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Identification of a novel phospholipase D gene and effects of carbon sources on its expression in *Bacillus cereus* ZY12[§]

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In the present study, a new strain, *Bacillus cereus* ZY12, producing phospholipase D (PLD) was identified. The expression of *PLD* in this strain was found to be induced by its substrate, phosphatidylcholine (PC), and completely silenced by other carbon sources, such as glucose, fructose, and maltose, which are generally used in microbial growth cultures, thus presenting a unique expression pattern different from other PLD-producing microorganisms. This study is the first to report on the ability of *B. cereus* to produce PLD, and successfully clone its PLD-coding gene and identify its function, extending the knowledge on PLD distribution and evolution in microorganisms.

Keywords: *Bacillus cereus*, phosphatidylcholine, Phospholipase D, substrate-induced gene expression

Introduction

Phosphatidylserine (PS) is a phospholipid widely used in many pharmaceutical and functional food industries. Recent studies have shown that PS-supplemented diet helps in revitalizing brain cell membranes and improving memory performance in patients with age-associated memory impairment or Alzheimer's disease (Vakhapova *et al.*, 2014; Tarfarosh *et al.*, 2017). Besides, PS can also act as an effective athletic nutrient for relieving exercise-induced stress by regulating the cortisol levels that increase following exercise (Li *et al.*, 2016). To date, most of the PS is obtained from extracts of animal organs, soybeans, vegetable oils, and egg yolk. However, low accumulation of PS in these substances and the risk of transmission of infectious diseases from animals to humans limit the production of PS (Damnjanović *et al.*, 2016). Unlike the low accumulation of PS in most of the natural materials, the levels of other phospholipids, such as phosphatidylcholine (PC), have been noted to be relatively high. For example, in egg yolk, PC and PS account for 68–86% and only 2% of phospholipids, respectively, making it extremely inefficient to obtain PS by direct extraction from natural materials (Pokorný and Schmidt, 2011).

In living organisms, PS is synthesized by phosphatidylserine synthase (CDP-diacylglycerol: L-serine 3-sn-phosphatidyltransferase, EC 2.7.8.8) (PSS) by combining CDP-diacylglycerol and L-serine (Salzberg *et al.*, 2008; Tamura *et al.*, 2013; Tong *et al.*, 2016). However, limited availability of CDP-diacylglycerol makes it impossible to produce PS by using PSS for commercial purposes. When compared with PSS, phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) (PLD) is widely found in most of the microorganisms, plants, and animals, and exhibits both hydrolytic and transphosphatidylation activities to synthesize less abundant phospholipids such as PS from highly abundant phospholipids such as PC (Fig. 1). When PC is used as substrate in the presence of L-serine, the phospholipid head group of choline can be replaced with L-serine to form PS under the catalysis of PLD. Therefore, conversion of PC to PS by PLD is considered to be the most feasible way to produce PS (Choojit *et al.*, 2016; Zhou *et al.*, 2017).

Comfurius and Zwaal (1977) were the first to report on the conversion of PC to PS by PLD, and in the past few decades, many PLDs have been cloned from various microorganisms, plants, and animals. When compared with plant and animal PLDs, microbial PLDs have shown broader substrate specificity and relatively higher transphosphatidylation/hydrolytic activity, making them suitable for potential application in PS production. So far, many microorganisms have been identified for their PLD activity, such as *Streptomyces* sp. (Liu *et al.,* 2014), *Corynebacterium* sp. (Hodgson *et al.*, 1990), *Escherichia coli* (Cole *et al.,* 1974), *Ochrobactrum* sp. (Hu *et al.,* 2013), *Haemophilus parainfluenzae* (Ono and White, 1970), *Pseudomonas aeruginosa* (Wilderman *et al.*, 2001), *Salmonella typhimurium* (Procyk *et al.*, 1999), and *Acinetobacter radioresistens* (Mao *et al.*, 2017). Among these PLD-producing microorganisms, *Streptomyces* sp. has been reported to produce PLD with the best transphosphatidylation activity (Shimbo *et al.*, 2014).

Although microbial PLDs have potential application in PS production, their high toxicity causing severe cell lysis by degrading phospholipids in cell membrane limits PLD production and identification of new microorganisms with high PLD activity (Zambonelli *et al.*, 2003). In the present study, a microbial strain with PLD activity was isolated from the soil of a soybean oil factory, identified as *Bacillus cereus*, and

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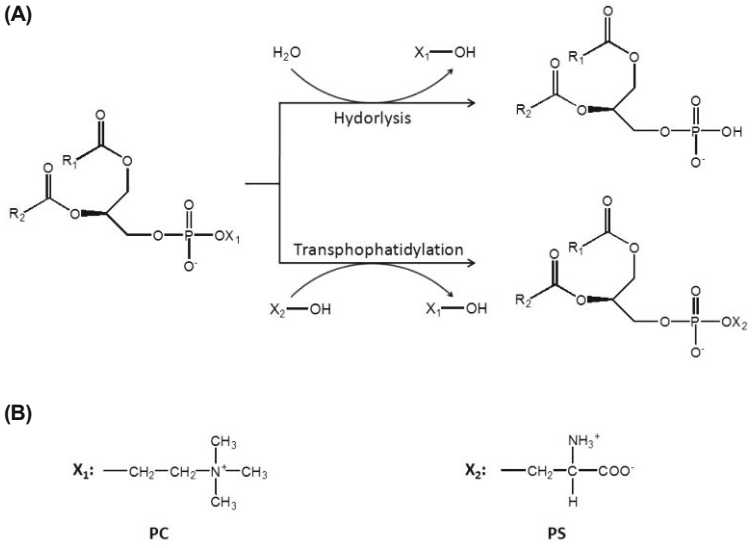

Fig. 1. Hydrolysis and transphosphatidylation activities of PLD (A) and phospholipid head groups for PC and PS (B).

designated as *Bacillus cereus* ZY12. Further investigation showed that the PLD activity in *B. cereus* ZY12 was regulated by carbon sources, and that its substrate PC could initiate the enzyme activity; however, no PLD activity could be detected in the presence of other common carbon sources, such as glucose, fructose, and maltose. To the best of our knowledge, the present study is the first to report on such severe impact of carbon sources on PLD activity in microorganisms. Furthermore, the *PLD* gene from *B. cereus* ZY12 was cloned and its function was examined through its heteroexpression in *E. coli*. The results obtained in the present study could deepen our understanding on the distribution of *PLD* gene family in microorganisms and provide a novel perspective on screening new PLD-producing microorganisms by using optimized medium, especially, appropriate carbon sources.

Materials and Methods

Isolation of bacterial strains and culture conditions

The bacterial strain was isolated from the soil samples collected from a soybean oil factory in Qiqihar city, China (latitude 48°15′N, longitude 123°56′E). In brief, the collected soil samples were cultured in enrichment (ER) medium at 30°C and 200 rpm. After 5–10 days of incubation, 1 ml of the culture suspension was transferred to isolation (IL) medium and incubated at 30°C and 200 rpm for 3–5 days. Then, the culture suspension was diluted 10 folds with sterile water and spread onto solid IL medium plate and incubated for 72 h at 30°C. To ensure purity of the isolated strain, individual colony was picked out and incubated on a separate IL medium plate. The final single colony obtained was preserved on IL medium plate for further study.

The composition of the ER medium was as follows: 20 g of yolk, 5 g of glucose, 5 g of yeast extract, 5 g of polypeptone, 2 g of K_2HPO_4, and 0.5 g of $MgSO_4{\cdot}7H_2O$ in 1 L of water (pH 7.0). The IL medium comprised 20 g of yolk, 2 g of KNO_3, 1 g of K_2HPO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, and 10 mg of $FeSO_4{\cdot}7H_2O$ in 1 L of water (pH 7.0). The Glc medium consisted of 5 g of glucose, 10 g of polypeptone, 2 g of KNO_3, 1 g of K_2HPO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, and 10 mg of $FeSO_4{\cdot}7H_2O$ in 1 L of water (pH 7.0). The Fru medium comprised 5 g of fructose, 10 g of polypeptone, 2 g of KNO_3, 1 g of K_2HPO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, and 10 mg of $FeSO_4{\cdot}7H_2O$ in 1 L of water (pH 7.0). The maltose medium comprised 5 g of maltose, 10 g of polypeptone, 2 g of KNO_3, 1 g of K_2HPO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, and 10 mg of $FeSO_4{\cdot}7H_2O$ in 1 L of water (pH 7.0). The PC medium contained 5 g of phosphatidylcholine, 10 g of polypeptone, 2 g of KNO_3, 1 g of K_2HPO_4, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, and 10 mg of $FeSO_4{\cdot}7H_2O$ in 1 L of water (pH 7.0).

Phylogenetic analysis based on *16S rRNA* gene sequence

Genomic DNA of the isolated bacterial strain was prepared according Jin and Komagata (1984). The *16S rRNA* gene was amplified with specific primer according to Lee and Whang (2015), and ligated to the pGEM-T vector (Promega) with T4 DNA ligase (TaKaRa) at 4°C overnight. The ligated plasmid DNA was transformed into competent cells (*E. coli* DH5α, Transgen Biotechnology) and positive colonies were identified by colony PCR and restriction enzyme digestion. The *16S rRNA* gene was then sequenced, and blast of the resulting sequence comparing with sequences from EzBioCloud (Kim *et al*., 2012) was performed. Subsequently, phylogenetic analysis was performed by using MEGA5.0 software (Tamura *et al*., 2011). A phylogenetic tree was constructed by neighbor-joining (Saitou and Nei, 1987) tree-making algorithm, and the topology of the resultant tree was evaluated using bootstrap resampling method of Felsenstein (1985) with 1,000 replicates.

Measurement of PLD activity

The isolated bacterial cells were cultured in a 500 ml flask containing 100 ml of IL medium at 30°C in a shaker at 200 rpm for 2 days. Then, both the cells and medium were col-

lected by centrifugation at 10,000 rpm and 4°C for 20 min for further PLD activity examination. For evaluating the PLD activity in a different growth medium, the microbial cells were also grown in Luria-Bertani (LB) medium at 30°C and 200 rpm for 2 days and the PLD activity was determined. To investigate whether IL medium could initiate PLD activity, the microbial cells pre-cultured in LB medium were inoculated into IL medium and grown at 30 C and 200 rpm for 2 days, and the PLD activity was examined.

For intracellular PLD activity measurement, 10 ml of the cultured cells (isolated bacterial strain or transformed *E. coli*) were collected by centrifugation at 10,000 rpm and 4°C for 20 min, then washed thrice with sterile water, and suspended in 10 ml of phosphate buffered saline. Cells were disrupted by ultrasonication treatment and the ultrasonication was carried out using 10 ml cell suspension in a 50 ml falcon tube for 15 min, which was kept in a salt-ice bath to prevent overheating. An Ultrasonic Homogenizer Scientz-II E (Ningbo Xinzhi Biotechnology Co., Ltd) was used at 20 kHz in the experiment and the acoustic power was 300 W. The duty cycle was 2 sec, with the generator on for 1 sec and off for 1 sec. After ultrasonication the cell fragments were separated by centrifugation at 10,000 rpm and 4°C for 20 min, and the supernatant was used for PLD activity examination. For extracellular PLD activity measurement, 10 ml of the cell culture were centrifuged at 10,000 rpm and the supernatant was directly used for PLD activity measurement. The PLD activity assay was performed according to the method of Uhm *et al.* (2005).

Cloning of *PLD* gene from the isolate

The prediction of putative *PLD* gene was performed based on the analysis of *B. cereus* genomic sequences. A gene from both *Bacillus cereus* ATCC 4342 and *Bacillus cereus* D17 was found to belong to *PLD* family according to the results of computational annotation of *B. cereus* genes from both the genome sequences. This putative *PLD* gene was PCR-amplified according to *B. cereus* ATCC 4342 genomic sequence by using the genomic DNA of the isolated bacterial strain as template. The primers used were as follows: 5′-TGGAA GAAATAATGATGCGA-3′ (Sense primer) and 5′-CTTTG ATGAGCCTGGAGTAAT-3′ (Antisense primer). PCR was conducted with *pfu* DNA polymerase (Fermentas) under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, and finally, 72°C for 5 min. After gel purification and A-tailing of the PCR product by using ExTaq DNA polymerase (TaKaRa), the purified DNA was cloned into pGEM-T easy vector (Promega) and transformed into *E. coli* DH5α (Transgen Biotechnology) competent cells. Subsequently, the PCR product was sequenced and full-length cDNA was cloned into pET30b vector by adding *Nde*I and *Hin*dIII restriction sites at the 5′- and 3′-end of the *PLD* gene by PCR amplification with specific primers (Sense: 5′-CGCATATGATTAAAAAAAT ATTGCG-3′; Antisense: 5′-CGAAGCTTTCACAAATAA AAATCAATCC-3′ with stop codon and 5′-CGAAGCTTA

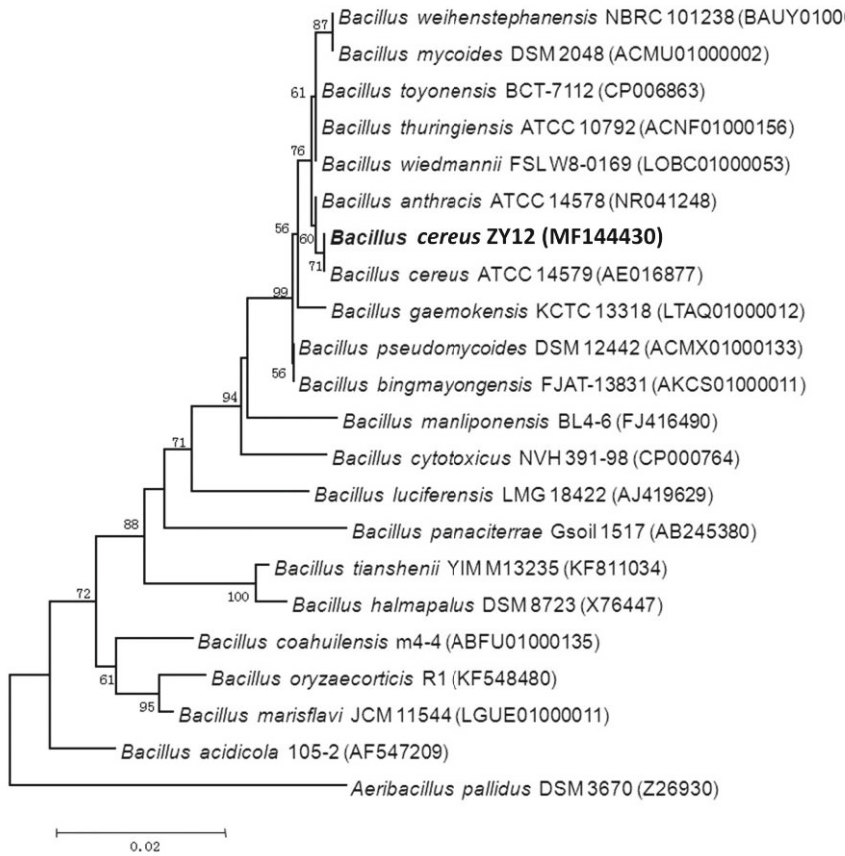

Fig. 2. Neighbor-joining phylogenetic tree showing the position of the bacterial isolate within *B. cereus* based on *16S rDNA* gene sequence data. GenBank accession numbers are shown in parentheses. Bootstrap values expressed as percentage of 1,000 replicates are given at the branching points. The scale bar represents 1% sequence dissimilarity.

AACAAATAAAAATCAATCC-3′ with mutated stop codon; TGA to TTT for adding His-tag at the 3′-end of *PLD*). Then, *PLD* and His-tagged *PLD* were expressed in *E. coli* BL21 DE3 cells for enzyme activity assay.

Analysis of *PLD* expression in the isolate

The bacterial cells were collected by centrifugation and total RNA was extracted with TRIzol reagent (Invitrogen). After DNaseI digestion of the RNA samples, reverse transcription was conducted with random primers by using AMV reverse transcriptase (Promega). Gene-specific primers (Sense: 5′-GAGATTGGTTATACGGGTGG-3′; Antisense: 5′-ATGTA TTCCCTTTGCTAGCC-3′) were designed according to the *PLD* sequence of the isolate for real-time PCR analysis. The primers of internal control gene (Sense: 5′-CAGCTCGTG TCGTGAGATGT-3′; Antisense: 5′-CGTGTGTAGCCCA GGTCATA-3′) were designed according to the *16S rRNA* gene sequence of the isolate. Real-time PCR was performed by using SYBR® Fast qPCR Mix (TaKaRa) in accordance with the manufacturer's instruction. The PCR conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 20 sec, 50°C for 20 sec, and 72°C for 15 sec, and finally, 72°C for 5 min. The expression levels of *PLD* were normalized to *16S rRNA* gene and calculated by using $2^{-\Delta\Delta Ct}$ method.

Results

Bacteria isolation and phylogenetic analysis

The PLD-producing strains were screened on IL medium plate with yolk as the sole carbon and nitrogen source. After 3–5 days of incubation, several colonies were observed on the plates with distinct transparent zones. Among them, 10 colonies were randomly selected and their *16S rRNA* genes were amplified by PCR for sequencing. The sequencing results showed that all the sequences of the *16S rRNA* genes were identical, suggesting that they belonged to the same bacterial strain. Subsequently, the nucleotide sequencing data were analyzed by using BLASTn online analysis tool to identify the closest phylogenetic relatives in GenBank database. The isolated bacterial strain showed 100% *16S rRNA* gene sequence similarity to *Bacillus cereus* FRI-35 and 99.97–99.99% similarity to other known *B. cereus 16S rRNA* gene sequences. A neighbor-joining dendrogram was generated as shown in Fig. 2, in which the isolated strain formed a phylogenetic cluster with *B. cereus*. The strain was subsequently designated as *B. cereus* ZY12 for further investigation.

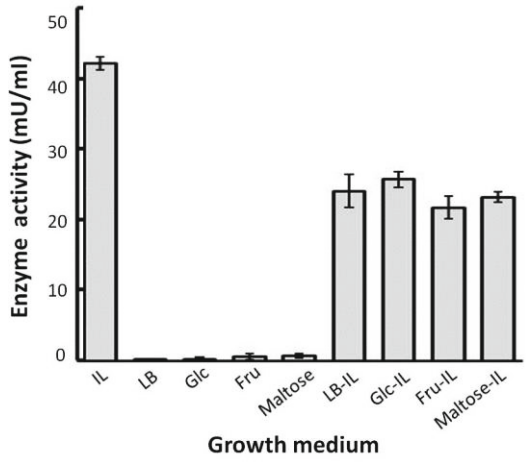

Fig. 3. Extracellular PLD activities of strain *B. cereus* ZY12 grown in different media. LB-IL, bacteria pre-cultured in LB medium and sub-cultured in IL medium; Glc-IL, bacteria pre-cultured in Glc medium and sub-cultured in IL medium; Fru-IL, bacteria pre-cultured in Fru medium and sub-cultured in IL medium; Maltose-IL, bacteria pre-cultured in maltose medium and sub-cultured in IL medium. Error bars represent standard deviations of three biological replicates.

Examination of the activity of PLD produced by *B. cereus* ZY12

To examine the effect of carbon sources on PLD production, the *B. cereus* ZY12 cells were grown in IL, LB, Glc, Fru, and maltose media, respectively. After 2 days of cultivation, both the cells and medium were collected by centrifugation for examining the intracellular and extracellular PLD activities (Fig. 3). Similar to many bacterial PLDs, intracellular PLD activity was not detected in cells grown in the above-mentioned media, whereas extracellular PLD activity was only observed in IL medium. To determine whether IL medium was critical for initiating PLD activity, the *B. cereus* ZY12 cells were pre-cultured in LB, Glc, Fru, and maltose media, respectively, and inoculated into IL medium, and the extracellular PLD activity was determined after 2 days of cultivation. The results showed obvious extracellular PLD activity after subculturing *B. cereus* ZY12 cells from LB, Glc, Fru, and maltose media to IL medium.

As shown in Fig. 4A and B, maximum biomass was obtained at about 36 h after inoculation in LB and IL media, respectively. However, the cell growth rate and maximum biomass were much lower in IL medium than those in LB medium, which might probably be owing to the initial toxicity of PLD to *B. cereus* ZY12 cells in the IL medium. Furthermore, PLD activity was not detected during 52 h of cultivation of *B. cereus* ZY12 cells in LB medium, similar to the previously obtained results. In contrast, the IL medium could successfully initiate PLD production and the highest enzyme activity was observed at 36 h after inoculation, when the cell growth was at the beginning of stationary phase. Besides, investigation of the effects of pH and temperature on PLD activity revealed that the highest PLD activity was obtained at pH 8 and 30°C, respectively (Fig. 4C).

Identification of *PLD* gene in *B. cereus* ZY12

The full-length *PLD* gene was PCR-amplified, and the sequenced PCR product has been submitted to NCBI (GenBank No. KY852313). Sequence similarity analysis revealed that the obtained *PLD* gene from *B. cereus* ZY12 shared 91–98% amino acid sequences similarity to its homologs in other available *B. cereus* genomes.

To verify the activity of PLD produced by *B. cereus* ZY12, full-length *PLD* and His-tagged *PLD* genes were mobilized into pET30b vector for their expression in *E. coli* (Fig. 5A). After 12 h of induction by 0.2 mM IPTG at 16°C, both PLD production and activity were examined. No extracellular PLD

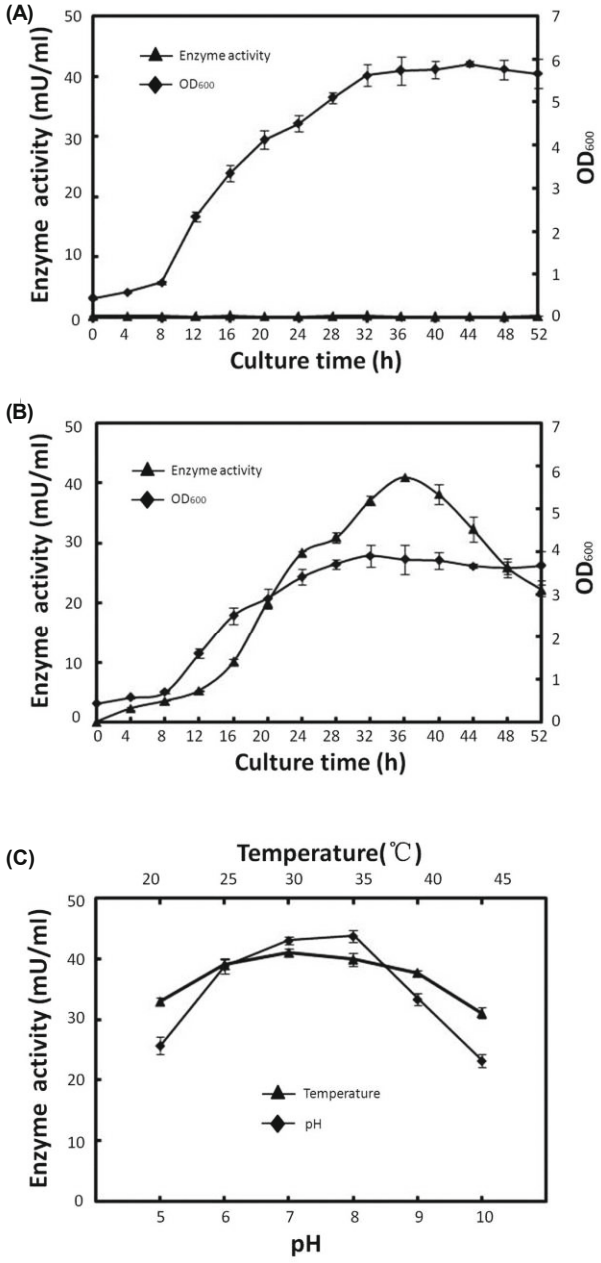

Fig. 4. Cell growth (◆) and extracellular PLD activities (▲) of *B. cereus* ZY12 in LB (A) and IL (B) medium. Effects of temperature (▲) and pH (◆) on extracellular PLD activities of *B. cereus* ZY12 cultured in IL medium at 30°C and 200 rpm for 2 days (C). Error bars represent standard deviations of three biological replicates.

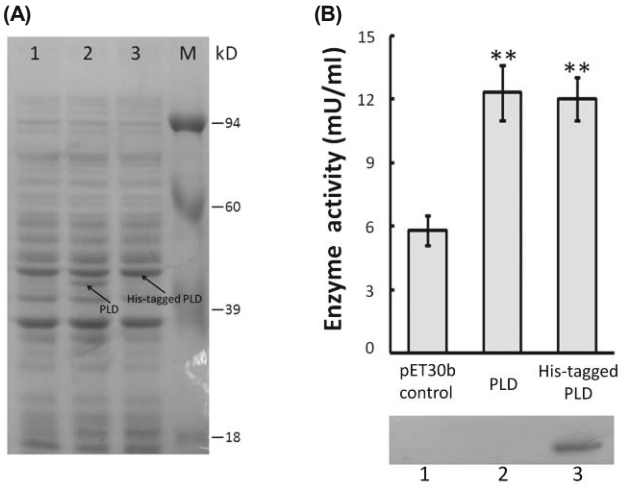

Fig. 5. Heterogenous expression of *B. cereus* ZY12 *PLD* in *E. coli*. (A) SDS-PAGE of PLD or His-tagged PLD expressed in *E. coli*; (B) intracellular PLD activity in transformed *E. coli*; (C) western blot analysis of His-tagged PLD expression in *E. coli* by using His-Tag antibody. Lanes: 1, protein extracted from *E. coli* carrying empty pET30b vector; 2, protein extracted from *E. coli* overexpressing recombinant ZY12 PLD; 3, protein extracted from *E. coli* overexpressing recombinant His-tagged ZY12 PLD; M, protein molecular weight markers. Error bars represent standard deviations of three biological replicates, and asterisks indicate statistically significant differences, when compared with empty vector (control). ** $P < 0.01$.

activity was detected in all the three transformed cell lines, whereas intracellular PLD activity was observed in all the cell samples because *E. coli* could produce its own PLD with transphosphatidylation/hydrolytic activity (Matsumoto, 1997; Zhang *et al*., 2008). However, cells expressing *PLD* or His-tagged *PLD* genes showed about two fold higher PLD activity, when compared with those expressing empty vector (control), confirming the function of *PLD* gene cloned from *B. cereus* ZY12 (Fig. 5B). Western blot analysis showed that the level of recombinant PLD produced by *E. coli* was not high, which could be an explanation for the relatively low PLD activity in the transformed *E. coli* cells, when compared with that in *B. cereus* ZY12 (Fig. 5C). In addition, the growth rate of *E. coli* cells expressing *PLD* was low, when compared with that of the control, indicating the toxicity of recombinant PLD, even at low concentration, to the cells.

Expression and activity of PLD in *B. cereus* ZY12

Based on the obtained *PLD* sequence, real-time PCR was performed to verify *PLD* expression in *B. cereus* ZY12. Figure 6A shows that the *PLD* transcript level was abundant in cells grown in IL medium, whereas no *PLD* transcript was detected in cells grown in LB medium. The IL medium could successfully induce the expression of *PLD* in cells pre-cultured in LB medium, revealing the importance of IL medium for inducing *PLD* expression in *B. cereus* ZY12. For determining whether PC is the major component to induce the expression of *PLD*, the *B. cereus* ZY12 cells pre-cultured in LB medium were inoculated into PC medium, with PC as the sole carbon source. As illustrated in Fig. 6B, PC could obviously induce the expression of *PLD*; in contrast, no changes in *PLD* expression were noted in cells continually sub-cultured in LB medium. Furthermore, analysis of PLD activity after transfer of *B. cereus* ZY12 cells pre-cultured in LB medium to LB or PC medium showed obvious enzyme activity after transfer of the cells to the PC medium, consistent with the real-time PCR results.

Discussion

PLD has significant potential for application in PS produc-

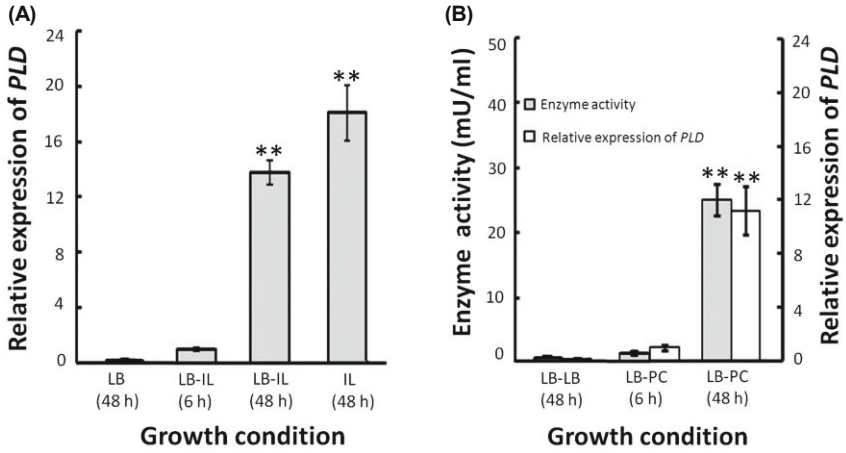

Fig. 6. Relative expression of *PLD* in *B. cereus* ZY12 cultured in LB and IL media, respectively, or pre-cultured in LB medium and sub-cultured in IL medium (LB-IL) for 2 days (A). Relative expression of *PLD* and extracellular PLD activities of *B. cereus* ZY12 pre-cultured in LB medium and sub-cultured in LB and PC media, respectively, for 2 days (B). LB-LB, bacteria pre-cultured in LB medium and sub-cultured in LB medium; LB-PC, bacteria pre-cultured in LB medium and sub-cultured in PC medium. Real-time PCR results are normalized to *16S RNA* gene and shown relative to the level in 6 h of cultivation in LB-IL or LB-PC. Error bars represent standard deviations of three biological replicates, and asterisks indicate statistically significant differences, when compared with LB or LB-LB. ** $P < 0.01$.

tion, and microbial PLDs are considered to be the best option owing to their broader substrate specificity and relatively high transphosphatidylation/hydrolytic activity. However, microbial PLDs are extremely toxic and their continuous accumulation is harmful to the cells, which is generally recognized as detrimental to enzyme production. Moreover, although many microbial PLDs have been identified, their activities are still not adequately high for their application in economical large-scale PS production. Therefore, it is necessary to find microorganisms producing PLD with higher activity that could be utilized for PS biosynthesis with higher production efficiency. However, for cell survival, these toxic genes (especially high-activity PLD-coding genes) are usually expressed at very low level or silenced by removing appropriate inducer (such as carbon sources), resulting in overlooking of many positive colonies during isolation based on PLD activity.

The present study is the first to confirm PLD activity in a *B. cereus* strain, *B. cereus* ZY12. Comparison of the *B. cereus* ZY12 PLD sequence with those of other widely investigated *Streptomyces* PLDs showed that the *B. cereus* ZY12 PLD contains two conserved HKD domains and several important amino acids related to PLD activity (shown in light gray regions in Supplementary data Fig. S1). Based on previous studies, three key amino acids responsible for PLD activity in *B. cereus* ZY12 PLD were identified and labeled as His155, Asp189, and His326. Furthermore, phylogenetic analysis of *B. cereus* ZY12 PLD with other reported bacterial PLDs revealed that this novel PLD is close to *A. radioresistens* PLD (Supplementary data Fig. S2), which has been reported to exhibit very good transphosphatidylation conversion rate and selectivity (Mao *et al.,* 2017).

Further investigation showed that yolk is necessary for inducing *PLD* expression and PC is the major component responsible for this induction in *B. cereus* ZY12. Previous studies have demonstrated that carbon source is very important for regulating *PLD* expression in microorganisms (McLain and Dolan, 1997). Similarly, the results of the present study showed that except PC as the sole carbon source, enriched carbon sources such as glucose, fructose, and maltose, which are generally used for microbial growth, could completely silence the expression and activity of PLD in *B. cereus* ZY12 cells (Fig. 7). In general, phospholipases are secreted by microorganisms for phosphate and carbon source acquisition, and, in some cases, as virulence factors. Considering its cell toxicity, it can be presumed that bacterial PLDs must be produced under certain extreme conditions, such as nutritional deficiency. When yolk or PC is used as the sole carbon source for cell growth, the bacterial cells may produce necessary enzymes, including PLD, and secrete them into the medium to convert unavailable carbon sources to available carbon sources for growth. However, in the presence of glucose, fructose, and/or maltose in the growth medium, the bacterial cells use these carbon sources rather than PC, thus resulting in no toxic *PLD* production. This hypothesis was confirmed in the present study by analyzing the expression of *PLD* homologs in other *B. cereus* strains based on the available microarray data from NCBI. The microarray results showed

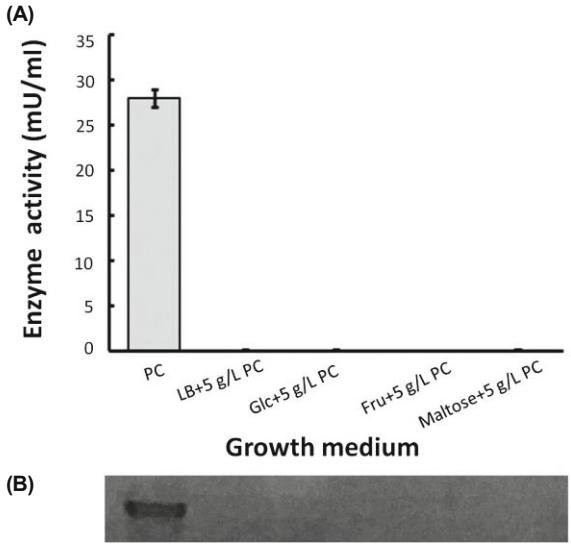

Fig. 7. PLD activities (A) and RT-PCR for the identification of *PLD* expression (B) in *B. cereus* ZY12 grown in different media for 2 days. PC, PC medium; LB + 5 g/L PC, LB medium supplemented with 5 g/L PC; Glc + 5 g/L PC, Glc medium supplemented with 5 g/L PC; Fru + 5 g/L PC, Fru medium supplemented with 5 g/L PC; Maltose + 5 g/L PC, Maltose medium supplemented with 5 g/L PC. Error bars represent standard deviations of three biological replicates.

that *PLD* homologs in other *B. cereus* strains were also completely silenced when the cells were grown in medium containing enriched carbon sources, indicating the general silenced expression pattern of *PLD* in *B. cereus* under normal growth conditions.

Heterogenous expression of *PLD* for recombinant enzyme production has been considered as a feasible method for large-scale conversion of PC to PS. However, cell toxicity of PLD limits the efficiency of enzyme production, suggesting that simple heterogenous expression of *PLD* with general approach might not be appropriate and more strategies should be considered for optimized PLD production, such as inclusion body expression for decreasing cell toxicity of the recombinant enzyme (Ramón *et al.,* 2014). Owing to the severe cell toxicity of PLDs, microorganisms must have a precise system for controlling the expression or activity of these enzymes for survival. In the present study, although *B. cereus* ZY12 *PLD* gene was not efficiently expressed in *E. coli*, trace of active PLD still caused obvious toxicity to cells, resulting in decreased growth rate.

Previous reports have shown that the activities of PLDs secreted by *Streptomyces* sp. reached 400–3,400 mU/ml medium (Hagishita *et al.,* 2000; Nakazawa *et al.,* 2010; Zhao *et al.,* 2010). However, in the present study, the highest activity of PLD produced by *B. cereus* ZY12 was 43.8 mU/ml medium, which is much lower than those of PLDs from *Streptomyces* sp. As the expression of *PLD* in *B. cereus* ZY12 cells is regulated by certain carbon sources, PLD could not be effectively produced and only a small amount of the enzyme was synthesized and secreted into the medium, where low PLD activity was observed.

By using yolk and PC as the sole carbon source in the growth medium for examining *B. cereus* ZY12 PLD activity, the present study is the first to identify a PLD-producing *B. cereus* strain, and this finding could extend our understanding of PLD distribution among microorganisms. Moreover, the results obtained confirmed that the expression of *PLD* gene could be completely silenced in some microorganisms in the absence of certain carbon sources. As most of the lipases and phospholipases are inducible enzymes, the strategy for functional identification of PLD from isolated PLD-producing strains should be reviewed to select appropriate carbon source during the isolation process to avoid possible overlooking of positive colonies possessing high-activity PLD-coding gene(s), which could be silenced under inappropriate growth conditions.

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