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International Journal of Pharmaceutical and Phytopharmacological Research

Int.J.Pharm.Phytopharmacol.Res. 2011, 1(2): 56-66

ISSN 2249 - 6084

Extraction, Partial Purification and Characterization of 5'-Phosphodiesterase from Germinated Phaseolus mungo (Mung Bean)

Nilesh Mahadeo Khutle[1]*, Dr.C.Vijaya[2], Harshal Pawar[1], Priti Jodhe[1], Ashwini Gaikar[3]

[1]Dr.L.H.Hiranandani College of Pharmacy, Ulhasnagar, Maharashtra,India
[2]Ultra College of Pharmacy, Madurai, Tamilnadu,India
[3]Sharadchandra Pawar College of Pharmacy, Otur, Pune,Maharashtra,India

Received on: 06/10/2011 **Accepted on:** 16/10/2011

ABSTRACT
5'-Phosphodiesterase (5'-PDE) is an enzyme that hydrolyses RNA to a mixture of ribonucleotide, from *which 5'-guanosine monophosphate (5'-GMP) and 5'-Inosine monophosphate (5'*-IMP) can be isolated. In *the present work 5'PDE was extracted and partially purified from germinated mung bean. 5'*-PDE activity was monitored using bis-(p-nitro phenyl phosphate) disodium salt as the substrate. The enzyme acts on substrate and releases the p-nitrophenol, which is measured at 420 nm. Purification was carried out by centrifugation, Ammonium sulphate precipitation followed by membrane filtration and heat shock treatment which gave 6.41-fold concentration. The enzyme had a pH optimum of 6.0 and showed good stability over pH range from 4 to 7.The optimum temperature for enzyme activity was found to be 60^0C and was also found stable at 4^0*C.5'*-PDE activity was enhanced up to 147% in the presence of Mg^{++}. Michelis-Menten equation with km 0.48 and Vmax 19230 was determined from Lineweaver Burke plot. This partially purified enzyme could be used for hydrolysis *of RNA to produce 5'-GMP and 5'*-adenosine *monophosphate, a precursor of 5'*-IMP.

Key Words: 5'-Phospodiesterase, Germinated mung bean, Purification, 5'-GMP, 5'-IMP

INTRODUCTION

A Phosphodiesterase is any enzyme that breaksa phosphodiester bond. These enzymes regulate the localization, duration, and amplitude of cyclic nucleotide signaling within subcellular domains. Phosphodiesterases are therefore important regulators of signal transduction mediated by these second messenger molecules. Phosphodiesterase enzymes are often targets for pharmacological inhibition due to their unique tissue distribution, structural properties, and functional properties.
An extracellular enzyme, 5'-Phosphodiesterase was first identified by Kuninaka et al[1]. 5'-Phosphdiesterase is the enzyme which hydrolyzes RNA to form 5'-nucleotides as 5'-GMP and 5'-IMP and hence there is a considerable commercial interest in the preparation of 5'-Phosphodiesterase[2].
5'-nucleotides have been widely used in pharmaceutical and food industry[3-5]. They can be used to synthesize the antivirus and anticancer drugs as the acridine.The nucleotide derivatives have important uses in the treatment of the illness of human central nervous system and circulatory system. The interest in 5'-Phosphodiesterase has grown over the last few years due to the application of 5'-nucleotides in antivirus and anticancer treatment[6].

The present investigation deals with extraction, partial purification and characterization of 5'-Phosphodieterase from germinated mung beans (Phaseolus mungo).Literature survey revealed that no such work has carried out till date on mung beans[7-10].

MATERIALS AND METHODS

Materials

Mung beans were purchased from the local market of Erode, Tamilnadu, India. Bis-(p-nitrophenyl phosphate) disodium salt, p-nitrophenyl phosphate and p-nitrophenol were purchased from SRL Pvt. Ltd., Mumbai. All other chemicals required for assay of enzymes as well as extraction, characterization and purification were obtain from S.D.Fine Chemical Ltd., Mumbai and were of analytical grade.

Production of 5'-PDE from germinated Mung bean

Mung bean were soaked in water for 4-5 hr and then allowed to germinate for 1 to 4 days at room temperature in closed chamber. Water was sprinkled on mung bean twice a day. The germinated mung bean were crushed in the mixer, and slurry was made in 4 parts of precooled water containing sodium benzoate (4.8%) and methyl p-hydroxy benzoate (0.9%) as preservatives. Slurry was stirred for 30 minute to ensure complete extraction of enzyme followed by passing through the muslin cloth to separate the extracted enzyme from crushed mung bean. It was kept for 1 hr at 8-12°C in a refrigerator. The large solid particles and starch were separated by centrifugation at 9000rpm at 8-10°C for 15 minutes and then clarified by passing through a vacuum filter.

Optimization of germination period

For optimizing the germination period of mung bean for maximum activity of 5'-PDE, four batches of 25 gm of presoaked mung bean were kept for germination in closed chamber at room temperature. After every day enzyme was extracted from one batch of germinated mung bean and enzymatic assay was performed as mentioned below.

Enzyme Assay of 5'-PDE

To 0.1 ml of appropriately diluted enzyme solution, 0.8 ml of acetate buffer of pH 6.0 (50 mM), 0.1 ml of substrate i.e. 10 mM bis-(p-nitro phenyl phosphate) disodium salt were added. The reaction mixture was incubated at 60°C for 10 min. The reaction was stopped by adding 0.3 ml of 5 % sodium carbonate. Final volume was made up to 3 ml by distilled water and vortexes to achieve a homogeneous mixture. The yellow color produce due to p-nitrophenolate ions was measured spectrophotometrically as absorbance at 405 nm using UV-Visible spectrophotometer (Make:ELICO,SL-164).A blank was prepared without adding enzyme solution. Standard graph of p-nitrophenol in the range of 0-200 Nmol was prepared using UV-Visible spectrophotometer .One unit of the enzyme activity was defined as the amount of enzyme required to release 1 Nmol of p-nitrophenol per min at 60°C at pH 6.0.

Assay of Phosphomonoesterase (PME)

Assay of phosphomonoesterase (PME) was carried out using the same procedure used for assay of 5'-PDE, except that the substrate used were 10 mM p-Nitro phenyl phosphate (p-NPP).

Determination of protein for measuring specific activity

Protein was determined by Bradford method using bovine serum albumin as a standard.The units/ml for 5'-PDE and PME and mg/ml of protein were determined, from which the specific activity (Units/mg) was calculated, also the purification fold ,% recovery and 5'-PDE: PME ratio was determine at each stage of purification.

Partial purification of the crude 5'-PDE

The extracted enzyme was preserved at 4°C and assayed for the 5'-PDE activity. It was further purified as follows:

Ammonium sulphate Precipitation

50 ml of filtrate was taken in the beaker, the beaker was kept in ice bath at 4°C, to this 5.67 gm of powdered ammonium sulphate was added to give a saturation of 20%.The salt was added slowly with mild

stirring by magnetic stirrer, over a period of 30-40 minutes and allowed to stand for 30 minutes, then solution was centrifuge at 7500 rpm at 10°C for 20 minutes, the small amount of precipitate formed was collected and dissolved in minimum amount of 50 mM acetate buffer (pH 5).The supernatant was brought to 40%, 60%,80% and 100% saturation by adding ammonium sulphate under the same condition .All the five precipitates were collected separately and dissolved in minimum amount of 50mM acetate buffer(pH 5.0) and dialyzed. For dialysis pretested membrane having pore size of 2.4 nm and made of generated cellulose that was partially permeable and had low MWCO of 12000-14000 was used. The bags were seamless and had average diameter of 21.5 mm and capacity of 3.63 ml cm^{-1}.Required length of the membrane were taken from roll. Both the sides of cut pieces were opened. One end was tied with thread to form a pouch for the enzyme solution and it was kept in contact with 50 mM acetate buffer (pH 5.0).The buffer was stirred greatly with magnetic stirrer to improve the solute exchange. The buffer were tested for ammonium sulphate every 1 hr.initialy and then after 30 minutes, using Nessler's reagent which gives brown color with ammonium sulphate the buffer was changed continuously with fresh buffer. After the complete removal of ammonium sulphate (7-8 hr.) the dialyzed bag was removed and partially purified enzyme was used for the further study.

Heat shock treatment

It was reported that PME activity could be destroyed at 60°C for 15 minutes. Hence the dialyzed sample of each fraction was further purified by destroying other heat labile proteins like PME. The enzyme from each dialyzed bags were taken separately in sample tube and shaken at 60 ± 1 °C for 15 minutes and there after cooled to 12-20°C by keeping in ice bath. After cooling the 0.1 ml of appropriately diluted enzyme preparation was taken and analyzed.

Determination of purification fold and enzyme recovery

Purification fold was calculated as the ratio of specific activity of the final extract to that of the initial extract. Enzyme recovery was calculated as the ratio of total final enzyme units to that total initial enzyme units.

Characterization of 5'-Phosphodiesterase

5'PDE was characterized for optimum pH, Optimum temperature, pH stability, thermostability, effect of metal ion and kinetic study.

Determination of optimum pH for activity

The optimum pH for activity was determined in the pH range from 3.0 to 10.6. 50 mM acetate buffer was used in the pH range of 3.0 to 5.5; 50 mM Phosphate buffer for pH range range 5.8 to 8.0; whereas 50 mM Tris-chloride buffer was used in the pH range of 8.6 to 10.6.Assay was carried out with 0.1 ml of appropriately diluted enzyme at 60°C for incubation period of 10 minutes.

Determination of optimum temperature for activity

For determination of optimum temperature for activity of the enzyme, the assay was carried out by using appropriately diluted 0.1 ml enzyme solution, for the incubation temperature of 30°C to 100°C at optimum pH 6.0 with an incubation period of 10 minutes.

Determination of optimum pH for stability

For optimization of pH for stability of enzyme buffer solution of pH range from 3.0 to 10.6 were prepared; 50 mM acetate buffer was used in the pH range of 3.0 to 5.5; 50 mM Phosphate buffer for pH range range 5.8 to 8.0; whereas 50 mM Tris-Cl buffer was used in the pH range of 8.6 to 10.6, then the partially purified enzyme was added to each buffer solution in 1:1 proportion. These solutions were kept in stand for 24 hr. at room temperature (28 c); assay was carried out for each buffer solution containing enzyme preparation by using 0.1 ml of appropriately diluted enzyme at 60°C for incubation period of 10 minutes.

Determination of Thermostability

Enzyme preparation was incubated in water bath at 60°C for the period of 10 minutes to 200 minutes and for 4°C in refrigerator for the period of 1 day to 30 days. After each time duration 0.1 ml of appropriately diluted enzyme was assayed by assay procedure as mentioned earlier at pH 6.0, temperature 60°C for incubation period of 10 minutes.

Effect of metal ions and other compounds

The effect of various metal ions like Mg^{++}, Zn^{++}, Cu^{++}, Mn^{++} and EDTA on enzymatic activity was carried out by adding different concentration 1, 5 and 10 mM. The volume was taken in reaction mixture described in the assay and the assay was carried out at 60°C and pH was taken 6.0 with an incubation period of 10 minutes.

Optimization of substrate concentration

Substrate, bis-(p-nitro phenyl phosphate) disodium salt, in the range of 0.05 mM to 10 mM was prepared in 50 mM acetate buffer (pH 6.0).Then the assay was carried out as mentions earlier by using 0.1 ml substrate,0.1 ml of diluted enzyme at optimum pH 6.0 and optimum temperature 60 °C with an incubation period of 10 minutes.

Kinetic study of partially purified 5'-PDE

Line weaver-Burke plot was plotted to determine the Km and Vmax.

RESULTS AND DISCUSSION

Mung bean presoaked for 4-5 hr and germinated at room temperature 27°C in closed chamber showed fungal growth after 4 days. Hence germination was not continued beyond 4 days.

The enzymatic activity was assayed every day after grinding the germinated mung bean in acetate buffer 5.0, centrifugation and filtration. The enzymatic activity was increased markedly with increasing period of germination. The crud extract of 3 days germinated mung bean (before dialysis) shows enzymatic activity of 1622 U/ml. The results are tabulated in Table-1.

The crude extracts from germinated mung bean after 3 days of germination showing 5'-PDE enzyme activity of 1622 U/ml and PME 2182 U/ml .Hence the ratio of PDE:PME was found to be 0.56.The protein content was found to be 1.325 mg/ml and hence the specific activity of enzyme of 5'-PDE was 1224 U/mg. The presence of PME which is contaminating enzyme associated with 5'-PDE need to be purified for highest specific activity and increased PDE: PME ratio.

Although ammonium sulphate precipitation is widely used technique for purification of enzymes, no literature was found on ammonium sulphate precipitation as a purification step for 5'-PDE from germinated mung bean. Ammonium sulphate was added to the filtrate for 0-20%, 20-40%, 40-60%, 60-80% and 80-100% and 80-100% saturation and the fraction precipitates obtained after centrifugation at each level of saturation were collected and dissolve individually in the minimum amount of 50mM acetate buffer solution (pH 5.0).This precipitates was then dialysed against 50mM acetate (5.0) buffer (pH 5.0) and then assayed for the enzyme activity.

The precipitate collected after 60-80% saturation shows the maximum 5'-PDE activity of 16253U/ml and shows the PME activity of 10940 U/ml, the specific activity for PDE 7656 U/mg and for PME 5153 U/mg. The maximum enzyme recovery of 60.12% and 6.25 fold purification could be achieved. Also the increased ratio of PDE: PME up to 1.48 was obtained.

Thermolabile enzymes that degrades RNA can be reportedly removed by giving a heat shock at 60-65° C .Hence in the present work Heat shock treatment was given to the enzyme solution (after ammonium sulphate precipitation and dialysis) for 15 min at 60°C and then the enzyme was assayed for the specific activity for each fraction.

The enzyme solution in fraction of 60-80% saturation shows the maximum PDE activity of 16201 U/ml and PME activity of 2930 U/ml, the maximum enzyme recovery was obtained 57.93%.The specific activity for enzyme 5'-PDE was 7849 U/mg and for PME was 1420U/mg obtained and 6.41 fold purification could be obtained for enzyme 5'-PDE, with the improved ratio of PDE: PME 5.53 was achieved which indicates that heat shock treatment given to fraction collected from ammonium sulphate precipitation is worthful technique to partially purify the 5'-Phosphodiesterase with low content of Phosphomonoesterase.

The enzyme solution of 60-80% saturation of ammonium sulphate precipitation and the heat shock treatment was taken for the further characterization of the enzyme 5'-PDE. Optimization of pH for PDE was carried out for the pH range of 3 to 10.6.Table-2 and Fig.2 explain the result for the optimization of pH for 5'-PDE.

The graph shows the enzymatic activity increases with increase in pH from 3-6; at the pH 6.0 the enzymatic activity of PDE was maximum and found to be 16198 U/ml. After the pH 6.0 the enzymatic activity decreases and at pH 10.6 it destroyed drastically.

Hence optimum pH was found to be 6.0.It means that at pH 6.0 enzymes remains in its native confirmation. The groups in active site of 5'-PDE are available in the dissociated form for reaction with substrate. So further study was carried out at 6.0.

Table-3 and Fig.3 explains the result of the optimum pH for stability of 5'-PDE.Maximum enzymatic activity was found in the pH range from 4 to 7 .In this range more than 80% of the original activity was retained after 24 hr at room temperature.While at pH 7.6 and pH 10,15% and 95% of the original activity was lost respectively during 24 hr and at pH 10.6 enzymatic destroyed completely.

Table-4 and Fig.4 illustrates the optimization of temperature for 5'-PDE. As the temperature increased from 30° to 60° C, enzyme activity increased progressively. Above 60°C enzymes activity decreased gradually up to 95°C and at 100°C only 72 U/ml activities was found. Thus the optimum temperature for activity was found to be 60°C.

Table 5a, 5b and Fig. 5a, 5b illustrates the effect of temperature on enzymatic activity for 5'-PDE preparation at 60° C for different periods of time up to 200 minutes and at 4°C, the enzyme showing good stability even after 30 days the enzyme retained 67% activity.

These observations indicate the possibility that the enzyme after complete purification may be used for structural studies on nucleic acids under conditions where part of the helical structure start to melt.

Table-6 and Fig.6 explains the effect of different metal ions on enzyme activity of 5'-PDE.Of various divalent metal ions examined, 5 mM Mg^{++} stimulated the enzyme activity up to 147%. 1mM and 10mM Mg^{++} stimulated activity by 109% and 127%respectively. But the other entire metal ion caused slight inhibition even at 1 mM. EDTA inhibited the enzyme activity drastically. The activity with 10mM DETA was loosed up to 91%.

For the determination of the optimum substrate concentration of partially purified 5'-PDE synthetic substrate, bis-(p-nitrophenyl phosphate) disodium salt was added in the concentration range from 0.05mM to 12mM.Table-7 and Fig.7 indicated that as the substrate concentration was increased above 0.05mM, rate of reaction increased progressively up to 1mM. When concentration increased above 1mM, the rate of reaction remained constant suggesting that 5'-PDE obtained from germinated mung bean followed the Michaelis –Menten equation. Hence, optimum substrate concentration was found to be 1mM, justifying that at this concentration enzyme remained saturated with substrate for 10 minutes and rate of reaction was maximum at this concentration.

K_m and V_{max} was determined from the Lineweaver Burke plot as shown in fig. K_m was found to be 0.48 mM, where as V_{max} 19230 nm/min. It shows good affinity of 5'-PDE for synthetic substrate.

CONCLUSION

It can be concluded that Mung bean (Phaseolus mungo) is good source of 5'-Phosphodiesterase and phosphomonoesterase was associated with 5'-PDE.

With the two simple and low cost ingredients step of purification, 5'-phosphodiesterase can be partially purified with 6.41 fold purification which reduces overall cost of purified enzyme. The highest PDE: PME ratio could be achieved by heat shock treatment after ammonium sulphate precipitation. 5'-phosphodiesterase showing the good range of pH for stability and having thermo stability property, with good affinity for synthetic substrate (k_m 0.48).This enzyme could find promising application of hydrolysis of RNA to flavor nucleotides.

ACKNOWLEDGEMENT

Authors are very much thankful to Dr. (Mrs.) S.S. Bhalerao, Principal of Hyderabad Sindh National Collegiate Boards (HSNCB's) Dr.L.H.Hiranandani College of Pharmacy, Ulhasnagar for her continuous support and encouragement

TABLES AND GRAPH

Table - 1: Optimization of germination period for maximum activity

Sr. No.	Germination period (Days)	Activity (U/ml)*
1	1	1035 ± 11
2	2	1249 ± 14
3	3	1622 ± 24

Table -2: Optimization of pH for enzyme activity

Sr. No.	pH	Activity (U/ml)*	% Relative Activity
1	3	6010 ± 25	37.10
2	4	6957 ± 19	42.95
3	4.5	8414 ± 17	51.94
4	5	12016 ± 9	74.18
5	5.5	12860 ± 28	79.39
6	5.8	13827 ± 25	85.36
7	6	16198 ± 48	100.00
8	6.8	10602 ± 23	66.45
9	7	10123 ± 17	62.50
10	7.6	10037 ± 18	61.96
11	8	8247 ± 8	50.91
12	8.6	7930 ± 9	48.90
13	9	6231 ± 12	38.47
14	10	2135 ± 4	13.18
15	10.6	182 ± 4	1.12

Table -3: Optimization of pH for stability

SR. NO.	pH	Activity (U/ml)*	% Relative Activity
1	3	10070 ± 12	62.16
2	4	12317 ± 15	76.03
3	4.5	14376 ± 27	88.74
4	5	16190 ± 46	100.93
5	5.5	16059 ± 26	99.12
6	6	15866 ± 12	97.93
7	6.8	15247 ± 9	94.11
8	7	13731 ± 11	84.76
9	7.6	11167 ± 23	68.93
10	8	7457 ± 18	46.03
11	8.6	3414 ± 9	21.07
12	9	1651 ± 9	10.19
13	10	860 ± 7	5.31
14	10.6	-	-

Table -4: Optimization of temperature enzymes at 60° C

Sr. No.	pH	Activity (U/ml)*	% Relative Activity
1	30	6167 ± 18	38.07
2	40	8102 ± 25	50.02
3	45	9204 ± 18	56.82
4	50	11183 ± 24	69.04
5	55	14102 ± 18	87.05
6	60	16199 ± 22	100.00
7	65	12817 ± 9	79.12
8	70	12151 ± 24	75.01
9	75	11656 ± 23	71.96
10	80	10382 ± 18	64.09
11	85	8414 ± 17	51.94
12	90	4220 ± 18	26.05
13	95	194 ± 16	1.20
14	100	72 ± 7	0

Table-5b: Determination of thermostability of enzymes at 4° C

Sr. No.	pH	Activity (U/ml)*	% Relative Activity
1	1	16070 ± 25	99.20
2	2	15667 ± 19	96.71
3	3	15222 ± 17	93.96
4	5	15086 ± 9	93.12
5	7	14419 ± 28	89.01
6	10	13753 ± 25	84.90
7	30	10876 ± 28	67.14

Table-5a: Determination of thermostability of for enzyme activity

Sr. No.	pH	Activity (U/ml)*	% Relative Activity
1	10	16188 ± 19	99.52
2	20	16122 ± 58	99.51
3	30	14914 ± 25	92.06
4	40	14516 ± 28	89.60
5	60	13731 ± 9	84.75
6	80	10758 ± 28	66.40
7	120	7844 ± 19	48.41
8	140	3452 ± 16	21.30
9	160	360 ± 19	2.22
10	200	0	0

Table -6: Effects of metal ions and other compounds on activity of 5'-PDE

Sr. No.	Concentration (mM)	Relative Activity (%)				
		Mg^{++}	Cu^{++}	Zn^{++}	Mn^{++}	EDTA
1	1	109	85	84	90	42
2	5	147	57	82	87	34
3	10	127	45	60	65	9

Table -7: Optimization of substrate concentration

Sr. No.	Substrate concentration (mM)	Activity (U/ml)*	Sr. No.	Substrate concentration (mM)	Activity (U/ml)*
1	0	0	10	1	15262 ± 19
2	0.05	2124 ± 25	11	1.5	15321 ± 16
3	0.1	3296 ± 19	12	2	15423 ± 9
4	0.15	4376 ± 25	13	3	15642 ± 12
5	0.2	6344 ± 21	14	4	15716 ± 16
6	0.3	10263 ± 19	15	5	15845 ± 19
7	0.5	11333 ± 9	16	7	15923 ± 16
8	0.75	13489 ± 41	17	10	16200 ± 19
9	0.8	13785 ± 25	18	12	16254 ± 16

*Values are average ± SD of three observations

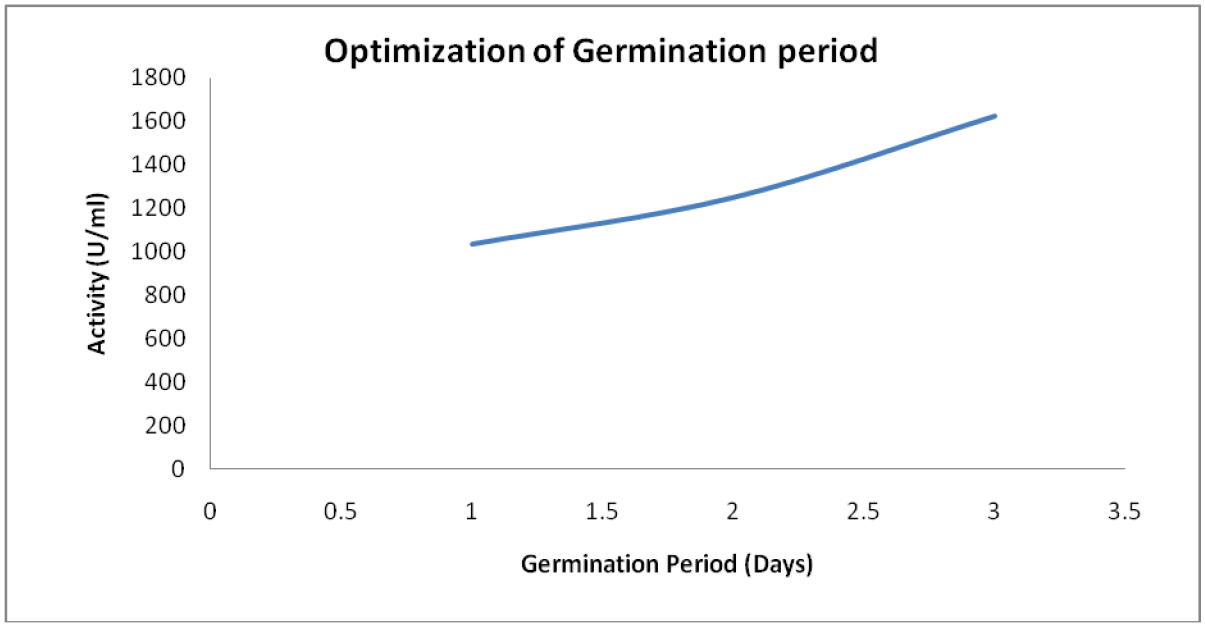

Fig.1: Optimization of germination period for maximum activity

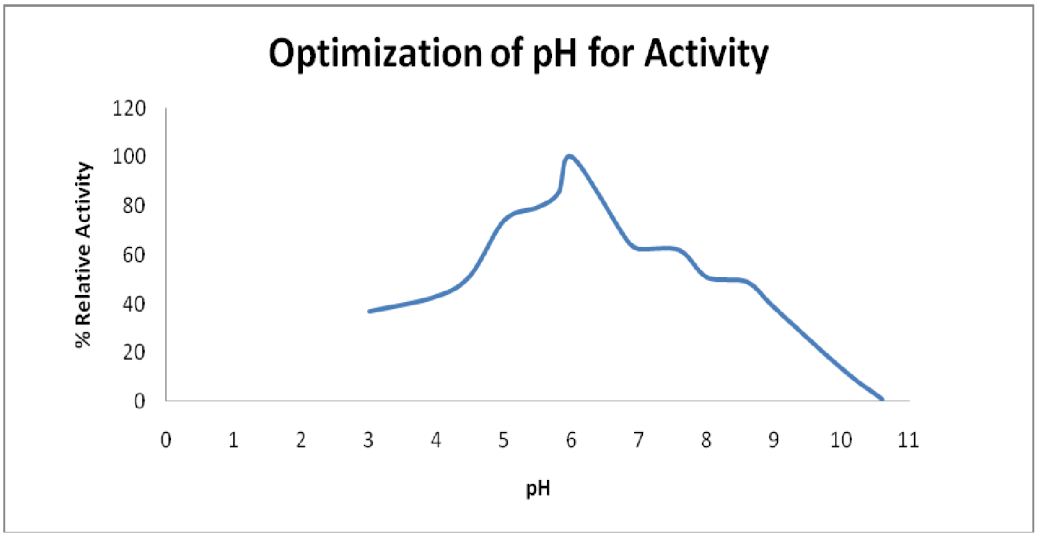

Fig-2: Optimization of pH for enzyme activity

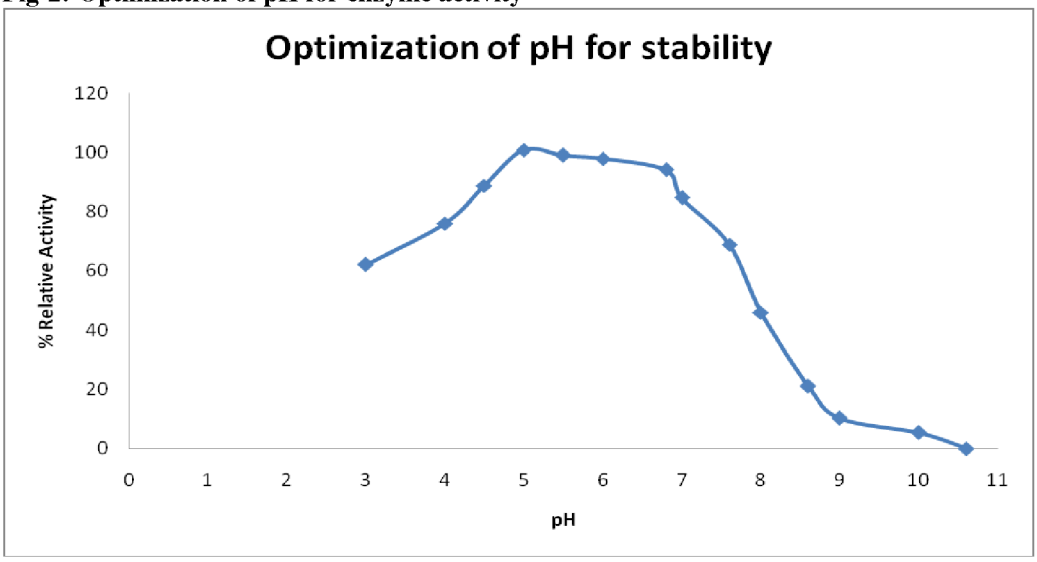

Fig-3: Optimization of pH for stability

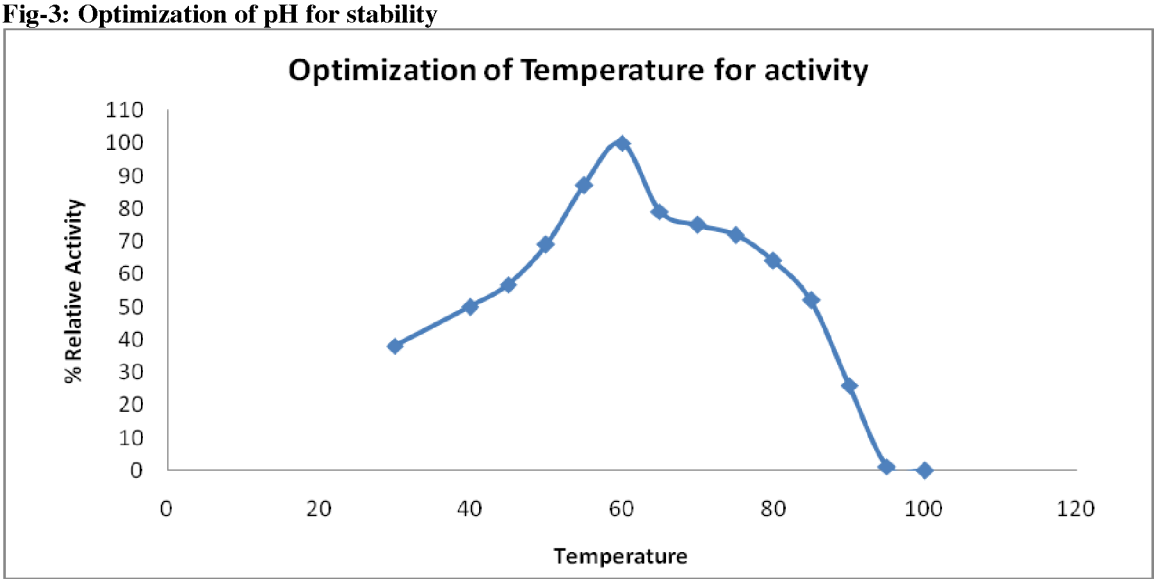

Fig-4: Optimization of temperature for enzyme activity

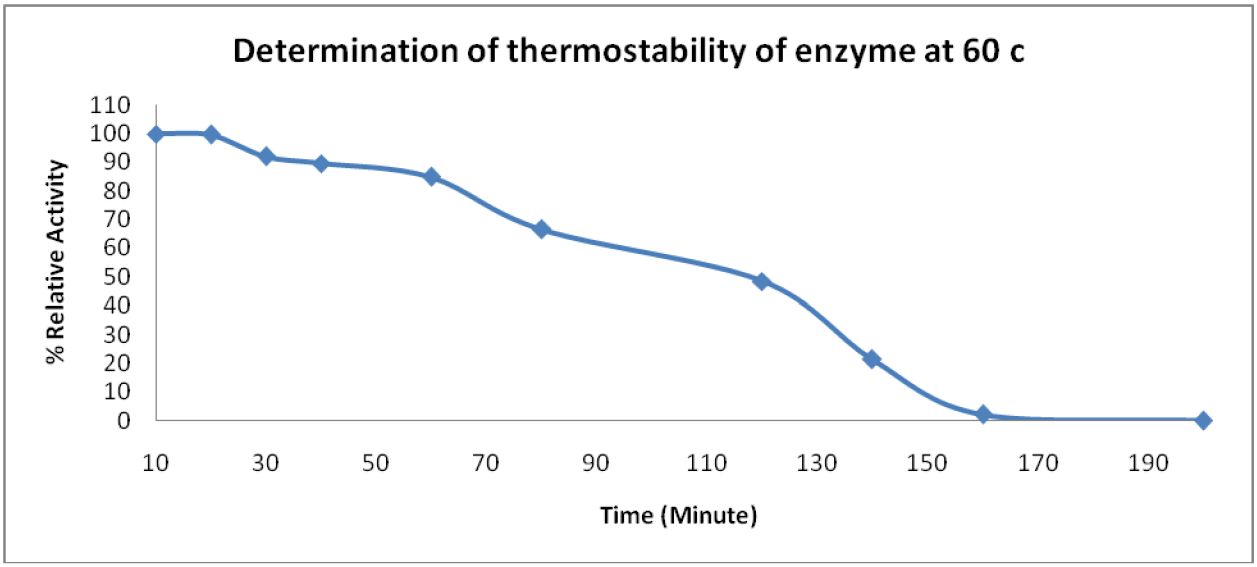

Fig. 5a: Determination of thermostability of enzymes at 60° C

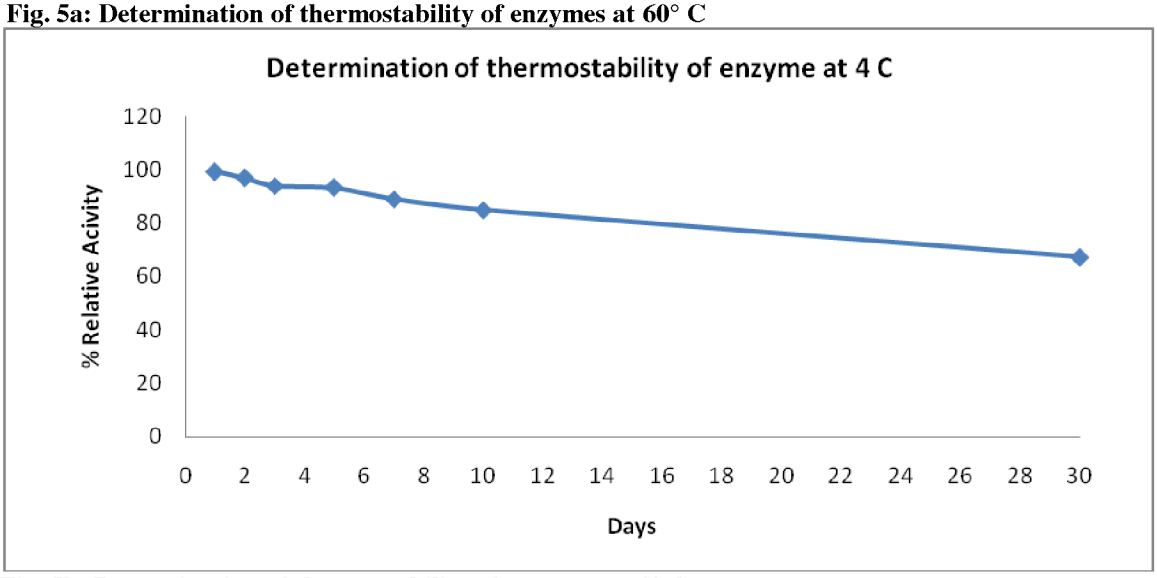

Fig. 5b: Determination of thermostability of enzymes at 4° C

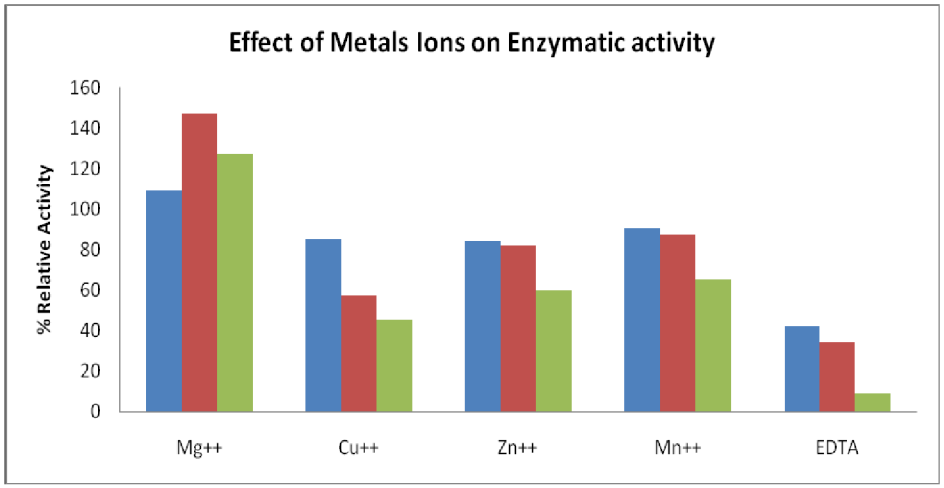

Fig. 6: Effects of metal ions and other compounds on activity of 5'-PDE

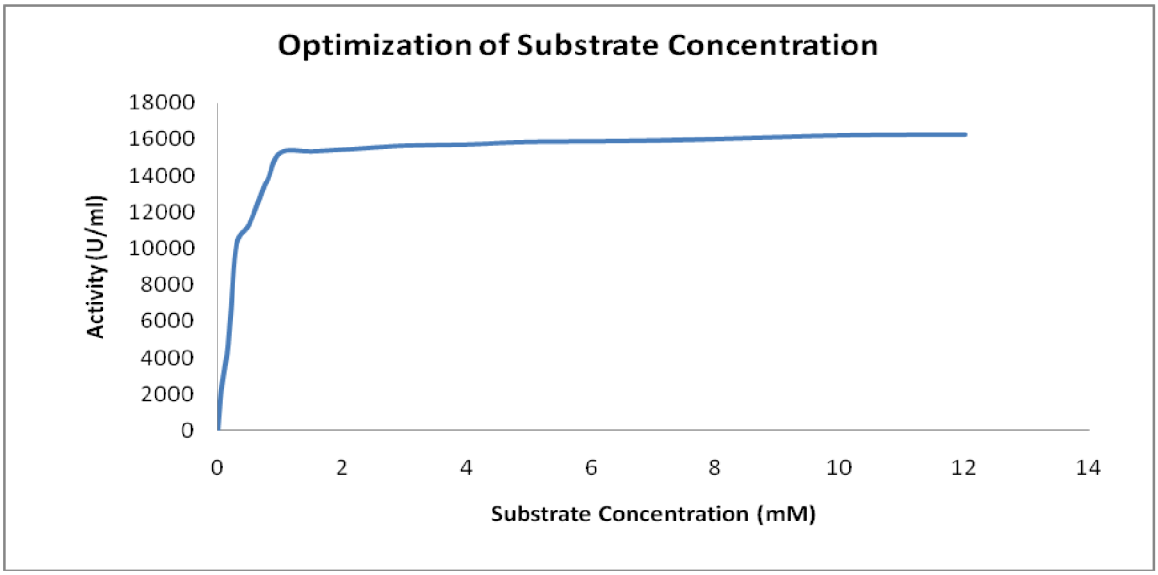

Fig. 7: Optimization of substrate concentration

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***Corresponding Author**: Mr.Nilesh Mahadeo Khutle,
Lecturer in Pharmacy,
Dr.L.H.Hiranandani College of Pharmacy,
Smt.CHM Campus, Opposite Railway Station,
Ulhasnagar, Dist.Thane, Pin-421003,
Maharashtra (India)
Telephone No. (0251)2561463
Fax No. (0251)2733693
Mobile No. +91 9702133634
Email ID: nilesh.bio@gmail.com