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Efficient Extracellular Expression of Phospholipase D in Escherichia Coli with an Optimized Signal Peptide

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Abstract. New secretion vectors containing the synthetic signal sequence (OmpA') was constructed for the secretory production of recombinant proteins in *Escherichia coli*. The *E. coli* Phospholipase D structural gene (Accession number:NC_018658) fused to various signal sequence were expressed from the Lac promoter in *E. coli* Rosetta strains by induction with 0.4mM IPTG at 28 ℃ for 48h. SDS-PAGE analysis of expression and subcellular fractions of recombinant constructs revealed the translocation of Phospholipase D (PLD) not only to the medium but also remained in periplasm of *E. coli* with OmpA' signal sequence at the N-terminus of PLD. Thus the study on the effects of various surfactants on PLD extracellular production in Escherichia coli in shake flasks revealed that optimal PLD extracellular production could be achieved by adding 0.4% Triton X-100 into the medium. The maximal extracellular PLD production and extracellular enzyme activity were 0.23mg ml^{-1} and 16U ml^{-1}, respectively. These results demonstrate the possibility of efficient secretory production of recombinant PLD in *E. coli* should be a potential industrial applications.

1. Introduction

Phospholipase D (EC 3.1.4.4) is one of the functional enzymes belong to phospholipase cluster which could hydrolysis a variety of substrates, including phosphatidylcholine, phosphatidylethanolamines and soluble phospholipids (Selvy *et al*. 2011). It is also capable of catalyzing transesterification of the polar head groups of phospholipids (Iwasaki *et al.*1994; Yang *et al.*1967; Eibl and Kovatchev 1981). Especially utilized lecithin and serine as ubstrates, phosphatidylserine which an important health care product prevent senile dementia could be efficient synthetized (Nakazawa *et al*. 2009). Therefore, Phospholipase D is of great potential for uses in the bio-pharmaceutical production of phosphatidylserine, which has the advantage of decreasing environmental pollution and energy consumption, when compared with the traditional chemical extraction methods carried out under high pH and organic reagent conditions (Abidi 1998).

Phosphatidylserine is one of the major constituents of the cell inner membrane of various species (including animals, plants and microbes) and is one of the most widely available phospholipid in nature(Matsumoto 1997). Therefore, Phospholipase D is commonly distributed in a variety of associated microbes too, but the content is extremely low in wild strains and only distributes in intracellular cytoplasm which is difficult to purification(Vance and Steenbergen 2005). So in order to obtain adequate PLD using in the industrial production of phosphatidylserine and simplified

downstream purification, we constructed an extracellular secretory overexpression system utilized *E.coli* as host strain which is exemplified by several exciting findings that show high levels for the production of recombinant proteins(Takemori *et al*. 2012)(Matsumoto *et al*. 2000; Su *et al*. 2012).

However, *E. coli* naturally does not secrete proteins into the extracellular medium under standard laboratory conditions(Jonet *et al*. 2012), so extracellular expression of secretory proteins in *E.coli* is indispensable mediated by a special leader peptide, also known as a signal peptide (Choi *et al*. 2000; Su *et al*. 2015). As we all know, different signal peptides have different secretion efficiency and they are generally not conserved among various species(Pournejati *et al*. 2014), but they share some common characteristic features which are conserved among different organisms(Velaithan *et al*. 2014). Most signal peptides are composed of three regions (Massahi and Çalık 2015), the positively charged N-domain with 2–10 amino acids which play a critical role in responding signal recognition particle on the membranes, the hydrophobic H-domain with 10 to 20 amino acids which direct the membrane translocation, and the C-domain that contains a cleavage site, which is most often an Ala-X-Ala sequence (Nakai 2000; Emanuelsson *et al*. 2000). In this paper, the secretion efficiency of signal peptides including OmpA, PelB, DsbA, MalE, TorA, OsmY (Qian *et al*. 2008) was researched and meanwhile a new synthetic signal peptide using N-domain of OmpA and H-domain of PelB named OmpA' was created and studied.

As research continued, we discovered that although the signal peptide could mediate extracellular production of PLD, but the expression yield was still low and plenty of recombinant PLD remained in periplasm. Hence, to improve the secretory ratio, the medium was supplemented with surfactants, which enabled the highly efficient extracellular production of target protein in *E. coli* (Duan *et al*. 2015) and the related expression condition process were also optimized (Wang *et al*. 2015; Liu *et al*. 2014). Finally, recombinant PLD production by this strategy was enhanced by optimizing of process conditions and the extracellular PLD enzyme activity was measured and reached 16U/mL suggesting the promising potential in industrial application.

2. Results and Discussion

2.1. Construction and Expression of Recombinant PLD Plasmids in E.coli.

To clone the PLD gene, the genomic DNA of *E.coli O104:H4* was used as a template. The forward primer 5'-GTGCCGCGCGGCAGCCATATGATGTTGTCAAAATTTAAG was designed to contain a *NdeI* site (underlined). The reverse primer 5'-TTGTCGACGGAGCTCGAATTCATACAGGATTCGGCTAAT was designed to contain a *EcoRI* site (underlined). The PCR product was purified by agarose gel DNA purification kit and ligated into *NdeI* and *EcoRI* site of pET-28a (+) which was digested with *NdeI* and *EcoRI* utilized one step cloning Kit. This plasmid was named as pETPLD. Seven different signal sequences OmpA, OmpA', pelB, OsmY, DsbA, MalE, TorA were incorporated at NcoI site of pET-PLD and several constructs were made, i.e., pET-PLD (1-7), respectively. Integration of all these recombinant plasmids were confirmed by evaluated DNA_sequences and then transformed into *E.coli* Rosetta (DE3) cells for expression studies grown in TB medium induced by 0.4 mM IPTG concentration at 28 ℃ for 48h. All of these expression conditions had been investigated previously, data not given here. To examine whether all of these signal sequences could be used for the secretory protein production in *E. coli*. The total extracellular protein of each construct was analyzed on 12 % SDS-PAGE. It was observed that PLD was successfully extracellular expressed with an accurate size of 53 kDa when it was linked with OmpA' signal sequence as shown in figure 1. SDS-PAGE analysis indicated that not all signal sequences had obvious effect on PLD secretion, only OmpA' could achieve tiny extracellular production of recombinant PLD. Result was consistent with our speculation, as OmpA' processed the N-domain of OmpA which could exactly identify the binding site on cytomembrane and meanwhile it also processed the H-domain of PelB which could efficient assist the transmembrane transport of recombinant protein. Therefore, OmpA' was an efficient signal peptide sequence that can secrete the recombinant enzyme out of the cell. Remarkablely, the control with no signal peptide obtained extracellular PLD which was attributed to cell autolysis in cultivation. Although signal peptide OmpA' could efficient guide PLD secreted into the medium, but the amount of extracellular PLD was

fractionary which was only 50mg/L. So we speculated that plenty of PLD remained in cytoplasm and periplasm. In an attempt to research this phenomenon, intracellular fraction was then obtained by sonication and the periplasmic proteins were purified by the modified osmotic shock method mentioned above. All of the fractions were purified by Ni-NTA agarose resin column affinity adsorption. The purified protein samples concentration measured by coomassie brilliant blue was 50mg/ml, 110mg/ml and 700mg/ml respectively. More than 90% of recombinant PLD remained in cytoplasm and periplasm, the amount of extracellular PLD was only about 5.8% of total PLD protein. It has been widely reported that extracellular protein expression in *E.coli* always distribute in periplasm (Choi *et al*. 2000). Although the reason for this is not clear, but there are lots of methods to solve this problem.

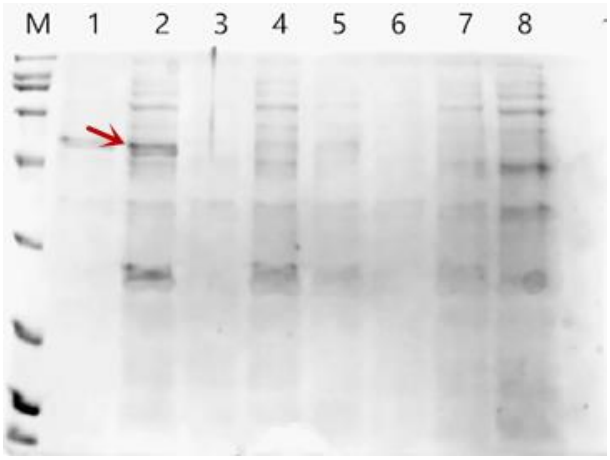

Figure 1. SDS-PAGE analysis of extracellular expression of recombinant PLD mediated by different signal peptide. Lane: M, molecular mass standard (From the top down: 200KD; 116KD;97KD; 66KD; 45KD; 29KD;20KD; 14KD; 6KD); 1, control (no signal peptide); 2, OmpA'-PLD; 3, OmpA-PLD; 4, DsbA-PLD; 5, TorA-PLD; 6, OsmY-PLD; 7, MalE-PLD; 8, PelB-PLD. Arrows indicate the recombinant PLD.

2.2. Effects of Surfactants on the Extracellular Production of PLD

Recombinant PLD could be successfully secreted expression in *E.coli*, but there was still amounts of PLD remained in the periplasm. However, to pursue this method of PLD production, the cells need to be disrupted through ultrasonication or homogenization prior to the surfactant treatment. Since it is well known that the cost of cell disruption is high, this process is not suitable for the production of industrial enzymes. However, it has been reported that surfactants can increase the permeability of cellular membranes and that they can be used to enhance the secretion of recombinant protein. Therefore, we considered that the surfactants could penetrate the outer membrane of *E. coli* and release the recombinant PLD in periplasmic space. In order to verify this hypothesis, effects of different surfactants including CaCI2, Glycine, SDS, Tween 80, and Triton X-100 on the secretion efficiency were researched respectively.

As shown in fig. 2, 0.2% of different surfactants were added into the culture medium, remarkably, not all of the five additives examined in this study promoted the secretion of recombinant PLD compared with the non-addition control. Notably, addition of SDS or Glycine into the medium resulted in a 1.88- or 1.74-fold increase in the extracellular production of PLD respectively compared to the control. Interestingly, the addition of Triton X-100, Tween 80 to the medium obviously enhanced the total extracellular PLD production which was 3.8- or 2.64-fold compared to the control. Noticeable, two nonionic surfactants TritonX-100 and Tween 80 boosted the extracellular release of recombinant PLD mainly because of the efficient impairment on outermembrane and enhanced the cell permeability. Then the recombinant PLD remained in periplasm could be released. Extracellular PLD concentration with different surfactants was shown in table 1. Triton X-100 facilitated about 21% of the total recombinant PLD secreted into medium which was much higher than Tween 80 (15%),

and satisfactorily, under the premise of total protein concentration was stable, Triton X-100 promoted the secretion efficiency much better than others. This result indicated that Triton X-100 worthy of further research.

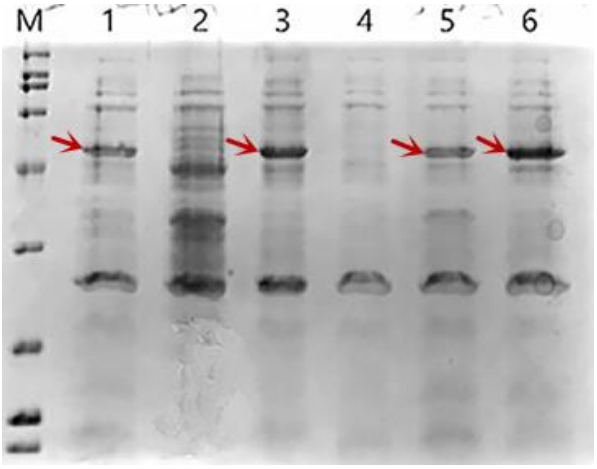

Figure 2. SDS-PAGE analysis of recombinant PLD extracellular production influenced by different surfactant. Lane: M, molecular mass standard (mentioned above); 1, control (no surfactant); 2, SDS addition; 3, Tween 80 addition; 4, CaCl2 addition; 5, Glycine addition; 6, Triton X-100 addition. Arrows indicate the recombinant PLD.

Table1. Effects of different surfactants on extracellular PLD production.

surfactants	PLD concentration(mg/L)		Extracellular PLD activity (U ml^{-1})
	Extracellular	intracellular	
Control	50	810	3.2
$CaCI_2$	57	800	2.8
Glycine	53	780	1.2
SDS	94	775	4.5
Tween 80	132	700	10.0
Triton X-100	190	720	13.5

2.3. The effect of Triton X-100 concentration on cell growth and PLD production in shake flasks

It was supposed that the surfactant Triton X-100 may possess dual functions when added to the medium. To balance the enhancement of PLD production and the threat to cell growth, it was necessary to investigate the optimal addition of Triton X-100 concentration on cell growth, protein production and enzyme activity.

The results showed that Triton X-100 not always inhibited the growth of *E.coli* in a dose dependent manner (Fig. 3A). The ODs of *E.coli* cultures supplemented increased from 0 to 0.4% of Triton X-100 addition, in contrast the ODs reduced from 0.4% to 0.8% which was not coincident to our expectation. Although Triton X-100 inhibited cell growth but PLD was a natural toxic protein for recombinant cell too. In summary, overexpress of PLD would seriously affect the growth of recombinant *E.coli*, however moderate addition of Triton X-100 enhanced extracellular expression of PLD to achieve a balance between the two element. Once Triton X-100 added in the medium the growth condition (ODs) were slightly better than the control. This result proved that Triton X-100 could restrain the cytolysis along with protein secretion. Effects of different addition of Triton X-100 on PLD extracellular expression and PLD enzyme activity were shown in fig. 3B and fig. 3C. According to the SDS-PAGE analysis in fig. 3B, 0.4% triton X-100 could achieve the most efficient extracellular expression of PLD, the extracellular protein concentration reached up to 230mg/L. In addition, with the adding amount increasing, PLD extracellular production was slight decrease due to cell growth inhibition.

Corresponding to this result, as shown in fig. 3C, the recombinant PLD exhibited the highest extracellular catalytic activity about 16U/ml after addition 0.4% Triton X-100 into the TB medium. The specific activity of recombinant PLD was about 70U/mg. Results indicated Triton X-100 actually increased PLD extracellular catalytic activity. Interestingly, 0.6% and 0.8% Triton X-100 addition could not enhanced PLD extracellular catalytic activity compared to 0.4% addition and 0.2% addition shown up lower enzyme activity compared to 0.4%. These results demonstrate that cell growth and extracellular production of recombinant PLD are affected by Triton X-100 addition. In designing further studies and consideration of cell growth condition, 0.4% Triton X-100 was chosen for further studies to improve the secretion of recombinant PLD.

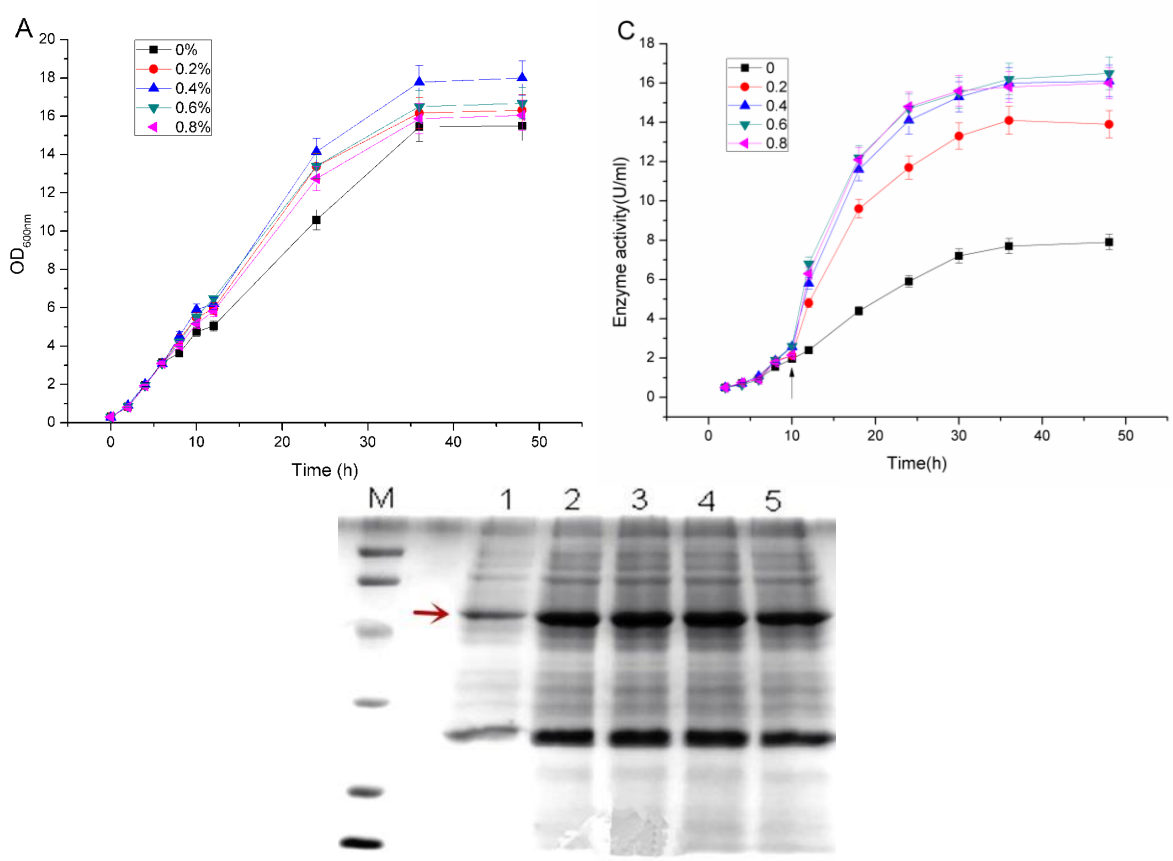

Figure 3. A Cell growth as a function of the time when Triton X-100 was added to the culture medium. Symbols: control with no Triton X-100 addition(■); 0.2% Triton X-100 addition(●); 0.4% Triton X-100 addition(▲); 0.6% Triton X-100 addition(▼); 0.8% Triton X-100 addition (◀). B SDS-PAGE analysis of PLD extracellular expression with different additive amount of Triton X-100. Lane: M, molecular mass standard (mentioned above); 1, control (no surfactant); 2, 0.2% Triton X-100 addition; 3, 0.4% Triton X-100 addition; 4, 0.6% Triton X-100 addition; 5, 0.8% Triton X-100 addition. Arrows indicate the recombinant PLD. C Time courses of extracellular PLD enzyme activity in the presence of different amount Triton X-100. Symbols describe as above 3A and arrow indicate the addition of Triton X-100.

2.4. Purification and Properties of the Recombinant PLD

The effects of pH and temperature on PLD activity were examined. As shown in fig.4A, the activity of recombinant PLD increased from 20℃to 35℃ and exhibited its maximum at 35 ℃, whereas it decreased sharply with the increase in temperature above 35 ℃. The recombinant PLD showed maximum activity at pH 8.0(fig.4B), the pH curve displayed a distribution of PLD activity at the pH range of 6.0 to 10.0 and peak values at pH 8.0. The PLD from the recombinant E. coli Rosetta cells

secreted in a functional form in cell-free medium. And the basic enzymic property had been studied and proved functional. Therefore, even the production of recombinant PLD in shake flask was inefficient, but its application can be significantly exploited for large-scale production in fermentor for industrial usages.

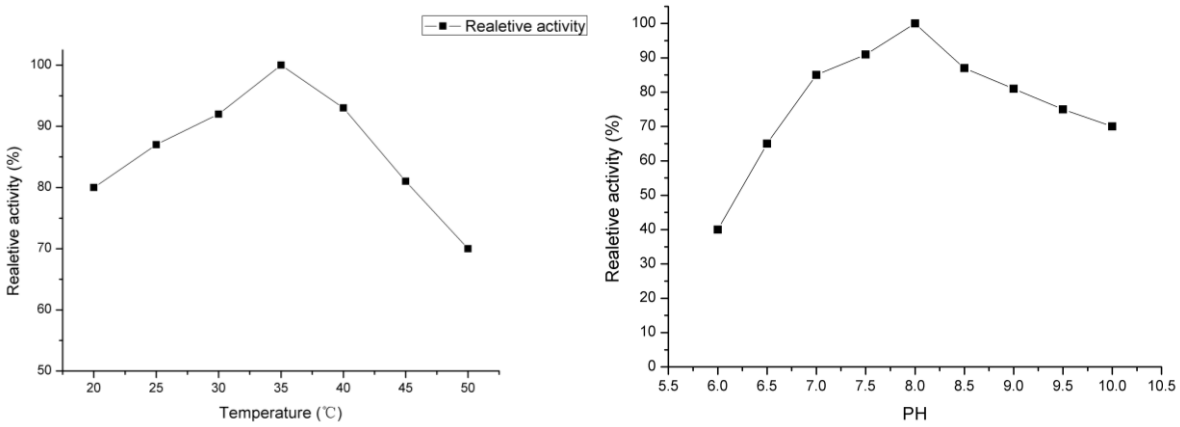

Figure 4. (A) Effect of temperature on the enzymatic activity of recombinant PLD. (B) Effects of pH on the enzymatic activity of recombinant PLD. The enzymatic activity was determined at 35 ℃ in 0.5 M PBS buffer (pH 6–10).

3. Materials and Methods

3.1. Bacterial Strains, Plasmids and Materials

E.coli DH5α strains were used for genetic manipulation host, *E.coli O104:H4* was used for the isolation of the genomic DNA and *E.coli* Rosetta was used for the expression of the PLD, respectively. The pET-28a (+) vector from Novagen was used as the expression vector.

3.2. Genetic Manipulation

For construction of different vectors with different signal peptide–PLD fusions, the PLD genes were amplified by the polymerase chain reaction (PCR) using the chromosomal DNA of E. coli E.coli O104:H4 as a template. Plasmid pET-28a was digested with NdeI and EcoRI. Then the PLD gene from E.coli O104:H4 was first cloned into pET-28a at the NdeI and EcoRI sites. In order to retain the C-terminal His-tag on the pET-28a plasmid, we operated site-directed mutation of PLD termination codon. This recombinant plasmid named as pET-28a-PLD was digested with NcoI. All signal peptides sequences obtained from the corresponding organism were cloned into pET-28a-PLD at NcoI site to yield the recombinant expressing plasmid and transformed into E. coli Rosetta (DE3) competent cells. The PLD gene was then located behind the leader signal sequences in frame. These sequences had been codon optimized and then examined by the SignalP 3.0 HMM software to predict the potential signal peptides (Bendtsen et al. 2004; Antelmann et al. 2001.

3.3. Design of the Signal Peptide

With the results of our study, OmpA and PelB could achieve extracellular expression of PLD. Generally, replacement of N-terminal positively charged amino acids results in increased secretion efficiency, due to the n-region affects the functions of SPs as intramolecular chaperones. Thus combining the advantages of them, we structured a signal peptide OmpA’ which utilized the N-terminal charged amino acid residuals of OmpA and the central hydrophobic core region of PelB. The main function of this new syncretic signal peptide is to enhance the secretion of target protein (PLD) in E.coli. In design development, we utilized N-terminal amino acid residue MKK of OmpA signal peptide which from E.coli outer membrane protein as the polar head. In the core hydrophobic domain of this syncretic signal peptide, the hydrophobic sequence LLPTAAAGLLLLAAQP of PelB signal peptide was used. PelB had been widely studied in the past decade years for holding fairly

strong hydrophobicity which could significantly assist protein transmembrane transport.

3.4. Cell Growth Conditions and Protein Expression

Luria-Bertani (LB) medium, which contained (g L^{-1}) NaCl 10.0,tryptone 10.0, yeast extract 5.0, was used for recombinant *E.coli* seed cultivation at 200 rpm and 37 ℃ for 10 h. A shake-flask culture was grown in Terrific Broth (TB) medium that consisted of (g L^{-1}) glycerol 5.0, tryptone 12.0, yeast extract 24.0, K2HPO4 16.4, and KH_2PO4 2.3. The resulting culture was then shaken at 200 rpm and 37 ℃. When the culture reached an OD_{600} of 0.8, isopropyl-β-d-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM to induce recombination protein expression. After induction, the culture was grown at 28 ℃ for 48h. To enhanced extracellular expression of PLD, we added different surfactants including Triton X-100, Tween-80, $CaCI_2$, Sarkosyl, SDS into the culture medium after induced 10 hours to research their effects on protein expression, cell growth and enzyme activity.

3.5. Cell Fractionation and Purified Recombinant PLD

The cultivation broth was centrifuged at 10,000 × g for 10 min at 4°C; the supernatant was frozen for further analysis of extracellular fraction. The periplasmic fractions were prepared by the modified osmotic shock method reported (Durrani *et al.* 2015) and the remaining cells were harvested and washed twice with 50 mmol l^{-1} phosphate buffer (pH 8.0). After that, the resuspended bacterial cells were sonicated thoroughly to release soluble intracellular enzyme. Then, the cell lysates were centrifuged at 10,000 × g for 10 min at 4 ℃ to collect the soluble intracellular fractionation. All the samples were subjected to SDS-PAGE analysis using 12% separating gel, followed by staining with coomassie brilliant blue G-250. All recombinant PLD activity was determined too. The recombinant PLD contained C-terminal His-tag, thus it could be purified by Ni-NTA agarose resin column affinity adsorption (Teng *et al.* 2011). At last, Protein concentration was measured by coomassie brilliant blue.

3.6. PLD activity determination

For measuring Phospholipase D (PLD) activity in vitro, a fluorescence microplate reader or fluorometer was utilized (Mohanty *et al.* 1997). PLD activity is monitored indirectly using 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. The activity unit (U) of PLD is defined as the amount of enzyme required to catalyst 1 umol of lecithin to phosphatidic acid and choline per minute at pH 8.0 at 37 ℃.

3.7. Properties of Purified Recombinant PLD

To estimate the optimal temperatures, activity of enzyme was determined using the standard assay in the temperature range from 20 to 50 ℃. For determining the enzyme activity, each enzyme was pre-incubated for 1h at the above temperature range in 50 mmol l^{-1} optimum pH phosphate buffer. Then the samples were rapidly added into the reaction system for 30 min and the activity of the samples was measured then.

The optimum pH was measured in phosphate buffer for pH 6.0-10.0. To determine the optimum pH of the PLD, the recombinant PLD was purified by Ni-NTA agarose resin column in different pH PBS buffers(6.0-10.0). Then the reaction was began at 35 ℃ for 30 min and the remaining activities of these treated enzymes were measured by the standard assay procedure.

4. Acknowledgments

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5. Conflict of Interest

All authors declare that they have no conflict of interest.

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