 0.92	2.23 ± 0.01
Sorbitol + Starch (1:1, w/w)	12.04 ± 0.64	27.06 ± 0.67	2.27 ± 0.52	15.88 ± 0.84	83.36 ± 0.80	4.17 ± 0.16

Recombinant *E. coli* strain pET-28a+PLD/BL21 (DE3) was induced with 2.5 g/L of lactose in TB with various carbon sources (1.0 %, w/v) at 25°C for 24 h and 36 h, respectively.

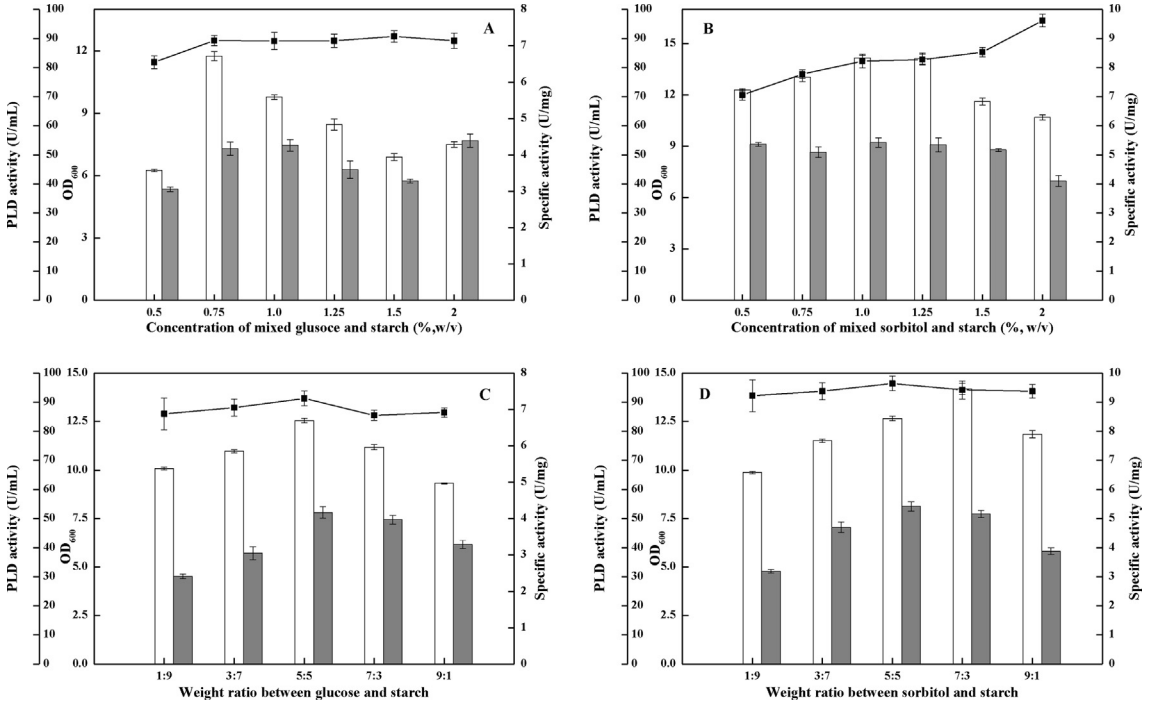

Fig. 5. Effects of concentration and weight ratio of mixed carbon sources on the recombinant *E. coli* growth and PLD expression. PLD activity (white area), cell density (■), and specific activity (light gray area). (A) Effect of mixed "glucose and starch (1:1, w/w)" concentration (0.5~2.0 %, w/v). (B) Effect of mixed "sorbitol and starch (1:1, w/w)" concentration (0.5~2.0 %, w/v). (C) Effect of weight ratio between glucose and starch at 0.75 % (w/v). (D) Effect of weight ratio between sorbitol and starch at 1.0 % (w/v). Recombinant *E. coli* strain pET-28a+PLD/BL21(DE3) was induced with 2.5 g/L of lactose in TB with varied concentration and weight ratio of mixed carbon sources at 25°C for 36 h.

Oxoid tryptone. In order to cut down the cost of culture medium, several cheap nitrogen sources such as peanut powder, corn steep liquor, ox bone peptone, soybean meal, and fish peptone were used to replace the Oxoid tryptone in TB. The results are shown in Fig. 6A. Among all the nitrogen sources tested, soybean meal was the worst nitrogen source, which resulted in the lowest PLD activity. Peanut powder could benefit the growth of recombinant *E. coli* strain, which led to the highest OD_{600}. But the highest cell density could not result in the highest PLD activity and specific activity. Compared to Oxoid tryptone, corn steep liquor and fish peptone could attain similar OD_{600} and slightly low PLD activity. However, the specific activity obtained by fish peptone was higher than that obtained by Oxoid tryptone. The specific activity obtained by corn steep liquor was the lowest among all the tested nitrogen sources. Therefore, the fish peptone could be used as the nitrogen source of TB to substitute the Oxoid tryptone. Furtherly, the effect of fish peptone at the concentration range from 0.6 % to 2.0 % (w/v) on the PLD expression was investigated. The highest PLD activity reached to 95.94 ± 0.43 U/mL at 1.0 % (w/v) fish peptone after 36 h induction (Fig. 6B).

The Oxoid yeast extract of TB medium was also replaced with 2.0–4.4 % (w/v) homebred Angel yeast extract to furtherly reduce the cost of culture medium. The results were shown in Fig. 6C. The maximum PLD activity achieved to 95.54 ± 1.09 U/mL at 4.0 % (w/v) of Angel yeast extract after 36 h induction. The PLD activity, specific activity, and OD_{600} obtained with 4.0 % (w/v) of Angel yeast extract were similar to those obtained with 1.2 % (w/v) of Oxoid yeast extract. Although 3.3-fold Angel yeast extract was required to obtain the similar PLD activity, the price of Oxoid yeast extract is 8 times higher than that of Angel yeast extract in the Chinese market. Therefore, Angel yeast extract was still considered to be a cheap substitute for Oxoid yeast extract in PLD production.

Phosphate is one of the components of *E. coli* cell membrane. It plays important roles in the functions of cell membrane. Phosphate can also affect the stability of recombinant plasmid and the expression of recombinant protein [36]. The total concentration of KH_2-

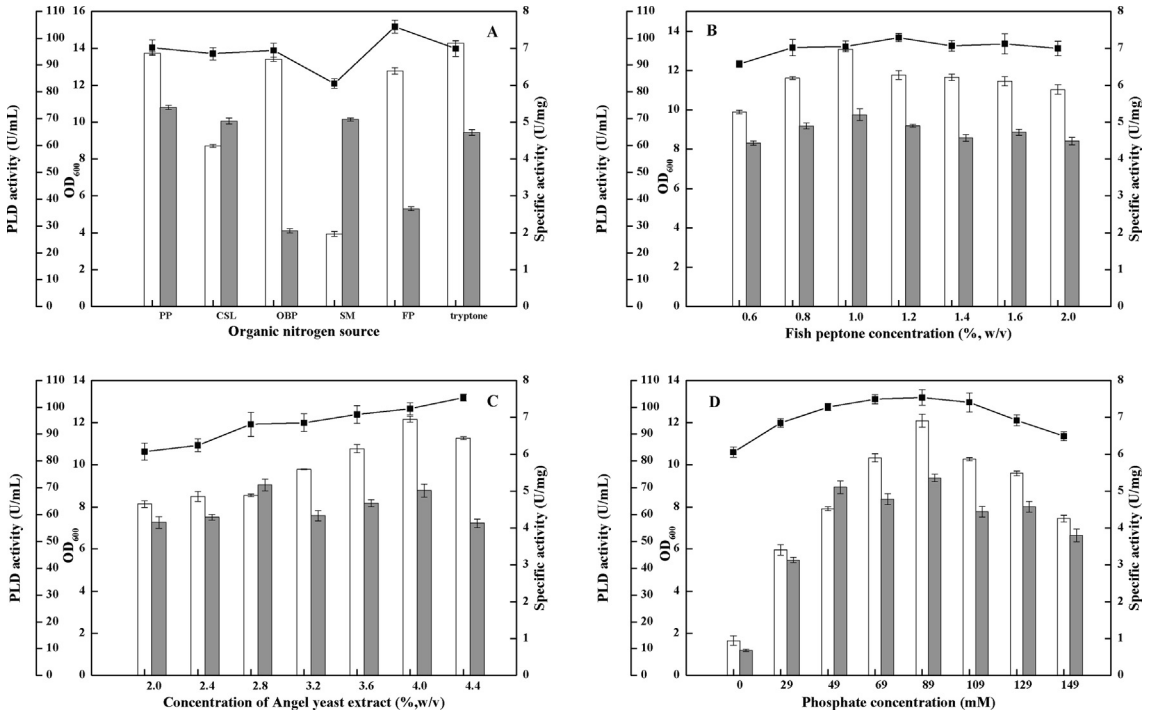

Fig. 6. Effects of nitrogen sources, Angel yeast extract and phosphate on the PLD expression. PLD activity (white area), cell density OD_{600} (■), and specific activity (light gray area). (A) Effect of various organic nitrogen sources, peanut powder-PP, corn steep liquor-CSL, ox bone peptone-OBP, soybean meal-SM, and fish peptone-FP. (B) Effect of fish peptone concentration. (C) Effect of Angel yeast extract concentration. (D) Effect of phosphate concentration. Recombinant *E. coli* strain pET-28a+PLD/BL21(DE3) was induced with 2.5 g/L of lactose at 25°C for 36 h.

PO_4 and K_2HPO_4 from 0 mM to 149 mM on the production of PLD was investigated. About 89 mM consisted of 72 mM K_2HPO_4, and 17 mM KH_2PO_4 could lead to the highest PLD activity, specific activity, and OD_{600} (Fig. 6D).

3.6. Time course of cultivation in shake flask and scale-up cultivation in a 5-L fermenter

After aforementioned optimization, the time course of pET-28a +PLD/BL21(DE3) cultivated in shake flask was investigated in the optimal medium consisted of 0.7 % (w/v) sorbitol, 0.3 % (w/v) starch, 1.0 % (w/v) fish peptone, 17 mmol/L KH_2PO_4, and 72 mmol/L K_2HPO_4 with 2.5 g/L lactose as inducer. The result was shown in Fig. 7. The highest PLD activity reached 104.28 ± 2. 67 U/mL after 32 h induction, which produced a productivity of 3.26 ± 0.083 U $mL^{-1}h^{-1}$, specific activity of 5.11 ± 0.13 U/mg, and OD_{600} of 13.15 ± 0.23 (Fig. 7A). The high-level soluble expression of PLD was fulfilled in the optimized medium (Fig. 7B).

Scale-up fermentation was performed in a 5-L fermenter to verify the optimal medium and culture conditions obtained in shake flask. A 5-L fermenter was loaded with 3.0 L of medium and inoculated with 50 mL of seed. The recombinant strain was firstly cultured at 37°C. When the OD_{600} attained about 0.8, the culture temperature was lowered to 25°C, and 2.5 g/L lactose was adopted to induce the PLD expression for 36 h. It could be found that the cell density OD_{600} increased very fast and attained the highest value after only induction for 4 h (Fig. 8A). However, high cell density could not lead to high PLD activity. The PLD expression level was largely lower than that attained in shake flask (Figs. 7A and 8A). When cultured in fermenter, the supply of oxygen and the distribution of nutrients and inducer were improved, which might be conducive to recombinant *E. coli* strain growth but not conducive to PLD expression. In order to slow down the growth rate of recombinant *E. coli* strain, changing the induction temperature from 25°C to 20°C was first attempted. It could be found that the cell growth rate had fallen down to a certain degree, but the PLD expression level was only improved a little (Fig. 8B). Because inducer lactose is also a carbon source, recombinant *E. coli* strain could consume it as a carbon source for cell growth, which might result in insufficient inducer for PLD expression. So, lactose feeding was next attempted. When the OD_{600} reached 7.0, the inducing temperature was reduced to 20°C and lactose was fed at a rate of 2.5 g $L^{-1}h^{-1}$. After feeding for 10 h, the feeding rate was changed from 2.5 g L^{-1}-h^{-1} to 0.625 g $L^{-1}h^{-1}$ for another 10 h. Then, when the OD_{600} was found to decline at 40 h, lactose was fed at a rate of 3.0 g $L^{-1}h^{-1}$ for 2.5 h. The results indicated that the maximal OD_{600} and PLD activity achieved 28.17 ± 1.53 and 122.94 ± 1.49 U/mL, respectively, which were higher than those obtained in shake flask (Fig. 8C). It was found that the recombinant *E. coli* strain still grew fast under the condition of feeding lactose although the inducing temperature was reduced to 20°C. Therefore, the mixed carbon source (0.7 % (w/v) sorbitol and 0.3 % (w/v) starch) in the optimized medium was substituted with 1.0 % (w/v) "slow" and cheap dextrin with the aim of slowing down the growth rate and cutting down the cost of medium. When the OD_{600} reached 0.8, the inducing temperature was reduced to 20°C and lactose was fed at a rate of 3.0 g $L^{-1}h^{-1}$ for 10 h. Then, when the downtrend of OD_{600} was found, a feeding medium (5 g of fish peptone, 3.5 g of sorbitol, 1.5 g of starch, 1.15 g of KH_2PO_4, and 8.2153 g of $K_2HPO_43H_2O$) that was prepared according to the components of the optimal medium in shake flask was added into the fermenter. The result is shown in Fig. 8D; the highest PLD activity reached 105.81 ± 2.72 U/mL, which was similar to that obtained in shake flask. However, the price of dextrin is 3 times lower than that of sorbitol in the Chinese market. Therefore, the cost of the medium was reduced, which can be conducive to the industrial production of PLD.

Finally, in this work, the high-level soluble expression of PLD from *S. chromofuscus* in *E. coli* was achieved in shake flask and fer-

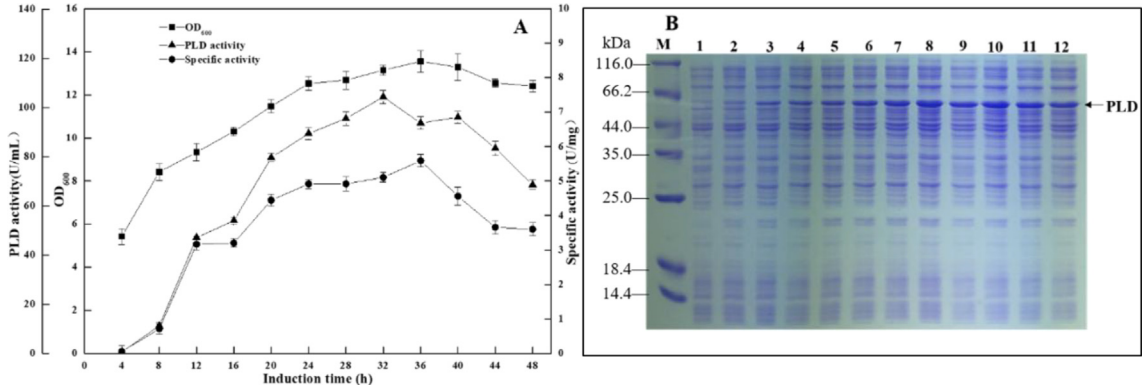

Fig. 7. The time course (A) and SDS-PAGE analysis (B) of recombinant *E. coli* strain pET-28a+PLD/BL21(DE3) cultivated in 250-mL shake flask containing 50 mL of the optimized medium with 2.5 g/L of lactose inducing at 25°C for 48 h. PLD activity (▲), specific activity (●), and cell density (■). Five microliters of sample of the supernatants (Lane 1–12) obtained after inducing at 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 28 h, 32 h, 36 h, 40 h, 44 h, and 48 h.

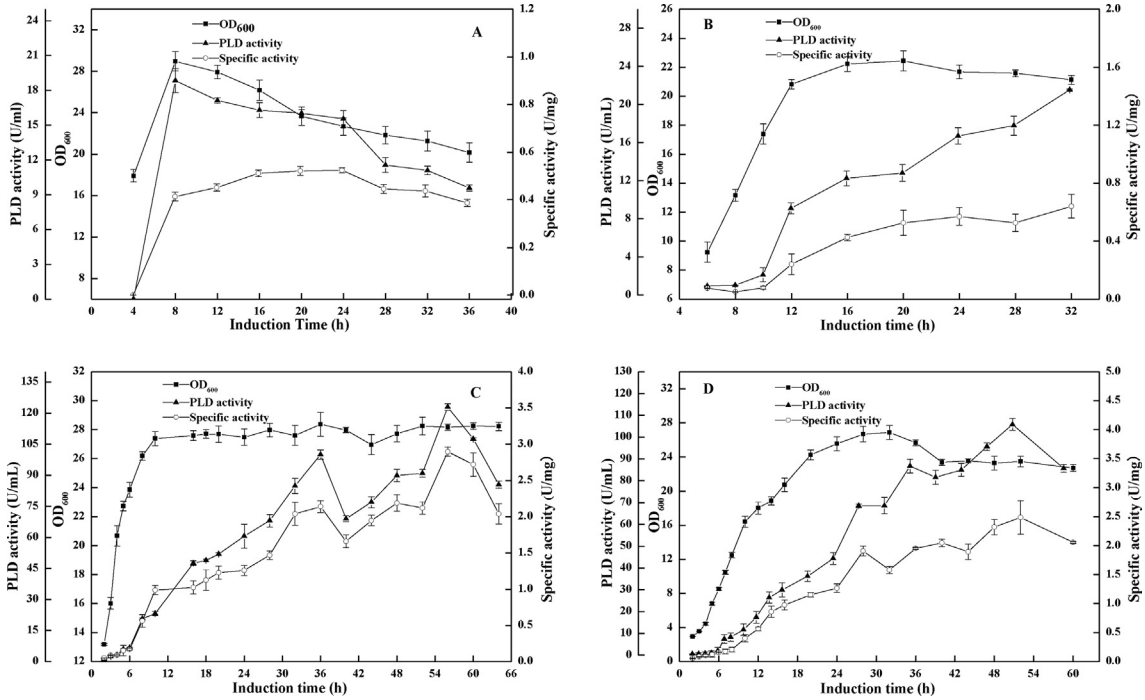

Fig. 8. Scale-up cultivation in a 5.0-L fermenter by adopting different strategies. PLD activity (▲), specific activity (○), and cell density (■). (A) Cultivation adopting the same medium and conditions as those in shake flask. (B) Cultivation adopting the same medium and conditions as those in shake flask except changing the inducing temperature from 25°C to 20°C. (C) Cultivation was carried out at 37°C until the OD_{600} reached about 7. Then, the inducing temperature was reduced to 20°C, and lactose was fed at a rate of 2.5 g/L/h. After feeding 10 h, the feeding rate was changed from 2.5 $gL^{-1}h^{-1}$ to 0.625 $gL^{-1}h^{-1}$ for another 10 h. When the OD_{600} was found to decline at 40 h, lactose was fed at a rate of 3.0 $gL^{-1}h^{-1}$ for 2.5 h. (D) The mixed carbon source (0.7 % (w/v) of sorbitol and 0.3 % (w/v) of starch) in the optimized medium was substituted with 1.0 % (w/v) of dextrin. Cultivation was carried out at 37°C until the OD_{600} reached about 0.8. Then, the inducing temperature was reduced to 20°C and lactose was fed at a rate of 3.0 $gL^{-1}h^{-1}$ for 10 h. When the downtrend of OD_{600} was found, a feeding medium (5 g of fish peptone, 3.5 g of sorbitol, 1.5 g of starch, 1.15 g of KH_2PO_4, 8.2153 g of $K_2HPO_4{\cdot}3H_2O$) was added.

menter. However, the high-level expression of PLD was still accompanied by the formation of some inclusion bodies. This problem has been taken into account and several measures will be employed to improve it. On the one hand, we plan to reduce the expression speed and improve the efficiency of correct protein folding by reducing induction temperature and adding molecular chaperones in our future works. On the other hand, the inclusion bodies can regain the biological activity via appropriate renaturation methods. We attempt to achieve inclusion bodies renaturation by developing new green solvents that can dissolve it. Therefore, it is promising to convert most insoluble inclusion bodies into soluble PLD.

4. Conclusion

Based on systematic optimization of expression plasmids, inducing conditions, and culture medium components, high-level expression of codon-optimized PLD from *S. chromofuscus* by an engineering recombinant *E. coli* strain pET-28a+PLD/BL21(DE3) was fulfilled in this work. The recombinant PLD activity reached 104.28 ± 2.67 U/mL in shake flask level. Scaling up in a 5.0-L fermenter, the PLD activity still reached 122.94 ± 1.49 U/mL by adopting 1.0 % (w/v) mixed carbon source and 105.81 ± 2.72 U/mL by using 1.0 % (w/v) cheap dextrin, respectively. As far as we know, this is the first report describing the high-level soluble expression

of PLD in *E. coli*. The PLD activity obtained at the fermenter level can adequately satisfy the requirements of industrial PLD production.

Conflict of interest

The authors declare no competing interests.

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Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2020.12.002.

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