High efficient extracellular production of recombinant *Streptomyces* PMF phospholipase D in *Escherichia coli*

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Research

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Abstract

Background Currently, *Streptomyces* is widely used in the preparation of phospholipase D (PLD) with high transphosphatidylation activity. However, the yield of PLD from Streptomyces was low and the culture period was long. Therefore, an efficient and cost-effective method is needed urgently.

Results Firstly, PLDs from Streptomyces PMF and Streptomyces racemochromogenes were separately over-expressed in *E. coli* to compare their transphosphatidylation activity based on the synthesis of phosphatidylserine (PS), and PLD_{PMF} was determined to have higher activity. To further improve PLD_{PMF} synthesis, a secretory expression system suitable for PLD_{PMF} was constructed and optimized with different signal peptides. The highest secretory efficiency was observed when the PLD_{PMF} gene was expressed together with its native signal peptide (Nat) and the signal peptide PelB from *E. coli*. For the application of recombinant PLD to PS synthesis, the PLD properties were characterized and 30.2 g/L of PS was produced after 24 h of bioconversion when 50 g/L phosphatidylcholine (PC) was added.

Conclusions We succeeded in over-expressing PLD from *Streptomyces* PMF in E. coli with high transphosphatidylation activity and enhanced the yield by secretory expression. The secreted PLD was successfully used in the production of PS. Our work makes the large-scale production of PLD and PS feasible.

Background

Phospholipase D (PLD, EC 3.1.4.4) catalyzes hydrolysis of the phosphodiester bond of glycerophospholipids to generate phosphatidic acid and a free headgroup. In addition to its hydrolytic activity, PLD can also catalyze the transfer of acyl groups to directly synthesize valuable phospholipid derivatives, such as phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG). These phospholipids have wide applications in the food, cosmetics and pharmaceutical industries [1]. PLD was first reported in 1947 and due to its special catalytic activity, research on PLD has recently increased [2]. PLD has been identified from plants [3], mammals [4], and bacteria [5]. However, these natural sources produce low levels of PLD that cannot meet the industrial demand [6]. Therefore, the production of PLD by microbial fermentation has attracted great attention due to its advantages of high unit activity and low cost.

PLD has been characterized in many microorganisms and is most commonly found in Streptomyces strains, such as Streptomyces PMF [7], S. lividans [8], and Streptomyces sp. YU100 [9]. For microbial production of PLD, Streptomyces strains are most widely used due to the high transphosphatidylation activity of native PLD and natural PLD secretion activity during fermentation. For example, Saovanee et al. isolated Streptomyces sp. SC734 from soil-contaminated palm oil, and the PLD it produced exhibited high activity with a conversion rate of phosphatidylcholine (PC) to PS of up to 94.7% in 100 min [10]. Ogino et al. constructed an overexpression system for secretory production of PLD in S. lividans and the amount of PLD secreted reached a maximum level of 118 mg/L [11]. However, the genetic-transfer systems for Streptomyces remain largely inefficient, which limits efficient production of PLD. Thus, the heterologous expression of Streptomyces PLDs in other model microorganisms, such as yeast or E. coli is highly desired.

Using Pichia pastoris as the host, Liu et al. developed a yeast cell surface display system to express PLD from S. chromofuscus, and the displayed PLD converted 67.5% of PC to PS within 10 h [12]. PLDs from different sources have also been successfully expressed with E. coli used as the host. For example, Carlo et al. expressed the PLD from Streptomyces PMF in E. coli BL21(DE3)pLysS, and 5 mg/L PLD was finally obtained with an enzyme activity of 15 mU/mL [13]. For the high-level and stable production of PLD, several engineering strategies were carried out in E. coli, including optimizing and tightly regulating promoter strength, optimizing codon usage and amino acid supplementation, and maintaining the best cellular state by supplementing nutrition. Finally, a large amount of PLD (81.5 mg/L) was obtained in batch culture [14]. Although there has been considerable progress in heterologous production of PLD, it is still not enough for industrial applications of PLD. Developing an efficient expression system for PLD production is urgently needed.

One of the biggest obstacles to efficient PLD production is that overexpressed PLD is toxic to the host, which may cause plasmid instability, cell lysis and PLD leakage [14]. Secretory production of heterologous proteins has great advantages compared with conventional cytosolic protein production, especially when the heterologous proteins are toxic. In addition, the secretory production of heterologous proteins could simplify the purification processes and reduce cost since cell disruption is not required. Many reports have proposed strategies for improving the secretory production of heterologous proteins, such as optimizing the environmental conditions [15], constructing leaky strains [16] or co-expressing the secretory pathway [17]. The production of PLD in the secretory form seems to be a promising approach to address this issue.

In this study, PLDs from Streptomyces PMF and Streptomyces racemochromogenes were separately overexpressed in E. coli to compare their transphosphatidylation activity based on synthesis of PS. Recombinant PLD_{PMF} exhibited higher activity. To further improve the synthesis of PLD_{PMF}, a secretory expression system suitable for PLD_{PMF} was constructed and optimized with different signal peptides. The highest secretory efficiency was observed when the PLD_{PMF} gene was expressed together with its native signal peptides (Nat) and PelB. After optimizing induction conditions including induction temperature, induction pH, IPTG concentration, induction time and addition of metal ions, 10.5 U/ml PLD was detected in the fermentation medium. For the application of recombinant PLD to PS synthesis, the PLD properties were characterized and 30.2 g/L of PS was produced after bioconversion for 24 h when 50 g/L PC was added.

Materials And Methods

Microorganisms and media

The strains used and constructed in this paper are listed in Table 1. The E. coli strains were cultured in Luria-Bertani medium (tryptone 10 g/L, NaCl 5 g/L and yeast extract 5 g/L) containing appropriate antibiotics at the following concentrations: 50 mg/L kanamycin (kana) and 100 mg/L ampicillin (Amp). Plasmid pET28a was used as the original plasmid.

Table 1
Strains and plasmids used in this study

Strains or plasmids	Characteristics	Sources
Strains		
Streptomyces PMF	Source of PLD_{PMF} gene	ATCC
Streptomyces racemochromogenes	Source of PLD_{SR} gene	ATCC
E. coli DH5α	Used as cloning vector	Invitrogen
E. coli BL21(DE3)	Used as expression host	Invitrogen
BL21(DE3)/pET28a-PLD_{PMF}	Express plasmid: pET28a-PLD_{PMF}	This study
BL21(DE3)/pET28a-PLD_{SR}	Express plasmid: pET28a-PLD_{SR}	This study
BL21(DE3)/pET22b-PLD_{PMF}	Express plasmid: pET22b-PLD_{PMF}	This study
BL21(DE3)/Nat-PLD*	Express plasmid: Nat-PLD*	This study
BL21(DE3)/OmpA-PLD*	Express plasmid: OmpA-PLD*	This study
BL21(DE3)/OmpA-Nat-PLD*	Express plasmid: OmpA-Nat-PLD*	This study
BL21(DE3)/OmpC-Nat-PLD*	Express plasmid: OmpC-Nat-PLD*	This study
BL21(DE3)/OmpF-Nat-PLD*	Express plasmid: OmpF-Nat-PLD*	This study
BL21(DE3)/OmpT-Nat-PLD*	Express plasmid: OmpT-Nat-PLD*	This study
BL21(DE3)/LamB-Nat-PLD*	Express plasmid: LamB-Nat-PLD*	This study
BL21(DE3)/PhoA-Nat-PLD*	Express plasmid: PhoA-Nat-PLD*	This study
BL21(DE3)/MalE-Nat-PLD*	Express plasmid: MalE-Nat-PLD*	This study
BL21(DE3)/PelB-Nat-PLD*	Express plasmid: PelB-Nat-PLD*	This study
Plasmids		
pET28a	pBR322 ori, P_{T7}, Kan^R	Our lab
pET22b	pBR322 ori, P_{T7}, OmpA signal peptide, Amp^R	Our lab
pET28a-PLD_{PMF}	pET28a derivative; $P_{T7-lacO}$- PLD_{PMF}	This study
pET28a-PLD_{SR}	pET28a derivative; $P_{T7-lacO}$- PLD_{SR}	This study
pET22b-PLD_{PMF}	pET22b derivative; $P_{T7-lacO}$-OmpA-PLD_{PMF}	This study
Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-Nat-PLD*	This study
OmpA-PLD*	pET22b derivative; $P_{T7-lacO}$-OmpA-PLD*	This study
OmpA-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-OmpA-Nat-PLD*	This study
OmpC-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-OmpC-Nat-PLD*	This study
OmpF-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-OmpF-Nat-PLD*	This study
OmpT-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-OmpT-Nat-PLD*	This study
LamB-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-LamB-Nat-PLD*	This study
PhoA-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-PhoA-Nat-PLD*	This study
MalE-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-MalE-Nat-PLD*	This study
PelB-Nat-PLD*	pET22b derivative; $P_{T7-lacO}$-PelB-Nat-PLD*	This study

Plasmid construction

All the primers used in this study are listed in Table 2. Two PLD gene fragments, PLD_{PMF} and PLD_{SR} were amplified from genomic DNA of Streptomyces PMF and Streptomyces racemochromogenes, respectively. The signal peptide genes OmpC, OmpF, OmpT, LamB, PhoA, MalE and pelB were synthesized by Sprin GenBioTech Co. LTD (Nanjing, China). The codon optimization procedure for the two PLD genes were conducted by Sprin GenBioTech Co. LTD (Nanjing,

China). We used the primers P1 and P2 to amplify the PLD_{PMF} gene and inserted it into the plasmid pET28a between the NcoI and EcoRI sites, yielding the recombinant plasmid pET28a-PLD_{PMF}. The PLD_{SR} fragment was amplified using the primer P3 with a NcoI restriction site and primer P4 with an EcoRI restriction site, and was ligated into pET-28a vector, yielding the plasmid pET28a-PLD_{SR}.

Table 2
Primers used in this study

Name	Primers	Sequences (5′-3′)
P1	NcoI-PLD_{PMF}-F	CATGCCATGGCAGCTGACTCTGCTACCCCG
P2	EcoRI-PLD_{PMF}-R	CCGGAATTCTCAGGCGTTGCAGATCCC
P3	NcoI-PLD_{SR}-F	CATGCCATGGGTGCGGAGGTGTGGTCGTAC
P4	EcoRI-PLD_{SR}-R	CCGGAATTCTCAGGCCTGGCAGAGG
P5	NdeI-Nat-PLD*	GGAATTCCATATGCTACATGGGTCACACCT
P6	XhoI-Nat-PLD*	CTCGAGCGGAGCGTTGCAGATACCAC
P7	NcoI-OmpA-Nat-PLD*	CCATGGGCTACATGGGTCACA
P8	XhoI-OmpA-Nat-PLD*	CTCGAGCGGAGCGTTGCAGATACCAC
P9	Eco-OmpA-PLD*	CATGCCATGGCAGCTGACTCTGCTACCCCG
P10	XhoI-OmpA-PLD*	CTCGAGCGGAGCGTTGCAGATACCAC
P11	LamB-F	GGAATTCCATATGATTACTCTGCGCAAACTTCCTCTGGCGGTTGCCGTCGCAGCGGGCGTAATGTCTGCTCAGGCAATGGCTCCATGGGCTACA
P12	MalE-F	GGAATTCCATATGAAAATAAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGACGATGATGTTTTCCGCCTCGGCTCTCGCCCCATGGG
P13	OmpC-F	GGAATTCCATATGAAAGTTAAAGTACTGTCCCTCCTGGTCCCAGCTCTGCTGGTAGCAGGCGCAGCAAACGCTCCATGGGCTACATGGGTCACA
P14	OmpF-F	GGAATTCCATATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGCTCTGTTAGTAGCAGGTACTGCAAACGCTCCATGGGCTACATGGGTCACA
P15	OmpT-F	GGAATTCCATATGCGGGCGAAACTTCTGGGAATAGTCCTGACAACCCCTATTGCGATCAGCTCTTTTGCTCCATGGGCTACATGGGTCACA
P16	PhoA-F	GGAATTCCATATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCCTGTGACAAAAGCCCCATGGGCTACATGGGTCACA
P17	PelB-F	GGAATTCCATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGCTACATGGGTCAC
P18	General reverse primer	CTCGAGCGGAGCGTTGCAGATACCAC

For secretory expression of PLD, PLD_{PMF} was cloned into plasmid pET22b together with different signal peptides. Primers P5/P6 were used for PCR amplification of the PLD_{PMF} gene containing the native signal peptide (Nat), while the primers P7/P8 were used to obtain the fragment OmpA-Nat-PLD*, and primers P9/P10 were used to obtain the fragment OmpA-PLD*. These three fragments were inserted into NcoI/XhoI sites of plasmid pET22b to yield the plasmids pET22b-Nat-PLD*, pET22b-OmpA-Nat-PLD* and pET22b-OmpA-PLD* respectively. To optimize the secretory efficiency, seven other signal peptides OmpC, OmpF, OmpT, LamB, PhoA, MalE and PelB were amplified using appropriate primers listed in Table 2 to replace OmpA of plasmid pET22b-OmpA-Nat-PLD*.

Protein expression and cell fractionation

The engineered E. coli was cultivated in 100 mL LB medium with 0.1 mM of appropriate antibiotics at 37 °C on a rotatory shaker (200 rpm). When the cell-culture density at 600 nm (OD_{600}) reached 0.6, 0.5 mM β-d-1-thiogalactopyranoside (IPTG) was added into the culture. Then the cells were incubated at 28 °C

for 12 h. Cells were harvested by centrifugation at 8000 rpm for 10 min. The supernatant was concentrated eight times using a rotary evaporator (20 °C, 15 mbar) and was then used as the extracellular fraction. The collected cells were resuspended in water to an OD_{600} of 20 and the crude extracts were prepared on ice by ultrasonication: 20 min pulsing (0.3 ms, 0.2 ms pause) at 40% amplitude.

Enzyme assay

The transphosphatidylation activity of PLD was measured according to the production of PS. One unit (U) was defined as 1 μmol PS produced per 1 min. To determine the PLD activity, a catalytic reaction was carried out in a two-phase system containing an aqueous phase and organic phase, and the volume ratio was 1:1. The aqueous phase contained 1 mL enzyme solution and 1 mL 0.2 M sodium acetate buffer (pH 5.5) containing 1.4 g/L serine. The organic phase was 2 mL methylene chloride containing 10 g/L PC. The molar ratio of substrate PC to serine was 1:60. The reaction mixture was incubated in a 200 rpm shaker at 30 ºC for 4 h. The reaction mixture was boiled for 5 min to completely arrest the reaction and the mixture was subsequently centrifuged at 6000 rpm for 5 min. After that, a 100 μL organic sample was taken from the mixture and diluted 10 times with a mixture containing chloroform and methanol with a volume ratio of 1:1. Then the diluted sample was determined by HPLC.

Analytical methods

The samples were analyzed by high-performance liquid chromatography (HPLC) (Agilent 1260, USA) equipped with a Chromachem evaporative light scattering detector (ELSD). The separation of PS and PC was performed on a ZORBAX Rx-SIL silica gel column (5 μm, 250 mm × 4.6 mm, Agilent). Mobile phase A contained 85% methanol, 14.5% water, 0.45% acetic acid, and 0.05% trimethylamine; mobile phase B contained 20% n-hexane, 48% isopropanol, and 32% mobile phase A. The flow rate was 1.0 mL min^{-1}. The elution conditions were as follows: initially 2% A; 5 min, 10% A; 9 min, 30% A; 14 min, 10% A; 17 min, 2% A. The column temperature, nebulizing temperature, and evaporating temperature were controlled at 38 °C, 72 °C, and 72 °C, respectively, and nitrogen was used as the nebulizing gas. The nitrogen gas flow rate was 2.0 SLPM (standard liters per minute). Each phospholipid was determined from the retention time using calibration solutions of corresponding phospholipids, and the concentrations of the phospholipids in the samples were calculated from the peak areas by integration.

Results

Intracellular expression of PLD in E. coli

The host strain E. coli BL21(DE3) is an efficient expression system for various recombinant proteins. Here we attempted to use it for the production of PLD. Two PLDs in the plasmids pET28a-PLD_{PMF} and pET28a-PLD_{SR} were separately introduced into BL21(DE3). Enzyme production was induced by the addition of IPTG and the activity of crude PLD extracts was compared. As shown in Fig. 1, both PLDs were functionally expressed in E. coli, and crude extracts of the strain BL21(DE3)/pET28a-PLD_{PMF} exhibited higher transphosphatidylation activity. Using the crude extracts of the strain BL21(DE3)/pET28a-PLD_{PMF} for the bioconversion of PC to PS, PS reached 0.37 g/L after 8 h, which is 1.4-fold higher than that of BL21(DE3)/pET28a-PLD_{SR}. Therefore, PLD_{PMF} was applied to further optimize expression.

Secretory expression of PLD in E. coli by optimizing signal peptides

To investigate the secretory expression of PLD, the signal peptides Nat (Native signal peptide from PLD_{PMF}), OmpA and the fused signal peptide OmpA-Nat were co-expressed with the PLD* (PLD_{PMF} without Nat sequence) gene (Fig. 2a). First, the effect of Nat and OmpA on the PLD secretory efficiency was compared. No PS was detected using the extracellular fraction of the strain BL21(DE3)/pET22b-Nat-PLD* for the conversion of PC to PS (Fig. 2b). In contrast, the PS yield reached 40.68% after bioconversion for 24 h using the extracellular fraction of the strain BL21(DE3)/pET22b-OmpA-PLD*, indicating that Nat is not functional for directing the secretion of heterologous PLD in E. coli. Subsequently, to identify whether the cleavage of Nat sequence in the N-terminus of PLD_{PMF} affected PLD activity, PLD* and PLD_{PMF} were separately expressed after being fused with OmpA in E. coli. We found that the PLD from the strain BL21(DE3)/pET22b-OmpA-Nat-PLD* exhibited higher transphosphatidylation activity with PS yield of 45.72% after bioconversion for 24 h. This result indicated that the Nat signal peptide is important for maintaining the transphosphatidylation activity of recombinant PLD expressed in E. coli. Thus, the expression of PLD_{PMF} directed by E. coli homologous signal peptides was best for the secretory expression of PLD in E. coli.

To further determine the optimum signal peptide for directing secretion expression of PLD in E. coli, seven different signal peptides were employed to replace OmpA (Fig. 3a). As shown in Fig. 3b, different signal peptides directed export of PLD with varying efficiencies. Compared with OmpA, the signal peptides OmpF, OmpT, LamB and MalE are less efficient and lower PS yield was observed. In contrast, more efficient PLD secretion was obtained with the signal peptides OmpC, PhoA and PelB resulting in higher PS yield. Among them, the highest level of extracellular PLD was found after expressing the plasmid PelB-Nat-PLD* in E. coli, where PS yield was increased by 86.51% compared to that from OmpA. Thus, the recombinant strain BL21(DE3)/pET22b-PelB-Nat-PLD* with the highest PLD secretory expression activity was selected for the following experiment.

Effect of fermentation conditions on the secretory expression of PLD

To further improve PLD synthesis, fermentation conditions, including induction temperature, induction pH, cell density at induction and IPTG concentration were optimized. For the control group, the induction temperature was 28 °C, IPTG concentration was 0.5 mM, cultivation pH was 7.0 and the induction OD_{600nm} was 0.6. Induction temperature is an important factor influencing heterologous protein expression in E. coli (Fig. 4a). The recombinant strain was incubated at a temperature ranging from 16 °C to 36 °C, and the maximum PLD activity was obtained at 20 °C with an increase of 19.4% compared to the control group (Fig. 4a). The effect of the concentration of IPTG was evaluated by varying the concentration from 0.4 mM to 0.8 mM. The highest PLD activity was achieved when 0.7 mM IPTG was added, which resulted in a 68.2% increase in PS yield (Fig. 4b). The optimal induction OD_{600} and time were also

determined at the induction OD_{600} of 1.4 after induction for 12 h (Fig. 4c and 4d). Varying pH of the growth environment change bacterial metabolic pathways, which might negatively affect the expression of heterologous protein in E. coli. In addition, pH also affects the charge state on the cell surface, and thus the permeability of the cell membrane, which has an important impact on the exchange of substances and the secretion of recombinant proteins. When the engineered BL21(DE3)/pET22b-PelB-Nat-PLD* was cultivated at pH ranging from 5.0 to 8.0, the PLD activity reached the highest level at pH of 6.5 (Fig. 4e).

To further improve the secretory expression of recombinant PLD, the effects of surfactant addition were evaluated. As shown in Fig. 4f, seven different surfactants were separately added into the fermentation medium. Compared to the control group, all surfactants benefitted the secretory expression of PLD, among which, the group with 3 g/L Span60 added exhibited the best PLD activity.

Characterization of the recombinant PLD activity

To characterize the recombinant PLD activity, the effects of reaction temperature, pH and metal ion additives were evaluated. To determine the optimal reaction temperature, bioconversion was carried out at 20, 25, 30, 35, or 40 °C (Fig. 5c). From 20 °C and 30 °C, the PLD activity clearly increased with increasing temperature, and reached the highest level at 30 °C. When the temperature was higher than 30° C, the PLD activity sharply decreased, indicating the temperature sensitivity of recombinant PLD. The PLD activity increased with a rise in reaction pH (from 4.0 to 8.0) and reached a maximum at pH 6.5 (Fig. 5b).

Several metal ions have been reported to play an important role in maintaining the activity of PLD [18]. To evaluate the effect of metal ions on recombinant PLD activity, metal ions including Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, K^{+}, Fe^{2+}, Mg^{2+} and Mn^{2+} were added. The reaction performed without any metal ions served as the control group. The addition of Co^{2+}, Ca^{2+} and Mg^{2+} showed positive effects on the PLD activity, and the addition of Ca^{2+} gave the highest level of PLD activity (Fig. 5c).

The application of recombinant PLD_{PMF} for the bioconversion of PC to PS

The optimum expression system, fermentation conditions and PLD traits were determined based on the above results. With the extracellular recombinant PLD produced by engineered E. coli, the capacity of producing PS from PC by PLD was tested under the optimal reaction conditions. The reaction was performed with PC substrate concentrations of 10 g/L, 30 g/L and 50 g/L. Samples were taken at reaction times of 4 h, 8 h, 12 h and 24 h to detect the amount of product PS and substrate PC (Fig. 6). In the case of 10 g/L PC, after bioconversion of 24 h, the production of PS reached 9.2 g/L with a molar yield of 88.05%. When the concentration of PC was increased to 30 g/L, the final PS titer of 18.2 g/L was obtained with a molar yield of 58.02% after bioconversion of 24 h. Further increasing PC concentration to 50 g/L, increased PS titer to 30.2 g/L with a molar yield of 57.81%.

Discussion

Phospholipase has achieved significant attention in recent years for its applications in the production of various high value rare phospholipids [19]. To achieve high production of PLD, overexpression of native PLD or heterologous expression of various PLDs in model microorganisms, including E. coli [20], yeast [21], and Bacillus subtilis [22] have been performed. Among them, E. coli is the most frequently used host strain for the expression of heterologous proteins due to its well-characterized genetics, high protein expression levels and rapid growth rate [23]. However, the toxicity from overexpressing PLD has limited its production in E. coli. In this work, after screening PLD sources, a secretory PLD expression system was developed and optimized by investigating the effects of different signal peptides for efficient PLD production.

After determining a suitable source of PLD, OmpA, a signal peptide that has been reported to guide the secretory expression of heterologous proteins with high efficiency in E. coli [24], was first employed to direct the secretory production of recombinant PLD. The effect of native signal peptide (Nat) in PLD sequence was also evaluated. Fortunately, the PLD production efficiency was largely enhanced with the secretory expression system. Moreover, the presence of Nat signal peptides resulted in a higher extracellular PLD activity, suggesting that the Nat sequence might contribute to the correct fold of recombinant PLD in E. coli. It is well known that the N-terminal signal sequence can guide the protein to the Sec-translocon through the post-translational SecB-targeting pathway or the co-translational signal recognition particle (SRP)-targeting pathway and then fold correctly [25]. However, there is currently no general rule in selecting a proper signal sequence for a given recombinant protein [26]. To identify a more efficient signal peptide to direct PLD secretion in E. coli, seven other signal peptides including OmpF, OmpT, OmpC, LamB, MalE, PhoA, and PelB were compared. The highest secreted PLD activity occurred with PelB (Fig. 3b), indicating the important role of signal peptide for the efficient production of recombinant PLD.

For the secretory production of recombinant proteins, membrane permeability might be a limiting factor since the cellular membrane often retards the entry of substrate into the cellular systems and prevents the product from being released from the cellular system for an easy recovery [27]. With the addition of 3 g/L Span60, the extracellular PLD activity was increased by 97.1%, indicating that cell membrane permeability is one of the key factors affecting secretory expression of recombinant PLD in E. coli. To address this issue, co-expression of the key secretion components, construction of leaky strains and utilization of different secretion pathways to enhance secretory production of heterologous PLD could be further carried out in future studies.

For applying PLD to the synthesis of high value added phospholipid, the properties of recombinant PLD were also characterized. As shown in Fig. 5a, recombinant PLD is sensitive to temperature changes. The best pH for PLD activity was observed under pH 5.5, which coincides with other reports in which PLD exerted high transphosphatidylation activity in a weak acid environment [28]. For the metal ion additives, the highest PLD activity was observed with the addition of Ca^{2+} [29]. Ca^{2+} binding to PLD has been reported to cause a conformational change in the PLD that enhances binding of protein to zwitterionic interfaces [10]. Ca^{2+} is also an activator when other soluble substrates are used [30]. Ca^{2+} possibly coordinates with enzymes, improving their stability. Finally, the recombinant PLD was applied for the bioconversion of PC to PS.

Previous reports on the enzymatic synthesis of PS focused on the use of PLDs expressed in Streptomyces. Duan and Hu compared five commercial PLDs in the synthesis of PS, and PLD derived from S. chromofuscus achieved 90% yield of PS after 12 h of bioconversion [31]. In our work, the recombinant PLD

converted 88.05% of PC into PS with a concentration of 10 g/L PC, indicating the high transphosphatidylation activity of the recombinant PLD expressed by E. coli. Under optimized conditions, 30.2 g/L PS was obtained with a yield of 57.81%. The recombinant PLDs obtained in E. coli are summarized in Table 3, and the highest PS concentration so far was obtained in our study.

Table 3
Production of recombinant PLD in E. coli and synthesis of PS

PLD origin	Expression Host	PS (g/L)	References
Streptomyces mobaraensis	E. coli	0.2	[29]
Streptomyces chromofuscus	E. coli	3.94	[32]
Streptomyces sp. YU100	E. coli	None	[9]
Streptomyces antibioticus	E. coli	None	[14]
Streptomyces PMF	E. coli	30.2	This study

Conclusions

In this study we cloned and expressed the PLD from Streptomyces PMF in E. coli; the strain BL21(DE3)/pET28a-PLD_{PMF} only exhibited intracellular PLD activity. In order to release the negative effects caused by the toxicity of PLD we constructed the strain BL21(DE3)/pET22b-PLD_{PMF} to secrete the PLD into the culture medium and the supernatant of the culture exhibited PLD activity producing 1.23 g/L PS in 8 h. Then, we investigated the effects of signal peptides and adding surfactants on the secretory production of PLD. Strain BL21(DE3)/PelB-Nat-PLD* showed the highest extracellular PLD activity. With the addition of 3 g/L Span60, the extracellular fraction was used for catalytic reaction, and the concentration of PS reached 4.51 g/L, 3.67-fold higher than strain BL21(DE3)/pET22b-PLD_{PMF}. After optimizing the induction conditions and catalytic situations, the recombinant PLD of the strain BL21(DE3)/PelB-Nat-PLD* produced 30.2 g/L PS in 24 h. With the advantages of simple operations, low cost of recycling the PLD and high activity of the enzyme, our work makes large-scale production of PLD and PS feasible.

Availability Of Data And Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The present manuscript has been seen and approved by all authors. They agree with its content and submission to Microbial Cell Factories.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contributions

XW, KC and PO develop the idea for this study. XW and SX design the research. XW, YP and JW did the majority of the lab work, plasmid construction, strain cultivation and product detection. JW did the literature review and prepared the manuscript. XW helped to revise the manuscript.

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Tables

Table 1. Strains and plasmids used in this study

Strains or plasmids	Characteristics	Sources
Strains		
Streptomyces PMF	Source of PLD_{PMF} gene	ATCC
Streptomyces racemochromogenes	Source of PLD_{SR} gene	ATCC
E. coli DH5α	Used as cloning vector	Invitrogen
E. coli BL21(DE3)	Used as expression host	Invitrogen
BL21(DE3)/pET28a-PLD_{PMF}	Express plasmid: pET28a-PLD_{PMF}	This study
BL21(DE3)/pET28a-PLD_{SR}	Express plasmid: pET28a-PLD_{SR}	This study
BL21(DE3)/pET22b-PLD_{PMF}	Express plasmid: pET22b-PLD_{PMF}	This study
BL21(DE3)/Nat-PLD*	Express plasmid: Nat-PLD*	This study
BL21(DE3)/OmpA-PLD*	Express plasmid: OmpA-PLD*	This study
BL21(DE3)/OmpA-Nat-PLD*	Express plasmid: OmpA-Nat-PLD*	This study
BL21(DE3)/OmpC-Nat-PLD*	Express plasmid: OmpC-Nat-PLD*	This study
BL21(DE3)/OmpF-Nat-PLD*	Express plasmid: OmpF-Nat-PLD*	This study
BL21(DE3)/OmpT-Nat-PLD*	Express plasmid: OmpT-Nat-PLD*	This study
BL21(DE3)/LamB-Nat-PLD*	Express plasmid: LamB-Nat-PLD*	This study
BL21(DE3)/PhoA-Nat-PLD*	Express plasmid: PhoA-Nat-PLD*	This study
BL21(DE3)/MalE-Nat-PLD*	Express plasmid: MalE-Nat-PLD*	This study
BL21(DE3)/PelB-Nat-PLD*	Express plasmid: PelB-Nat-PLD*	This study
Plasmids		
pET28a	pBR322 ori, P_{T7}, Kan^R	Our lab
pET22b	pBR322 ori, P_{T7}, OmpA signal peptide, Amp^R	Our lab
pET28a-PLD_{PMF}	pET28a derivative; $P_{T7\text{-}lacO}$- PLD_{PMF}	This study
pET28a-PLD_{SR}	pET28a derivative; $P_{T7\text{-}lacO}$- PLD_{SR}	This study
pET22b-PLD_{PMF}	pET22b derivative; $P_{T7\text{-}lacO}$-OmpA-PLD_{PMF}	This study
Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-Nat-PLD*	This study
OmpA-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-OmpA-PLD*	This study
OmpA-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-OmpA-Nat-PLD*	This study
OmpC-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-OmpC-Nat-PLD*	This study
OmpF-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-OmpF-Nat-PLD*	This study
OmpT-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-OmpT-Nat-PLD*	This study
LamB-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-LamB-Nat-PLD*	This study
PhoA-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-PhoA-Nat-PLD*	This study
MalE-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-MalE-Nat-PLD*	This study
PelB-Nat-PLD*	pET22b derivative; $P_{T7\text{-}lacO}$-PelB-Nat-PLD*	This study

Table 2. Primers used in this study

Name	Primers	Sequences (5′-3′)
P1	NcoI-PLD$_{PMF}$-F	CATGCCATGGCAGCTGACTCTGCTACCCCG
P2	EcoRI-PLD$_{PMF}$-R	CCGGAATTCTCAGGCGTTGCAGATCCC
P3	NcoI-PLD$_{SR}$ -F	CATGCCATGGGTGCGGAGGTGTGGTCGTAC
P4	EcoRI-PLD$_{SR}$ -R	CCGGAATTCTCAGGCCTGGCAGAGG
P5	NdeI-Nat-PLD*	GGAATTCCATATGCTACATGGGTCACACCT
P6	XhoI-Nat-PLD*	CTCGAGCGGAGCGTTGCAGATACCAC
P7	NcoI-OmpA-Nat-PLD*	CCATGGGCTACATGGGTCACA
P8	XhoI-OmpA-Nat-PLD*	CTCGAGCGGAGCGTTGCAGATACCAC
P9	Eco-OmpA-PLD*	CATGCCATGGCAGCTGACTCTGCTACCCCG
P10	XhoI-OmpA-PLD*	CTCGAGCGGAGCGTTGCAGATACCAC
P11	LamB-F	GGAATTCCATATGATTACTCTGCGCAAACTTCCTCTGGCGGTTGCCGTCGCAGCGGGCGTAATGTCTGCTCAGGCAATGGCTCCATGGGCTACATGGGTCACA
P12	MalE-F	GGAATTCCATATGAAAATAAAAACAGGTGCACGCATCCTCGCATTATCCGCATTAACGACGATGATGTTTTCCGCCTCGGCTCTCGCCCCATGGGCTACATGGGTCACA
P13	OmpC-F	GGAATTCCATATGAAAGTTAAAGTACTGTCCCTCCTGGTCCCAGCTCTGCTGGTAGCAGGCGCAGCAAACGCTCCATGGGCTACATGGGTCACA
P14	OmpF-F	GGAATTCCATATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGCTCTGTTAGTAGCAGGTACTGCAAACGCTCCATGGGCTACATGGGTCACA
P15	OmpT-F	GGAATTCCATATGCGGGCGAAACTTCTGGGAATAGTCCTGACAACCCCTATTGCGATCAGCTCTTTTGCTCCATGGGCTACATGGGTCACA
P16	PhoA-F	GGAATTCCATATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCCTGTGACAAAAGCCCCATGGGCTACATGGGTCACA
P17	PelB-F	GGAATTCCATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGCTACATGGGTCACA
P18	General reverse primer	CTCGAGCGGAGCGTTGCAGATACCAC

Table 3. Production of recombinant PLD in *E. coli* and synthesis of PS

PLD origin	Expression Host	PS (g/L)	References
Streptomyces mobaraensis	*E. coli*	0.2	[29]
Streptomyces chromofuscus	*E. coli*	3.94	[32]
Streptomyces sp. YU100	*E. coli*	None	[9]
Streptomyces antibioticus	*E. coli*	None	[14]
Streptomyces PMF	*E. coli*	30.2	This study

Figures

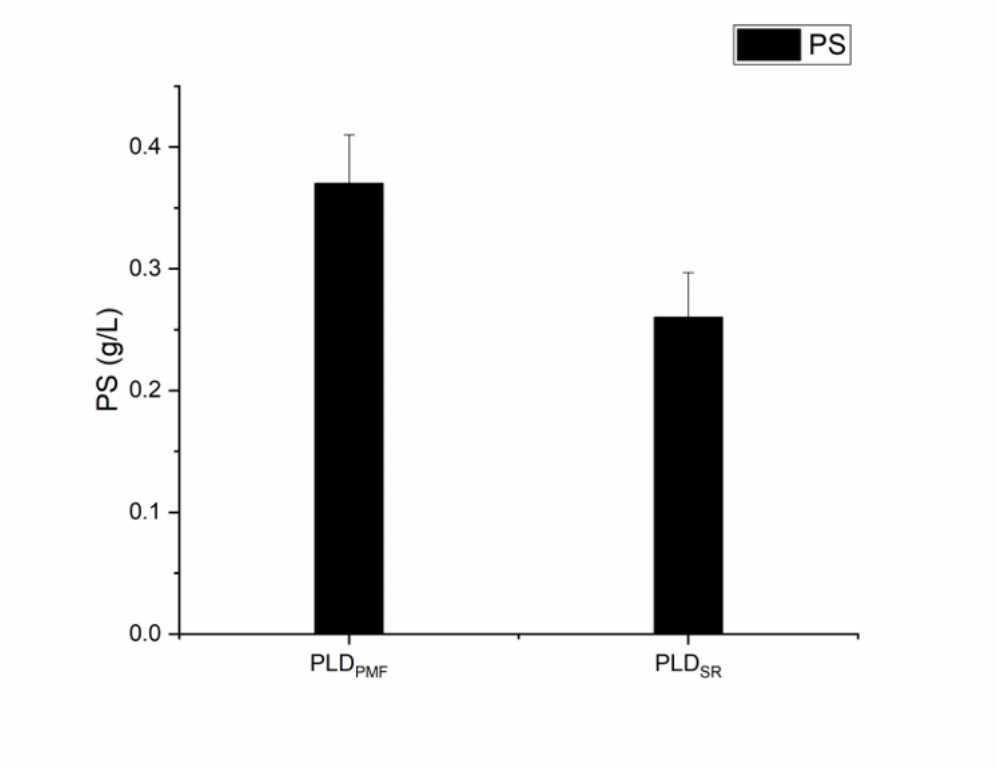

Figure 1

Comparison of the transphosphatidylation activity of the PLD coming from Streptomyces PMF and S. racemochromogenes when expressed in E. coli.

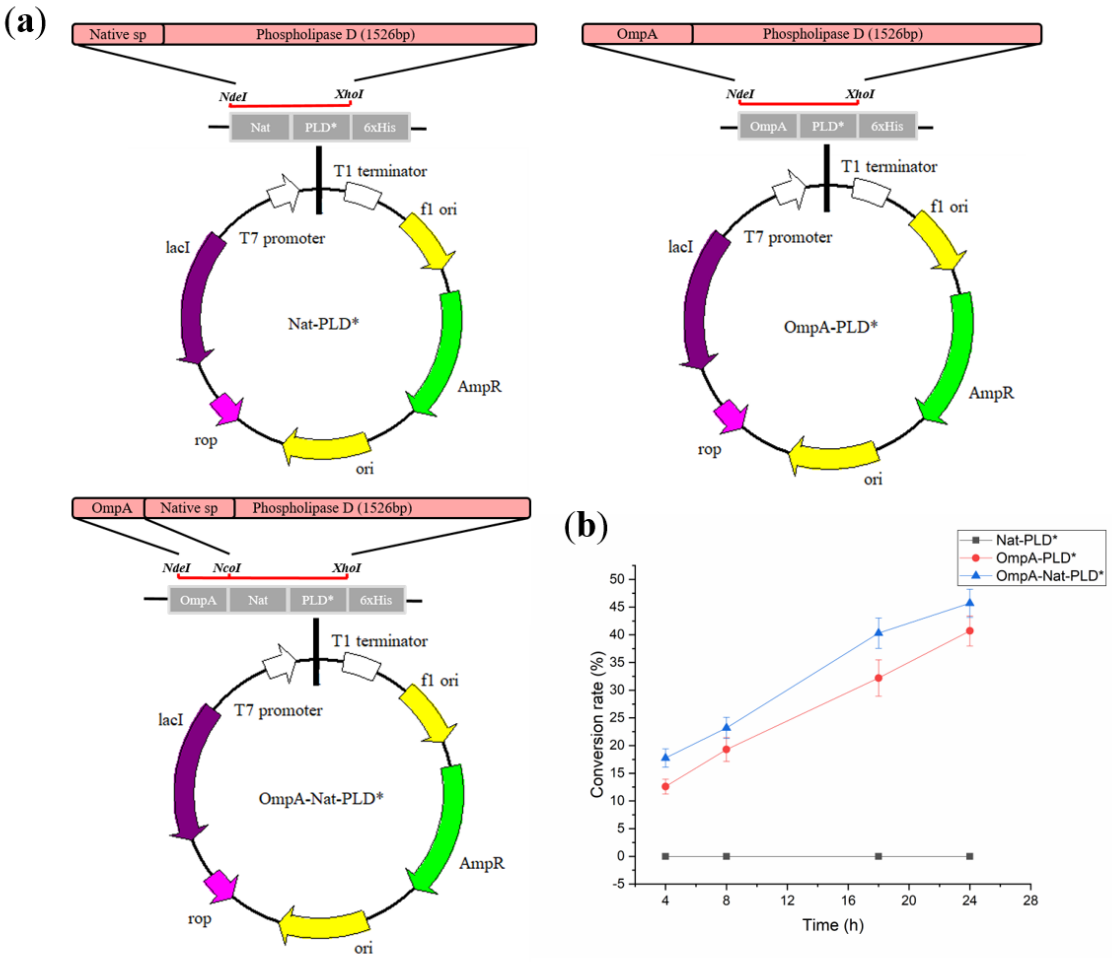

Figure 2

Effects of the Nat signal peptide on the transphosphatidyltion activity of recombinant PLDPMF expressed by E. coli. (a) Structure of the plasmids Nat-PLD*, OmpA-PLD* and OmpA-Nat-PLD*. (b) The conversion rates of PC to PS catalyzed by the extracellular PLD expressed by the strain: BL21(DE3)/Nat-PLD*, BL21(DE3)/OmpA-PLD* and BL21(DE3)/OmpA-Nat-PLD*.

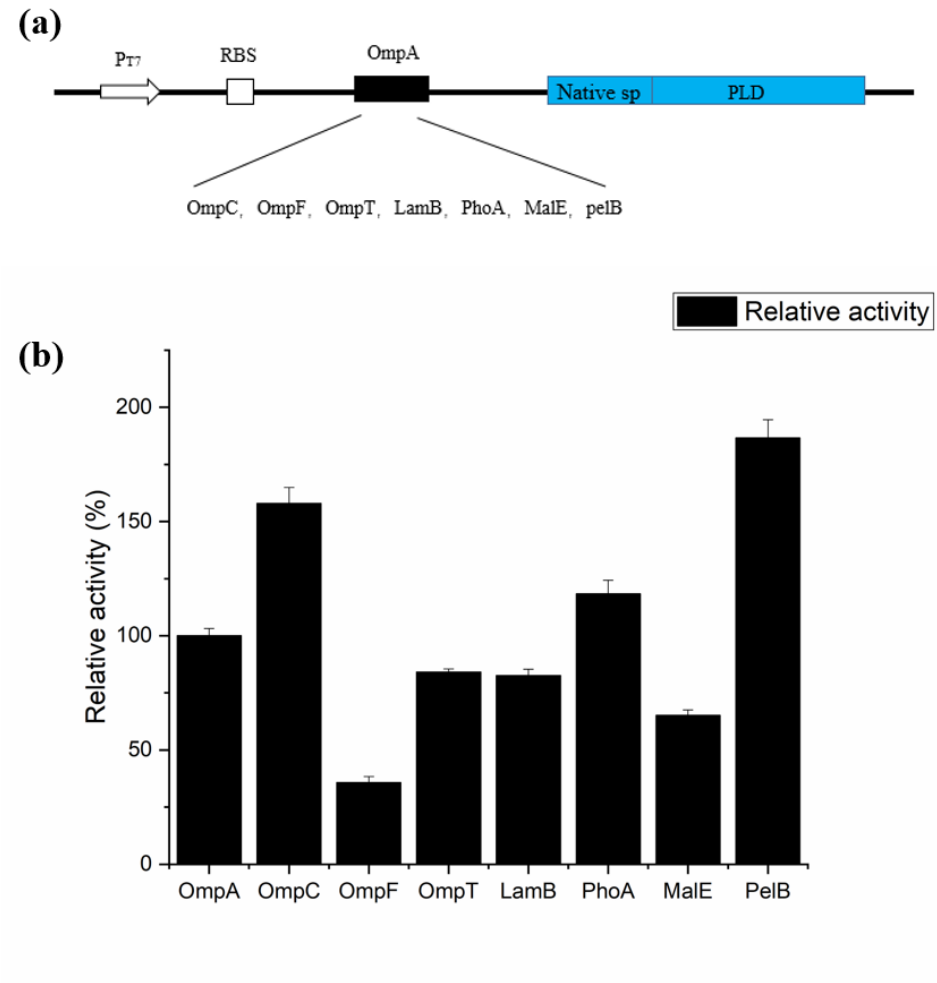

Figure 3

Optimization of the signal peptides for the secretory expression of PLDPMF in E. coli. (a) Using different signal peptides from E. coli to replace OmpA. (b) The relative transphosphatidylation activity of recombinant PLD when fused expressed in E. coli with different signal peptides.

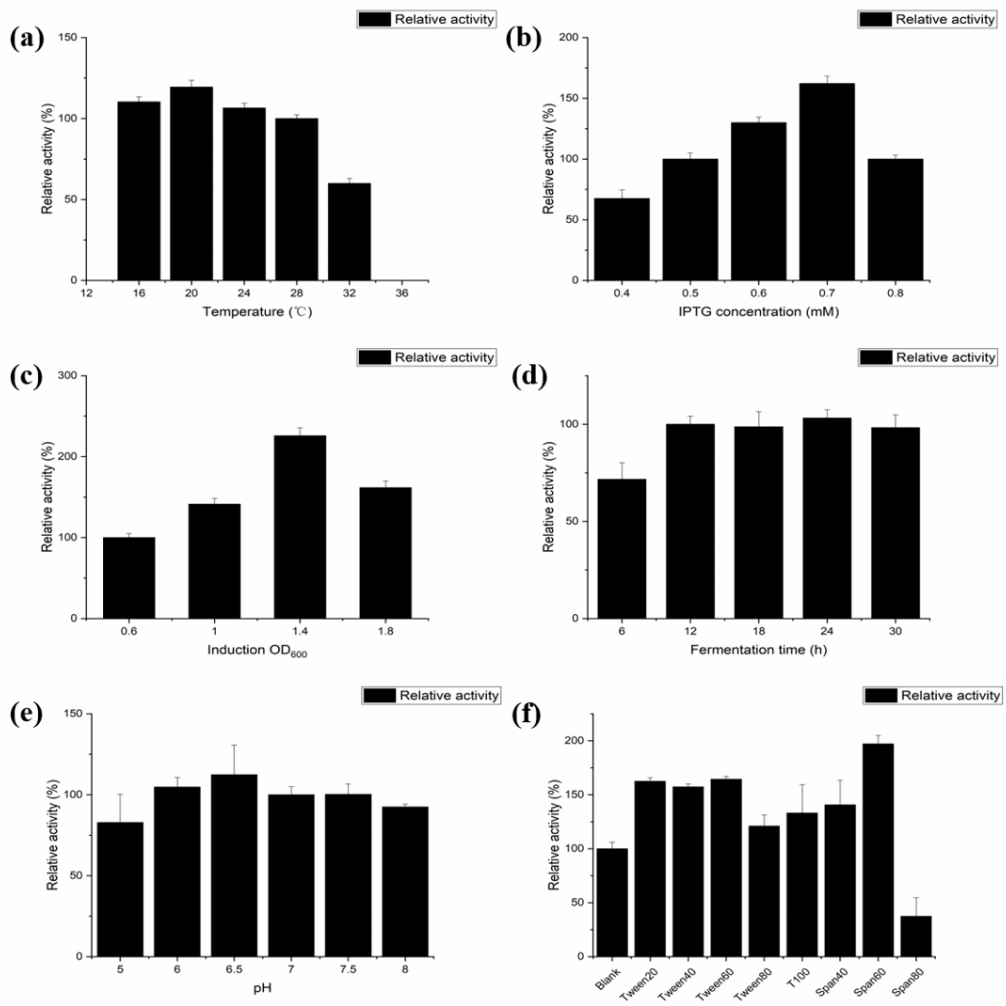

Figure 4

Optimization of the fermentation conditions for the secretory expression of PLDPMF. (a) The effect of induction temperature. (b) The effect of the concentration of IPTG. (c) The effect of the induction OD600. (d) The effect of the fermentation time. (e) The effect of the induction pH. (f) The effect of the addition of surfactants.

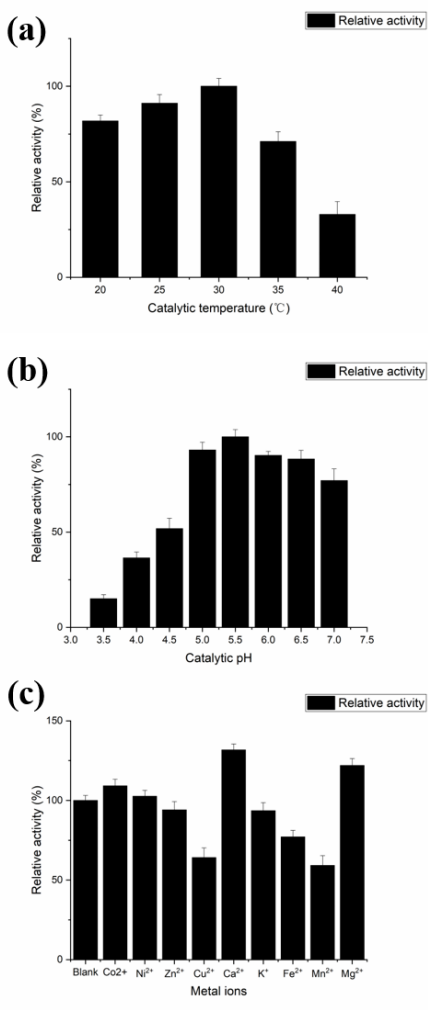

Figure 5

Characterization of the recombinant PLDPMF. (a) The effect of the catalytic temperature. (b) The effect of the catalytic pH. (c) The effect of the addition of metal ions.

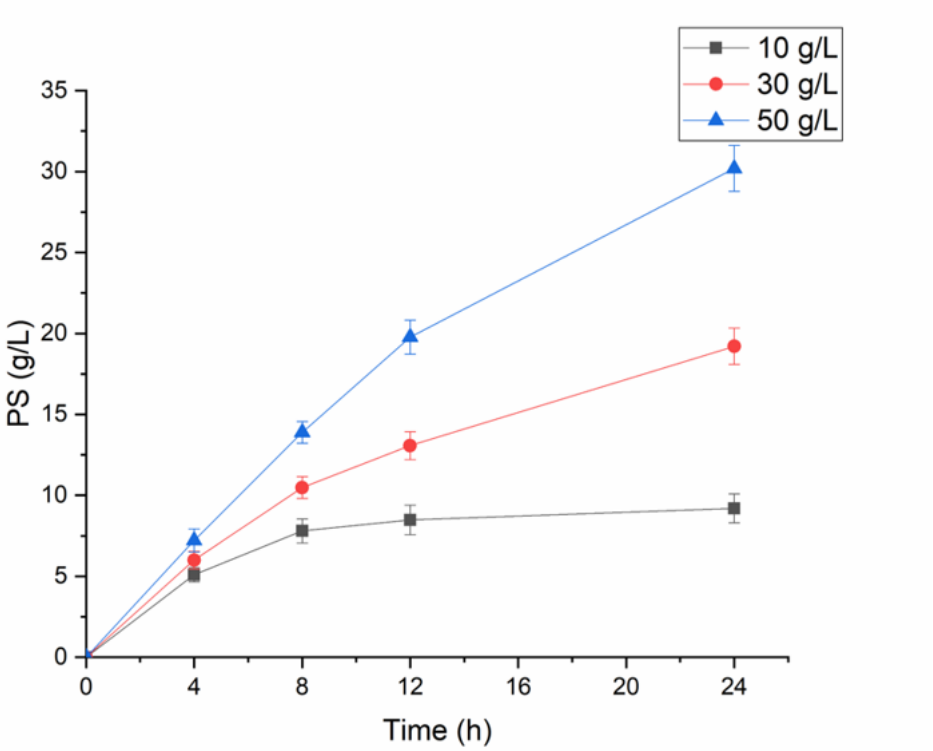

Figure 6

Using the recombinant PLDPMF for the bioconversion of PC to PS.

Supplementary Files

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