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Master Degree Thesis

# Purification and Characterization of Phospholipase D from *Streptomyces tendae*

Graduate School of Chosun University

College of Pharmacy

Poonam Mander

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Advisor: Prof. Jin Cheol Yoo

Thesis submitted for the degree of Master of Pharmacy

December 2008

Graduate School of Chosun University

College of Pharmacy

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# Abbreviations

| | |
|---|---|
| BSA | Bovine Serum Albumin |
| CHAPS | (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate) |
| DEAE | Diethyl aminoethyl |
| DGPP | Diacylglycerol Pyrophosphates |
| EDTA | Ethylenediaminetetraacetic Acid |
| PA | Phosphatidic Acid |
| PBut | Phosphatidyl Butanol |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PC | Phosphatidyl Choline |
| SDS | Sodium Dodecyl Sulfate |
| Tris | (Tris[hydroxymethyl]aminomethane) |

# ABSTRACT

## Purification and Characterization of Phospholipase D from *Streptomyces tendae*

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*Streptomyces tendae*, a member of actinomycetes isolated from Korean soil, produced an extracellular phospholipase D. The fermentation process was carried out along with its purification and characterization. Various concentrations of carbon, nitrogen and mineral sources were supplied to the culture medium for media optimization. It was able to produce maximum PLD activity when cultured in a medium containing 2% sucrose, 2% soybean, 0.05 % $CaCl_2$ and 0.1% $CaCO_3$. During fermentation process most of the PLD was secreted during early growth phase. The culture broth exhibited 3.7 U/ml of hydrolytic activity when cultivated at 28°C for 5 days. The enzyme was purified using Sepharose CL-6B column chromatography followed by DEAE Sepharose CL-6B ion exchange column chromatography. The enzyme was purified to apparent homogeneity giving a single band on SDS-PAGE with a molecular mass of 42.8 kDa. Optimal activity was found at 60°C and pH 8. Additionally, the enzyme activity remained virtually unaltered when treated at 60°C for 90 min and was stable at pH range of 8-10, suggesting that the enzyme is thermostable and slightly pH stable in alkaline range. Further, the enzyme activity was found to be enhanced by 1.5% Triton X-100 and 2 mM $Ca^{2+}$. This work presents the purification and characterization of an enzyme from *Streptomyces tendae*.

# 초 록

# *Streptomyces tendae* 에서 생산되는 Phospholipase D 의 정제 및 특성분석

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*Streptomyces tendae* 는 한국 토양에서 분리한 방선균의 일종으로, extracellular phospholipase D 를 생산하는 균주이다. PLD 를 생산을 위한 발효과정은 정제 및 특성분석과 함께 수행하였다.

최적배지 조건을 알아보기 위해 탄소원, 질소원, 금속이온을 다양한 농도에서 실험한 결과 2% sucrose, 2% soybean, 0.05 % $CaCl_2$, 0.1% $CaCO_3$ 배지 조성에서 최대의 PLD 활성을 나타내었다. 28°C 에서 5 일간 배양 시 배양액 속에 hydrolytic activity 가 3.7 U/ml 존재하였다. Sepharose CL-6B column chromatography 와 DEAE Sepharose CL-6B 이온교환 column chromatography 를 사용하여 정제된 효소는 10% SDS-PAGE 에서 42.8kDa 하나의 밴드를 나타내었다. 효소의 최적 온도는 60°C 이고, 최적 pH 는 pH8 이었다. 효소의 온도 안정성을 알아보기 위해 60°C 에서 90 분간 방치하였을 때 100% 안정함을 나타내었다. pH 8~10 에서 안정한 것으로 보아 알칼리성 PLD 임을 알 수 있었다. 계면활성제와 금속이온에 대한 영향을 살펴본 결과 Triton X-100 와 $Ca^{2+}$.에 의해 효소의 활성이 증가하였다.

# 1. Introduction

The genus *Streptomyces* affiliated to the Streptomycetaceae family is the largest antibiotic-producing genus in the microbial world discovered so far. Besides antibiotics, they are also reported to produce various types of enzymes. Convenience in enzyme production, higher transesterification activity and storage stability of the enzyme have made some microorganisms, particularly *Streptomyces* strains, an important source of phospholipase D in biocatalytic applications. The most traditional source of phospholipase D is cabbage while phospholipase D from *Streptomyces hachijoensis*, *Streptoverticillium cinnamoeum*, *Streptomyces lydicus*, *Streptomyces antibioticus*, and *Streptomyces* sp. strain PMF are the primarily studied microbial phospholipase Ds [1]. In the present study, *Streptomyces tendae* capable of producing novel thermostable serine protease enzyme [2] conserved in microbiology laboratory, college of pharmacy, Chosun University, is selected for production, purification and characterization of phospholipase D.

Phospholipase D is an enzyme which is abundantly found in bacteria, fungi, plants and animals [3]. Phospholipase D enzyme was first found and characterized in carrot and later in other plant such as cabbage, peanuts, castor bean etc. Among the all sources, phospholipase D from actinomycetes has been mostly investigated due to its higher transphosphatidylation activity compared to other sources [4, 5]. There is very low identity between plant, yeast and human phospholipase D gene. Bacterial phospholipase D show homology with each other, but no significant similarity with plant phospholipase D [6]. Based on homology in structure and mechanism, phospholipases D are considered to be a part of superfamily of protein known as phospholipase D super family, each member containing the highly conserved 'HKD' motif and other conserved sequences [7,8,9]. Phospholipase D can catalyze basically two types of reaction, hydrolytic and transphosphatidylation. In the hydrolytic reaction, phospholipids such as phosphatidyl choline (PC), which is considered to be major substrate for phospholipase D, is hydrolyzed to phosphatidic acid (PA) and choline by the action of phospholipase D. Furthermore phospholipase D performs a transphosphatidylation reaction using water or

primary alcohol as ethanol or 1-butanol as the nucleophile to generate PA, phosphatidyl ethanol or phosphatidyl butanol (PBut).

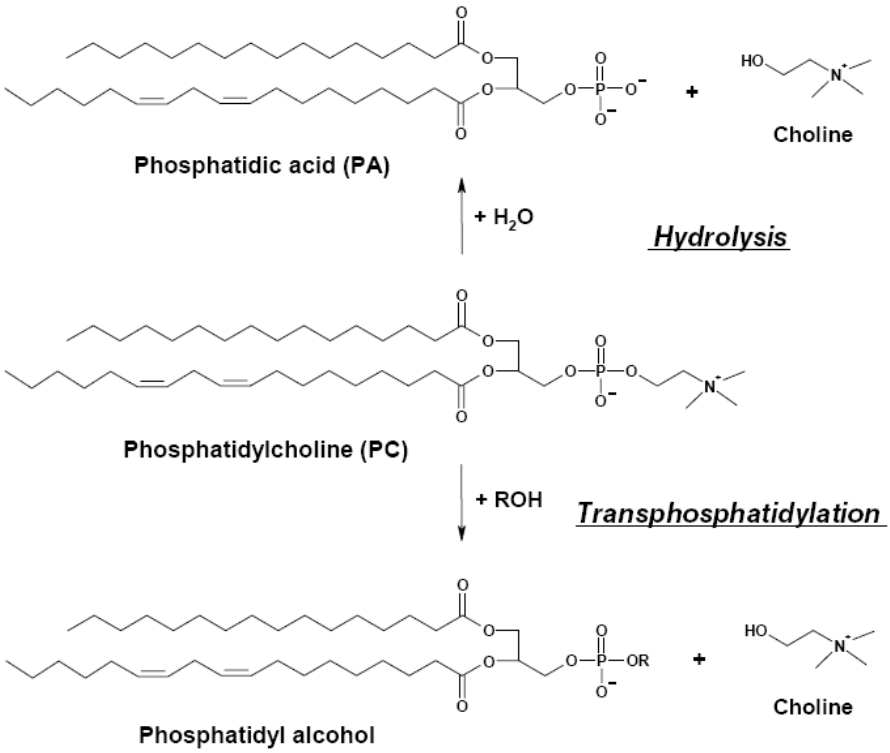


Figure 1: Hydrolytic activity and transphosphatidylation activity shown by phospholipase D [10]

The two reaction catalyzed by phospholipase D compete with each other and the product formed in transphosphatidylation reaction seems to be hydrolyzed by phospholipase D [11]. Microbial phospholipase D, especially from *Streptomyces*, show relatively higher transphosphatidylation activity compared to those produced by other organisms. The transphosphatidylation reaction is important process for the synthesis of scarce phospholipids like phosphatidyl ethanolamine, phosphatidylserine, phosphatidylglycerol or phosphatidyl inositol [12-14]. Some artificial phospholipids, which have very high demand in pharmaceutical and cosmetic industry, are also synthesized by such reaction. When talking about its importance, its performance is versatile, such as in case of mammalian cells, phospholipase D and its product phosphatidic acid (PA) are involved in number of signaling cascades, cell proliferation, membrane trafficking and defense response. While in plants, phospholipase D phosphorylates PA to produce diacylglycerol

pyrophosphates (DGPP), and this DGPP are newly discovered phospholipase D whose formation attenuates PA level, but itself is second messenger. Phospholipase D and PA role in plant signaling and provide the first demonstration that DGPP is formed during physiological conditions that evoke PA synthesis [15]. With advancement in molecular and structural biology of phospholipase D, the trend for recombinant production and genetic engineering of phospholipase D is highly increasing. Several phospholipase D have been produced by heterologus expression. For example, phospholipase D from *Streptomyces antibioticus*, *Streptomyces* PMF and Chimeric phospholipase D from *Streptomyces* sp were obtained as recombinant proteins expressed in *E. coli*. Thus phospholipase D from plant or microorganism can be used as biocatalyst in the transformation of phospholipids analog in both laboratory and industrial scale. From practical point of view phospholipase D enzyme is important for production of artificial phospholipids that are of some importance in pharmaceuticals and food industry and cosmetic industry. The schematic diagram for the purification scheme carried in this research work is shown in Fig. 2.

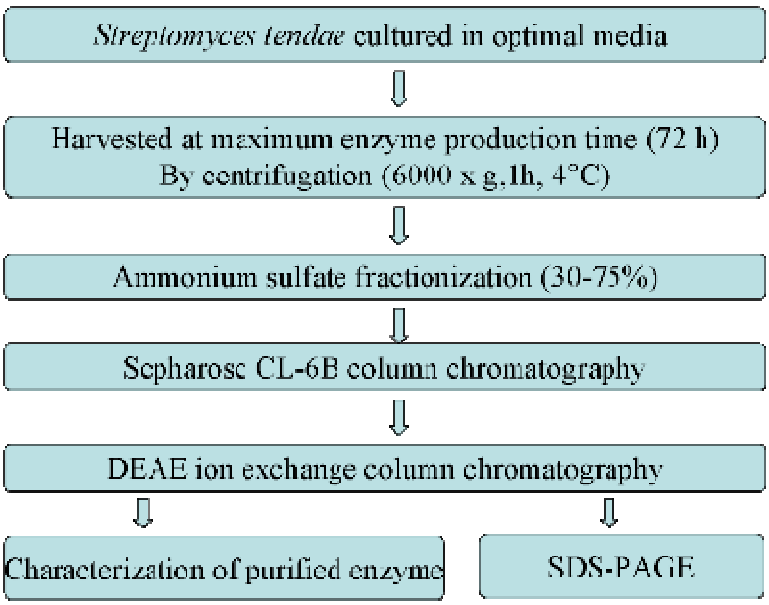


Figure 2: Schematic diagram of the purification scheme of phospholipase D

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Chemicals and Instruments

Choline oxidase (E.C.1.1.3.17, from *Alcaligenes* sp.), peroxidase (E.C.1.11.1., from horseradish), L-α-phosphatidylcholine from dried egg yolk, phenol, 4-aminoantipyrine, Triton X-100, as well as some other chemicals of analytical grade were purchased from Sigma. Sepharose CL-6B and DEAE Sepharose CL-6B were obtained from Pharmacia, and all other chemical used were of analytical grade.

### 2.1.2. Bacterial Strain

Actinomycetes is a non taxanomic term for a group of common soil microorganism sometimes called as “thread or ray fungi”. In growth habit many actinomycetes resemble fungi, but smaller and the term common to both are used to describe morphological features. The most common genus of actinomycetes in soil is *Streptomyces* that produces straight chains or coils of spores or conidia [16,17]. *Streptomyces tendae* belonging to actinomycetes group was the selected strain for the purification and characterization of phospholipase D enzyme. The considered bacterial strain was isolated from soil samples collected in Jeonnam province, Korea.

## 2.2. Methods

### 2.2.1. Culture Condition

*Streptomyces tendae,* isolated from Korean soil, maintained on OSYM agar medium (2% oatmeal, soybean meal 1%, dried yeast 1%, mannitol 1%). Spores from the strain were precultured at 28 °C and 160 rpm for 2 days in a rotary shaker with 50 ml of emersion media containing 0.4% beef extract, 0.1% yeast extract, 0.4% peptone, 1% glucose, and 0.25% NaCl. 2% of this per-culture was then used to inoculate 200 ml of medium

containing 2% sucrose as carbon source, 2% soybean as nitrogen source, 0.05% $CaCl_2$ as mineral source and 0.1 % $CaCO_3$, which is cultivated for 5 days at 28 °C and 160 rpm. To determine the optimal medium composition, various carbon and nitrogen sources in different concentrations were supplied in production medium. Culture samples were collected at interval of 24 hour and phospholipase D production was checked by measuring absorbance at 500 nm. Samples were centrifuged at 6,000 x g for 20-30 minutes at 4 °C.

### 2.2.2. Ammonium Sulfate Precipitation

*Streptomyces tendae* strain was grown in optimal medium for 5 days and harvested by centrifugation at 6,000 x g for 1 hour at 4 °C. Then ammonium sulfate (30-75%) was slowly poured into the supernatant, allowing the salt to dissolve slowly. The supernatant was continually stirred on ice bath until all salts were completely dissolved. Precipitates were recovered by centrifugation at 6000 x g for 1 hour at 4 °C and dissolved in 10 mM Tris-HCl (pH 8.5).

### 2.2.3. Sepharose CL-6B Column Chromatography

The dialyzed ammonium sulfate precipitate were concentrated using an ultra filtration (Amicon, Danvers, MA) through a membrane pore size of 30,000 MW. The resulting concentrated samples (5-20 ml) were applied to a Sepharose CL-6B column (116 cm length, 2.2 cm in diameter) at an elution speed of 4 cm/hr. Sample were eluted with 10 mM Tris-HCl buffer (pH 8.5). Fractions having phospholipase D activity were colleted and concentrated using cellulose membrane filter having pore size of 10,000 MW.

### 2.2.4. DEAE Sepharose CL-6B Ion Exchange Column Chromatography

The active fraction eluted through Sepharose CL-6B column chromatography was concentrated and then loaded onto a DEAE Sepharose CL-6B column (75 cm height and 1.2 cm diameter) at a flow rate of 2 ml/10min. Equilibrations and elution were performed first with 10 mM Tris-HCl to remove unbounded proteins and then with a linear salt

gradient ranging from 0 to 0.2 M KCl. Active fractions were pooled, concentrated and then used for characterization.

### 2.2.5. Enzyme Assay

The phospholipase D activity assay was performed by following the procedure described by Imamura and Horiuti (1978) [18]. It is based on the enzymatic determination of choline that is released during the hydrolysis of phosphatidylcholine (PC) by phospholipase D. The substrate solution PC, was prepared by mixing 200 mg of L-α-phosphatidylcholine (60%), 4 ml distilled water and 0.4 ml diethyl ether. And then it was sonicated on ice bath for 1 hour. Then PC emulsion is made by taking 200 µl PC, 100 µl 1M Tris-HCl (pH 8.5) and 66 µl 0.1 M $CaCl_2$. The final reaction contained 40 µl enzyme sample and 60 µl PC emulsion, followed by incubation at 55 °C for 20 minutes and arrested by the addition of 60 µl 1M Tris-HCl buffer (pH 8.5) containing 50 mM EDTA. The samples were boiled for 10 minutes and then chilled for 10 minutes in ice. Then to the above mentioned standard reaction, 150 µl of mixture containing 1.7 U peroxidase, 7.98 mM 4-aminoantipyrine, 4.3 mM phenol and 0.14 U choline oxidase was added and then incubated at 37 °C for 1 hour. The sample mixture was then treated with 0.5% Triton X-100 (v/v) to remove turbidity. The amount of quinoneimine dye formed during reaction was measured at 500 nm. A UV-1601 UV visible spectrophotometer (Shimadzu Corporation, Japan) was used to measure the absorbance. One unit of enzyme was defined as the amount of enzyme required to convert 1 µm of choline from PC.

### 2.2.6. Protein Assay

Protein concentration was determined by the Bradford method (1976) [19] using bovine serum albumin (Life technologies, Gaithersburg, MD, U. S. A.), as the standard.

### 2.2.7. Optimization of Temperature and Thermostability

For optimization of temperature, phospholipase D activity was determined at different temperatures (20 °C to 80 °C) by incubating the phospholipase D catalyzed standard reaction mixture at different temperatures for a time interval of 20 minutes. For the determination of thermal stability, enzyme samples were incubated at different temperatures (60 °C to 70 °C). Aliquots of samples were withdrawn at interval of 15 minutes and kept on ice bath before measuring the residual activity.

### 2.2.8. Optimization of pH and pH Stability

For optimization of pH, phospholipase D activity was determined at different pH values (3-11) by taking different pH buffers having 0.1 M concentration (citric acid and sodium phosphate buffer- pH 3.0, 4.0, 5.0, 6.0. 7.0 , Tris-HCl- pH 7.5, 8.0, 8.5, 9.0, sodium bicarbonate-sodium hydroxides – pH 10 and 11 in the phospholipase D catalyzed standard reaction mixture as described above.

For pH stability, aliquots of enzyme samples were treated with various pH buffer (0.1 M) for 24 hour at 4 °C. Then the residual enzyme activity was evaluated under the standard assay condition.

### 2.2.9. Effect of Metal Ions on Phospholipase D Activity

The effect of different 2 mM metal ions ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, EDTA) on the activity of phospholipase D was studied, using the above standard reaction mixture.

### 2.2.10. Effect of Detergents on Phospholipase D Activity

The effect of various detergents (Triton X-100, Tween 20, Tween 80, Polyethylene-4-Lauryether, Deoxycholic-acid, N-Lauryl Sarcosine, CHAPS and SDS) at 1.5 % concentration was studied on the phospholipase D activity by using the above standard reaction mixture.

### 2.2.11. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of phospholipase D from *Streptomyces tendae* was performed using different samples such as growth medium, filtered culture supernatant, ammonium sulfate, after GPC and DEAE column chromatography containing phospholipase D activity, which were carried out as described by Laemmli (1970) [20] using 5% stacking gel and 10% polyacrylamide resolving gels (1.5 mm thickness). Low molecular weight protein standards (Fermentas) was used as reference proteins. Samples of protein preparations were dissolved in a solution containing 60 mM Tris-HCl (pH 6.8), 14.4 mM β-mercaptoethanol, 2% SDS, 25% (v/v) glycerol, and 0.01% Bromophenol Blue and immediately heated at 100°C for 10 minutes.

Electrophoresis was carried out at a constant current of 150 V/40 mA until the tracking dye (Bromophenol Blue) reached at the bottom of the gel (approximately 1 hour total running time). SDS-PAGE gels were stained by using a Coomassie Brilliant Blue Stain.

# 3. Results and Discussion

## 3.1. Morphology of *Streptomyces tendae*

*Streptomyces tendae* was grown in OSYM agar plate. White colony with 2 mm diameter, white aerial mycelia, gray colored spores were observed after 4 days of incubation in 28 °C, as shown in Fig. 3.

## 3.2. Effect of Nutrients on Phospholipase D Production

Various carbon, nitrogen and mineral sources were supplied to the medium to evaluate their effect on production of phospholipase D. Thus, 2% of several different types of carbon source were supplied and production of phospholipase D was examined (Fig.4). The strain grew well on various tested carbon sources and sucrose showed the highest effect. Similarly 2% of several different nitrogen sources were tested for enzyme production, as shown in Fig. 5. Maximal enzyme production was shown by 2% soybean. Enzyme production was also enhanced by the addition of 0.05% $CaCl_2$ as mineral source (Fig. 6).

## 3.3. Fermentative Production of Phospholipase D Enzyme

The fermentation study was carried out in optimized media containing 2% sucrose, 2% soybean, 0.1 % $CaCO_3$ and 0.05 % $CaCl_2$. The fermentative production of phospholipase D was investigated in 200 ml batch culture system. In the fermentation study it was observed that, *Streptomyces tendae* secreted maximum phospholipase D in culture broth at 72 h of cultivation as shown in Fig. 7. Enzyme activity was found to be 3.8 Units/ml in broth.

## 3.4. Purification of Phospholipase D Enzyme

### 3.4.1. Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed as the first step of phospholipase D purification. Ammonium sulfate fractions collected at 30-75% saturation and assayed for specific phospholipase D activity after dialysis.

### 3.4.2. Purification

The broth was concentrated by ammonium sulfate precipitation at 30-75% saturation at 4 °C. The pellet collected by centrifugation at 6,000 x g for 1 hour at 4 °C was dissolved in 10 mM Tris-HCl (pH 8.5) buffer. After filtration through YM 30 (pore 30,000 MW), the supernatant was applied to Sepharose CL-6B column (2.2 cm x 116 cm). Sample was eluted by 10 mM Tris-HCl (pH 8.5). Fig. 8 shows the graph of fractions that collected from Sepharose CL-6B chromatography column. Sample collected from Sepharose CL-6B chromatography was applied on DEAE anion exchange column. Elution was carried out with a gradient of 0.2 M KCl at a flow rate of 10 cm/h (Fig. 9). Increase in the phospholipase D specific activity using ammonium sulfate was 1.7-fold. Subsequent increases in phospholipase D specific activity were 2.3 and 4-fold by using Sepharose CL-6B column chromatography and using DEAE column chromatography, respectively. The enzyme purified to homogeneity resulting in a 4-fold purification with a yield of 2% as presented in Table 1 was analyzed on SDS-PAGE (Fig.10). The molecular weight of phospholipase D (lane no. 5, Fig. 10) was estimated to be 42.8 kDa by comparing the reference proteins.

## 3.5. Optimization of Temperature and Thermo Stability

Using purified enzyme from *Streptomyces tendae* its reaction characteristics and stability were determined. The optimum reaction temperature was found to be 60 °C for hydrolysis of PC (Fig. 11).

When investigating the thermal stability, *Streptomyces tendae* exhibited thermal stability up to 60 °C when measured after 90 min, suggesting that it is a thermostable phospholipase D enzyme (Fig.12). Similar results have been observed from phospholipase D produced by *Streptomyces* sp. [21].

## 3.6. Optimization of pH and pH Stability

The optimum pH for *Streptomyces tendae* phospholipase D is pH 8 as shown in Fig. 13. In addition the enzyme exhibited high stability in alkaline range of pH(8-10). The enzyme activity was lost in highly acidic condition. This result indicates that the enzyme from *Streptomyces tendae* is more stable in alkaline range (Fig.14).

## 3.7. Effect of Detergents on Phospholipase D Activity

The effect of various ionic, anionic and zwitter-ionic detergents on *Streptomyces tendae* phospholipase D activity for was studied and the results were presented in Table 2. It was found that with the supplementation of 1.5% detergents, the reaction medium increased the enzyme activity from 1.8 to 2.7 fold under the control condition (without any detergent). The activity was inhibited in N-luryl sarcosine and SDS, which are anionic detergents. The enzyme activity was significantly enhanced by 1.5% triton X -100 which is non ionic detergent.

## 3.8. Effect of Metal Ions on Phospholipase D Activity

Various metal ions such as $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$ and KCl were used to examine their effect on phospholipase D activity. Among them, $Ca^{2+}$ was found to be the most promising metal ion and showed maximum activity at 2 mM concentration, as presented in Table 3. When different concentration of $Ca^{2+}$ ion was treated, the highest enhancement of the enzyme activity was found at 2 mM concentration (Fig.15).

Table 1: Purification of phospholipase D from *Streptomyces tendae*

| Steps | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purity Fold | Yield (%) |
|---|---|---|---|---|---|
| **Crude supernatant** | 199 | 948 | 4.8 | 1 | 100 |
| **Ammonium sulfate** | 84 | 742 | 8 | 1.7 | 78 |
| **Sepharose CL-6B** | 37.5 | 427 | 11 | 2.3 | 45 |
| **DEAE Sepharose** | 0.9 | 16 | 18 | 4 | 1.7 |

Table 2: Effect of Detergents on phospholipase D from *Streptomyces tendae*

| **Detergent** | **Type of ion** | **Relative activity %** |
|---|---|---|
| None | | 100 |
| **Triton X- 100** | Non ionic | **265** |
| Tween 20 | Non ionic | 233 |
| Tween 80 | Non ionic | 198 |
| Polyoxyethylene-4-laurylether | Non ionic | 210 |
| Deoxycholic acid | Non ionic | 216 |
| N-laurylsarcosine | Anionic | 25 |
| CHAPS | Zwitter ionic | 180 |
| SDS | Anionic | 0 |

Table 3: Effect of metal ions on phospholipase D from *Streptomyces tendae*

| **Metal ions** | **Relative activity %** |
|---|---|
| None | 3.5 |
| **$CaCl_2$** | **100** |
| $MgSO_4$ | 1 |
| $MnSO_4$ | 5 |
| $CoSO_4$ | 7 |
| $CuSO_4$ | 0 |
| $FeSO_4$ | 5 |
| $ZnSo_4$ | 0 |
| KCl | 0 |
| EDTA | 0 |

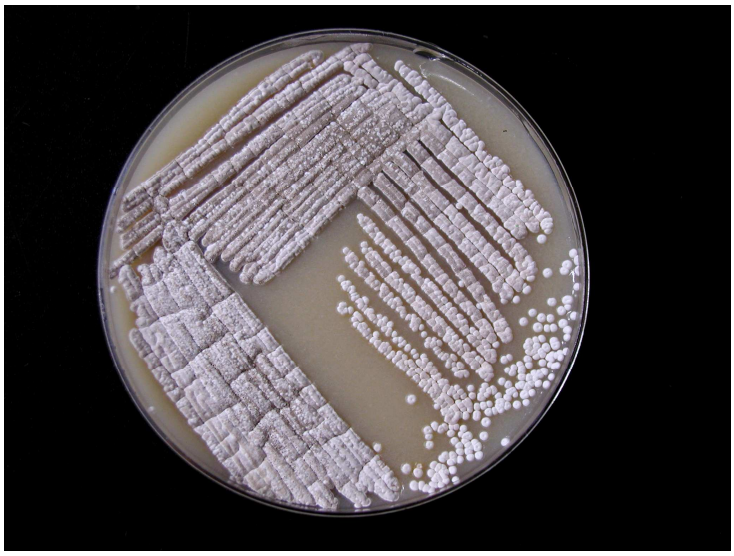

Figure 3: Colony morphology of *Streptomyces tendae*

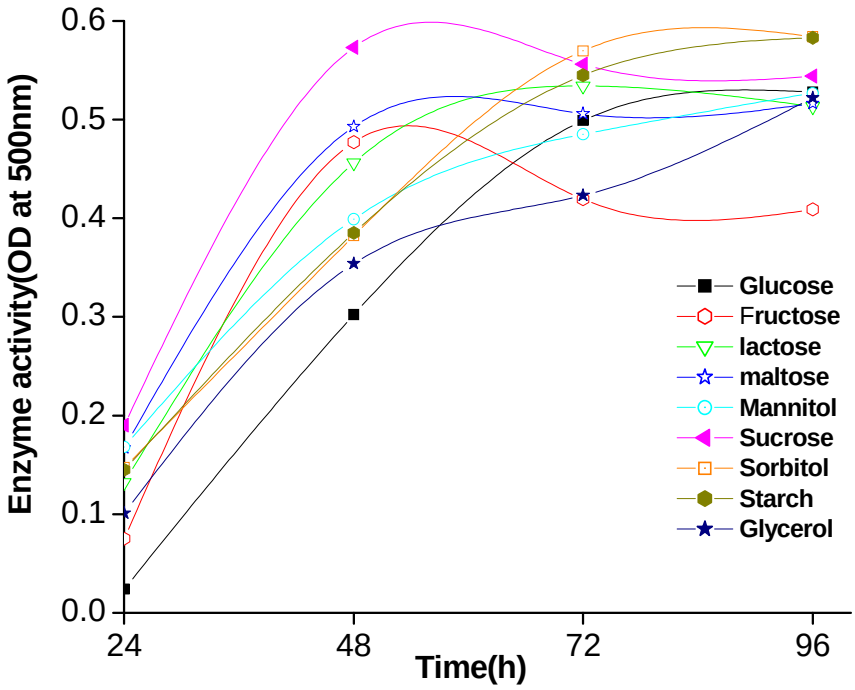


Figure 4: Effect of various carbon sources on the production of phospholipase D

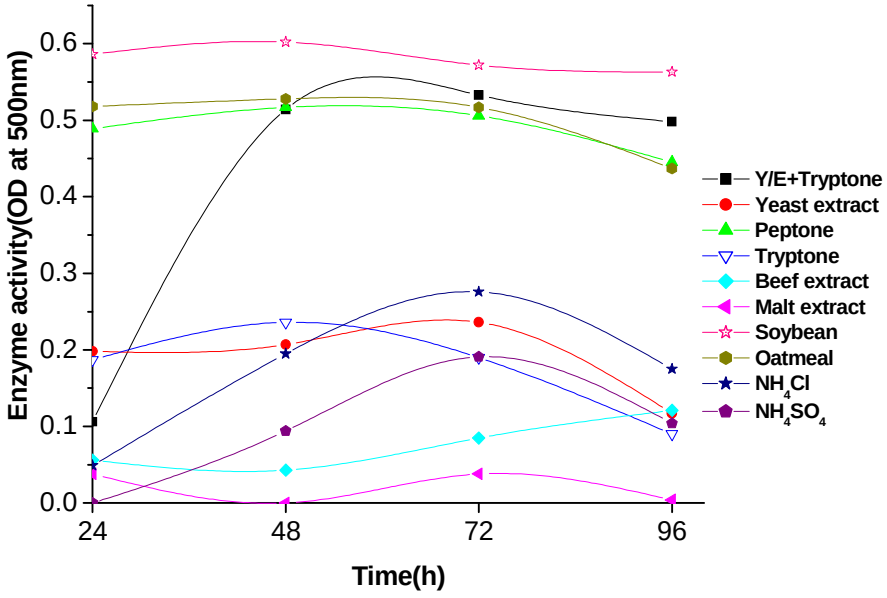


Figure 5: Effect of various nitrogen sources on the production of phospholipase D

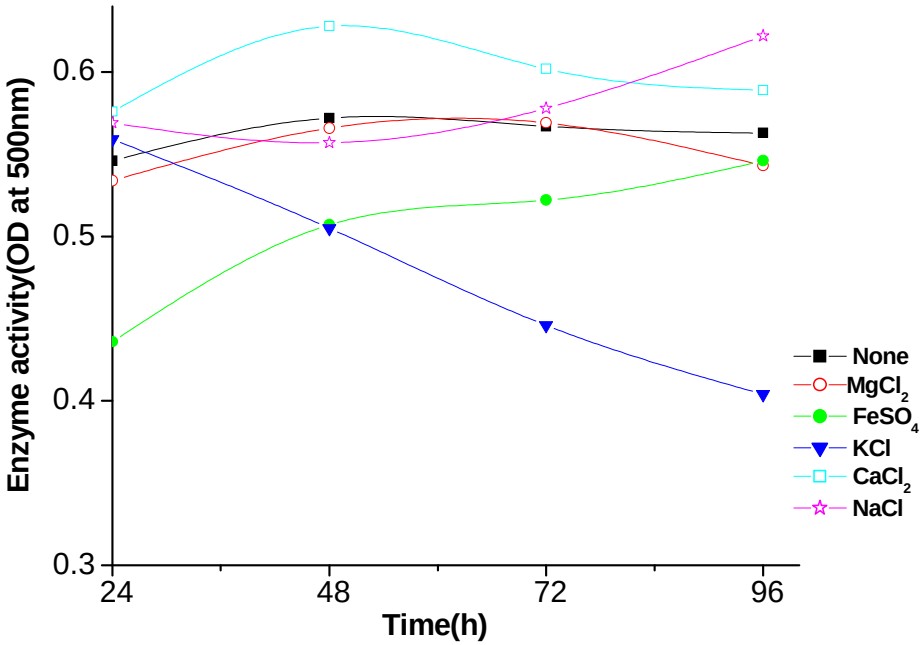


Figure 6: Effect of metal ions (0.05%) on the production of phospholipase D

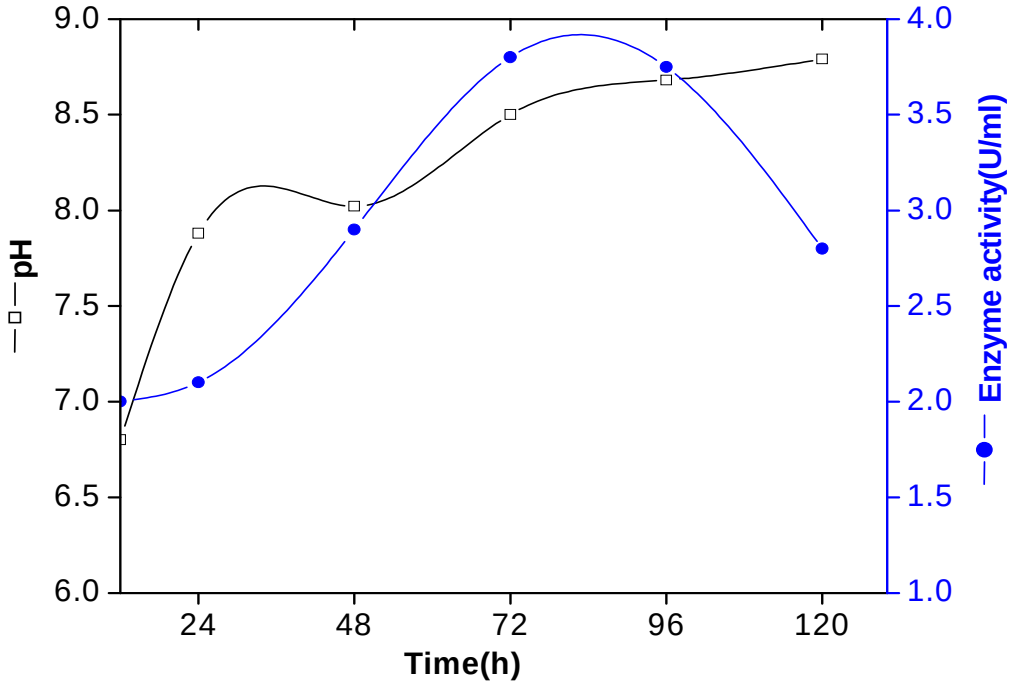


Figure 7: Fermentation profile for the production phospholipase D

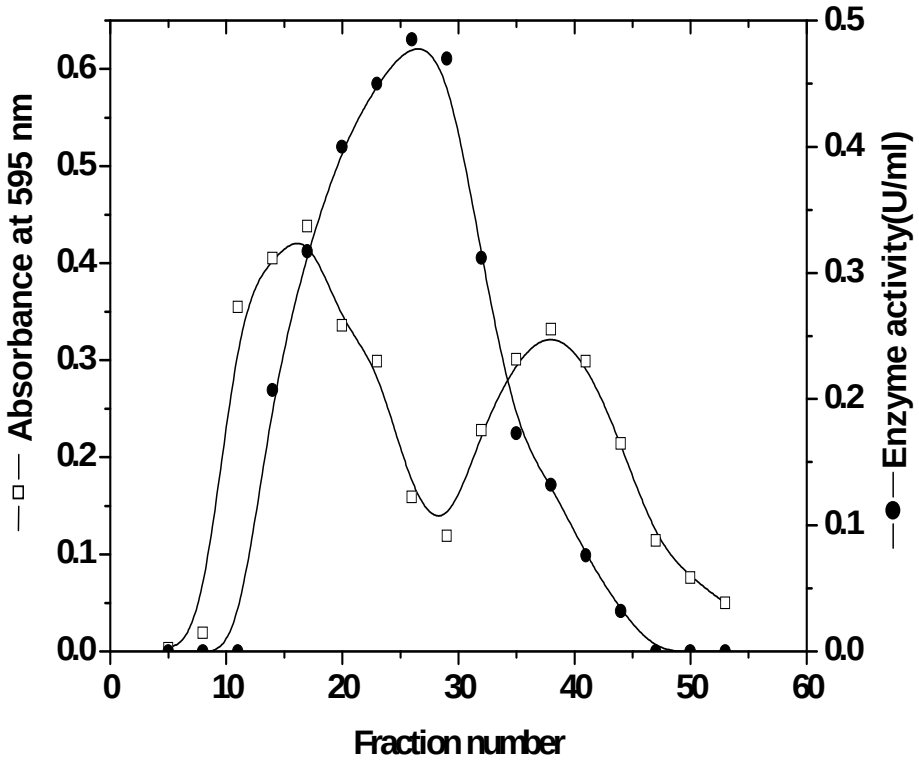


Figure 8: Elution profile of phospholipase D from Sepharose CL-6B column chromatography

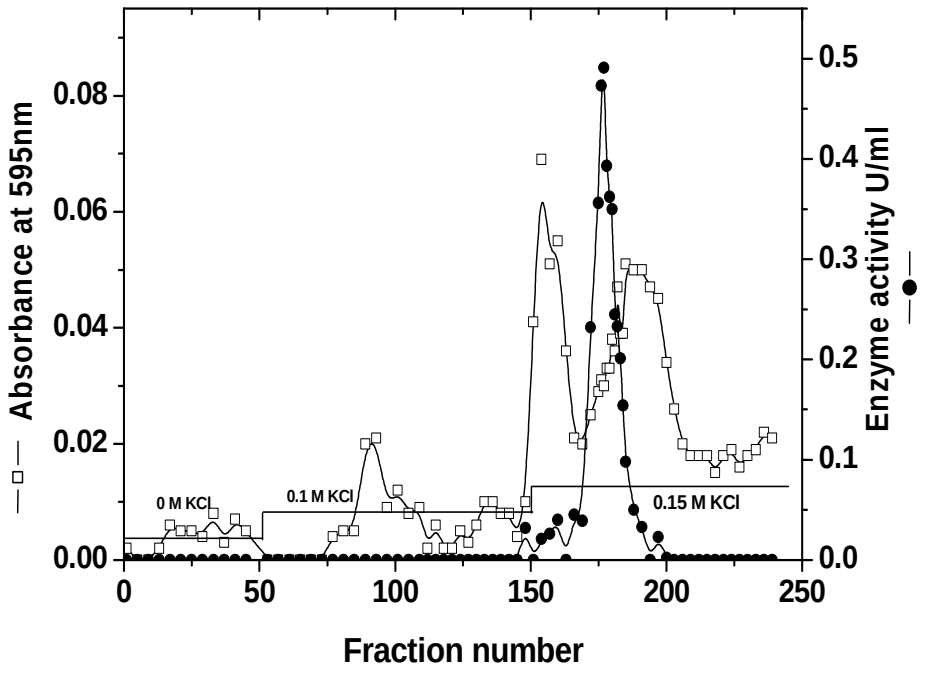


Figure 9: Elution profile of phospholipase D from DEAE ion exchange column chromatography

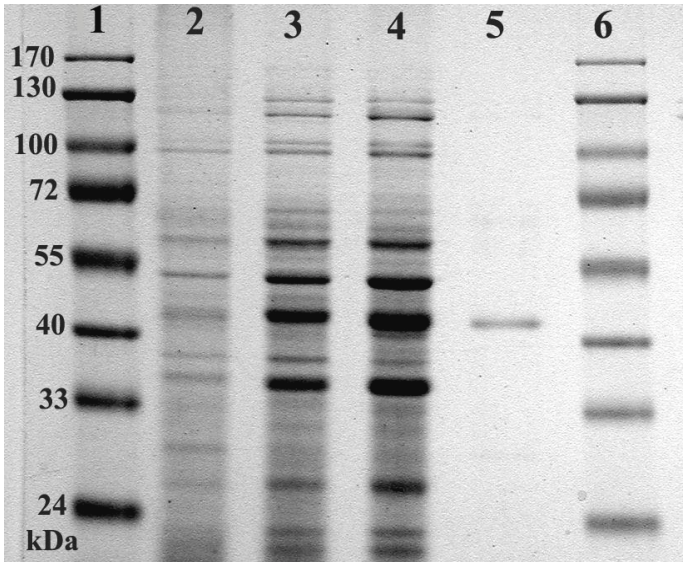


Figure 10: Molecular weight determination of phospholipase D by SDS PAGE

Lane 1 and 6: Protein size marker

Lane 2: Crude extract

Lane 3: Ammonium sulfate

Lane 4: Sepharose CL-6B

Lane 5: DEAE Sepharose CL-6B

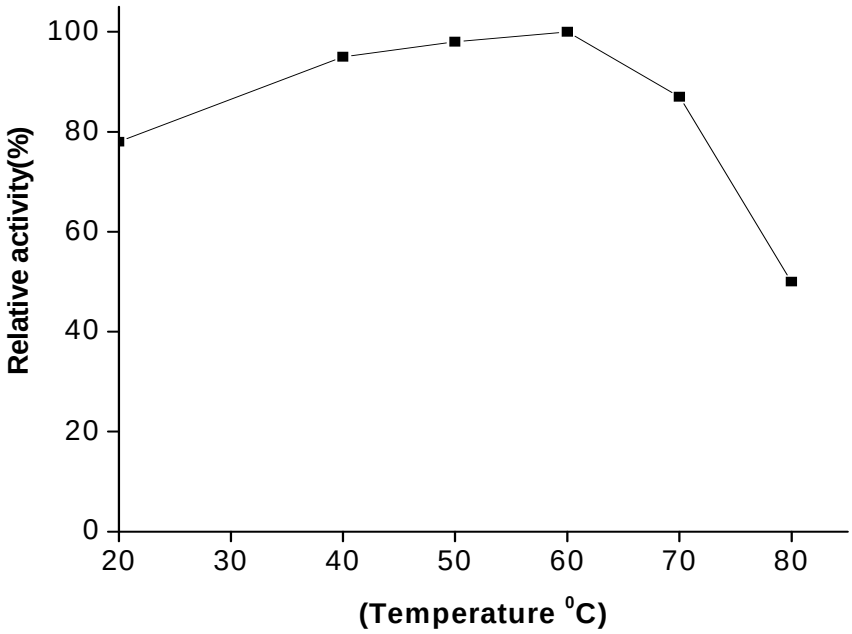


Figure 11: Effect of temperature on phospholipase D activity

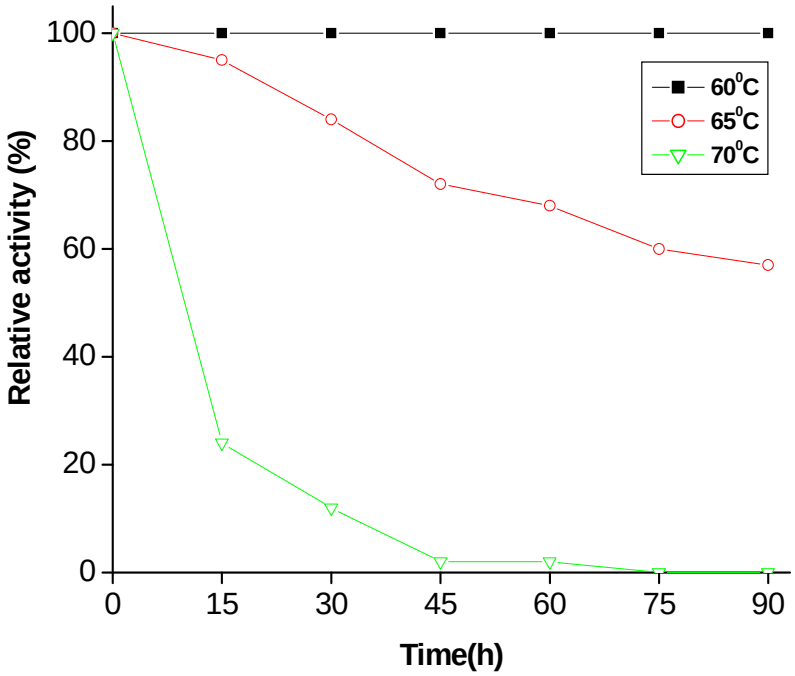


Figure 12: Effect of temperature on stability of phospholipase D

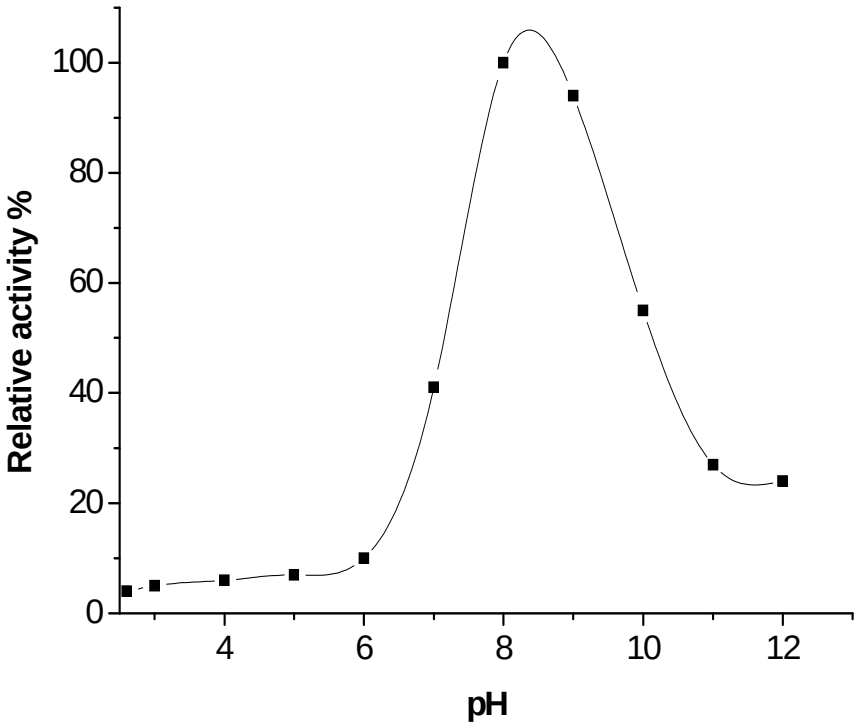


Figure 13: Effect of pH on phospholipase D activity

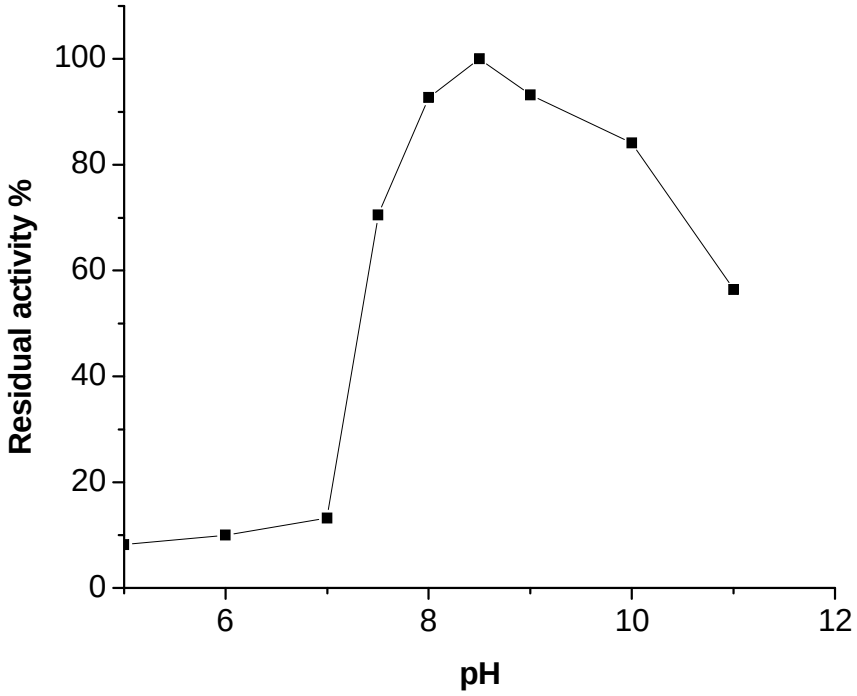


Figure 14: Effect of pH on stability of phospholipase D

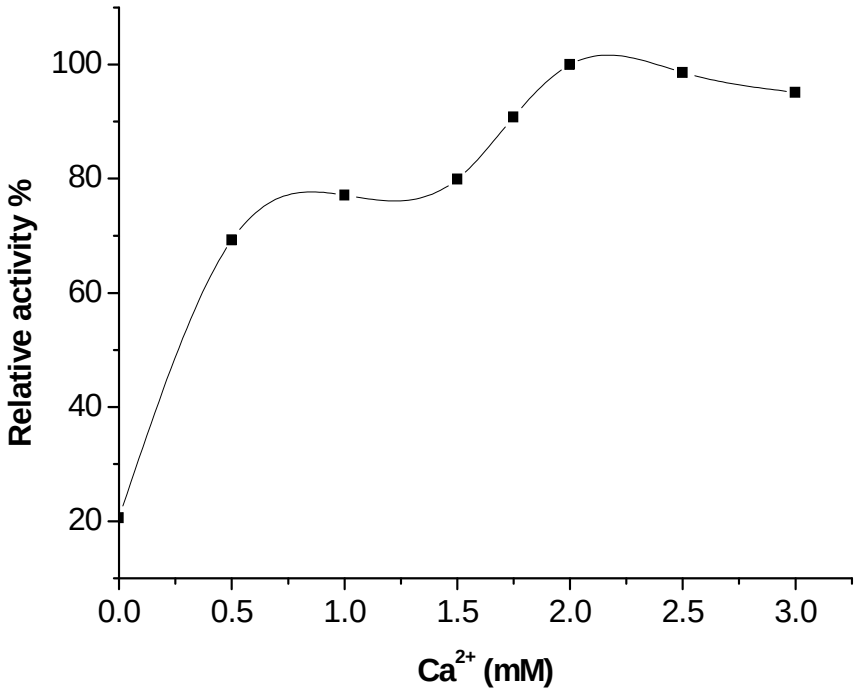


Figure 15: Effect of different concentration of $Ca^{2+}$ ion on phospholipase D

# 4. Conclusion

The production of Phospholipase D from *Streptomyces tendae* was highest in a medium supplemented with 2% sucrose, 2% soybean, 0.05% $CaCl_2$ and 0.1% $CaCO_3$ at 28 °C and 160 rpm/min for 3 days. Ammonium sulfate precipitation at 30-75% saturation, Sepharose CL-6B and DEAE ion exchange column chromatography were used for the purification of the phospholipase D enzyme, which resulted in a 4-fold purification with 1.7 % yield. Molecular mass of the phospholipase D was estimated to be 42.8 kDa. The enzyme activity was optimal at pH 8.5 and 60 °C. Like most conventional bacterial phospholipases D, Triton X-100 and divalent cation, $Ca^{2+}$ were required for enhancing the enzyme activity.

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