

Comparative analysis of the effect of different incubation periods, pH and temperature conditions on acid phosphatase activity of *Zea mays*, *Phaseolus vulgaris* and *Vigna mungo*

Prabhjot Kaur Khatkar* and Shelly Gupta¹

Department of Biochemistry, DAV University, Jalandhar, Punjab, India

¹School of Bio-engineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India

shelly.24846@lpu.co.in

Abstract

Acid phosphatases are the class of enzymes that are ubiquitously found throughout the plant and animal kingdom. Plant acid phosphatases play a crucial role in all tissues of the plant. They are activated at low pH and help in procuring inorganic phosphate from organic sources. Role of acid phosphatases in optimal seed germination enables better regeneration efficacy and plant development. Various parameters like pH and temperature maximize the activation of acid phosphatases in germinating seeds thereby culminating in optimal time and development of seedlings. In the current study, we aim to characterize the effect of different pH and temperature conditions on acid phosphatases isolated from three commercially important crops. This study would help in understanding the ideal conditions that would optimize seed germination

Introduction

Acid phosphatases are ubiquitous in nature and occur in numerous organisms and tissues. Although it is involved in catalysis of simple hydrolytic reactions, but its role in multiple physiological functions makes it an important system to study [1]. It is used by plants to procure inorganic phosphate from organic sources in soil under conditions of phosphate deprivation or other stress [2]. Moreover, it can degrade harmful pesticides found in soil [3].

Acid phosphatases show wide distribution among all living systems ranging from lower to higher organisms. A specific class of APases called purple acid phosphatases (PAP) are expressed in various plant parts, like in *Pisum sativum* cell wall [4] aphloem of *N. tobaccum* etc. Acid phosphatases are the family of enzymes that catalyze the hydrolysis of phosphate monoesters under acidic conditions of pH ranging between 4–7 [5]. Dysregulation of their function is associated with many diseases.

Depending on the optimum pH conditions, phosphatases can be classified as acid and alkaline phosphatases. Acid phosphatases are those which work below the pH 7 whereas alkaline phosphatases work above the pH 7 [5]. Most APases are active under low pH conditions. For example, AtPAP26 from *A. thaliana* is active within a pH range between 4.7–6.6 [6].

pH 7 has been observed to be optimum for immobilized acid phosphatase activity, whereas pH 5.0 is the optimum pH for soluble acid phosphatase. Further, at alkaline pH, immobilized acid phosphatase was shown to retain 40% relative activity, whereas for soluble enzyme, activity begins to decline at pH beyond 5.0 [7]. In most of the isolates, optimum pH has been observed to be 5.8 for acid phosphatase production for 67 % cases and for the remaining 33%, optimum pH observed was 4.8 [8].

Other than pH, thermal stability is another parameter which greatly affects enzyme activity. For example, studies on purified APases from *Artocarpus communis* showed a retention of 50 % of the activity at a temperature of 55 °C. However, at 37°C it showed stability and activity for approximately 150 min [9]. In a separate study, APases isolated from *Hypericum perforatum* showed stability and activity up to 80 °C whereas maximum activity was showed at 10°C [10]. These studies suggest that affect of temperature on enzyme activity is different for Apases isolated from different sources and need to be analysed separately for each species.

Difference in temperatures for optimal activity of the enzyme has also been observed for free and bound forms of the enzymes. For example, APases from a given source were found to be stable at 60°C whereas the temperature range for the immobilized enzyme was found to be broader. Enhanced stability can be attributed to the supporting matrix that can absorb some heat protecting the enzymes from the effects of high temperatures [11]. Tolerance range for an isolated APase in terms of its pH and temperature gives specific idea about the ideal conditions under which it can be optimally applied for industrial purposes.

In the present study, we aim to extract acid phosphatases from the seeds of three different plant species: *Zea mays* (maize), *Phaseolus vulgaris* (kidney bean) and *Vigna mungo* (black gram) and compare their activity under important parameters like pH and temperature.

Materials and Methods

Reagents: Sodium acetate (1M) stock solution, sodium hydroxide (0.2M) stock solution, 5ml of para-nitrophenyl solution (PNP, conc. 10mg/ml) stock solution, 1ml of para-nitrophenyl phosphate (PNPP) 0.5M stock solution, magnesium chloride (0.1M) stock solution, Biuret reagent.

Seeds used: *Zea mays* (maize), *Phaseolus vulgaris* (kidney bean) and *Vigna mungo* (black gram) which were commercially purchased and all the extractions were performed from the same batch.

Preparation of sodium acetate buffer: For making stock solution of 1M in 100ml, 8.2 gm of sodium acetate was weighed and dissolved in 100ml of distilled water and pH was adjusted to 4.6 using glacial acetic acid.

Preparation of NaOH (0.2M): Weighed 0.8g of NaOH and dissolved in 100ml of distilled water.

Preparation of PNP standard curve: For preparing 5ml stock solution with concentration 10 mg/ml, dissolved 50mg of PNP in 5ml of distilled water. Using stock solution of 10mg/ml in 0.1M sodium acetate buffer, six different dilutions were prepared likewise 0.25mg/ml, 0.5mg/ml, 1mg/ml, 1.5mg/ml, 2mg/ml. The optical densities of PNP solutions were measured at 405nm using UV spectrophotometer and a standard curve was plotted.

Preparation of enzyme extract: 10 gm each of maize, kidney bean, black gram seeds were weighed and soaked separately in petri dishes overnight in distilled water. Using 0.01M sodium acetate, enzyme extract was prepared and further it was centrifuged at 10,000 rpm for 10min at 4°C. Supernatant was collected and subjected to further analysis.

Preparation of biuret reagent: 1.5gm of copper sulphate, 4.5gm of sodium potassium tartrate was dissolved in 250ml of 0.2N sodium hydroxide solution. 2.5gm of potassium iodide was added in this solution and volume made up to 500ml with 0.2N sodium hydroxide.

Protein estimation by using biuret reagent: Prepared 10mg/ml stock solution of BSA (standard protein). Using stock solution, BSA dilutions were made as 0.1mg/ml, 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1mg/ml. The OD at 540nm was measured and standard curve was prepared. Three test samples using enzyme extracts were prepared in different test tubes

marked as T1 (maize), T2 (kidney bean), T3 (black gram). 1.5ml of biuret reagent was added in 1ml of test samples and incubated at 37°C for 10 min. OD was measured at 540nm.

Detection of acid phosphatase activity: After estimation of protein by biuret reagent, concentration of each sample was calculated and activity of phosphatases was detected in different plant species. To the reaction mix, 0.01M MgCl₂ and 0.05M PNP was added along with specific concentration of enzyme extract. Final volume was made to 1ml with acetate buffer and tubes were allowed it to incubate at 37°C for 15min. Following incubation, 2ml of sodium acetate buffer was added making volume of 3ml and again incubated at 37°C for 30min. Finally, 0.1M NaOH was added and measured OD at 405nm.

Analysis of effect of incubation period on acid phosphatase activity: Using different concentrations of 0.1mg/ml, 0.2 mg/ml and 0.5 mg/ml, enzyme extracts from all the three samples were incubated for different time periods; 0min, 5min, 10min, 15min and 30min. Following that activity was analyzed using the same procedure as described above and absorbance was measured.

Detection of acid phosphatase activity at different incubation temperatures: All the three enzyme extracts were taken at the same concentration of 0.1 mg/ml and reaction was prepared as described previously. Using same sample of each extract, different incubation temperatures of 4°C, 25°C, 37°C and 45°C were used and absorbance was measured

Detection of acid phosphatase activity at different pH range: As described above, enzyme activity was analyzed for all three extracts using different pH values; pH 3, pH 4.6, pH 5.5 and pH 8.2. Absorbance was measured at 405nm.

Results and discussions

1. Preparation of standard curve using PNP:

Using different concentration of PNP i.e. 0.25mg/ml, 0.5mg/ml, 1mg/ml, 1.5mg/ml and 2mg/ml, standard curve was plotted. Linear curve obtained with equation $y = 0.2759x - 0.0173$ and $R^2 = 0.994$.

Sr.no.	Vol. of PNP(ml)	Conc. of PNP(ml)	Acetate buffer(ml)	Incubate at 37°C for 30min	NaOH (μl)	OD at 450nm
Blank	0	0	3ml	37°C	200μl	0
1	1ml	0.25mg/ml	2ml	37°C	200μl	0.051

2	1ml	0.5mg/ml	2ml	37°C	200µl	0.134
3	1ml	1mg/ml	2ml	37°C	200µl	0.250
4	1ml	1.5mg/ml	2ml	37°C	200µl	0.376
5	1ml	2mg/ml	2ml	37°C	200µl	0.551

Table1: PNP Dilution Table

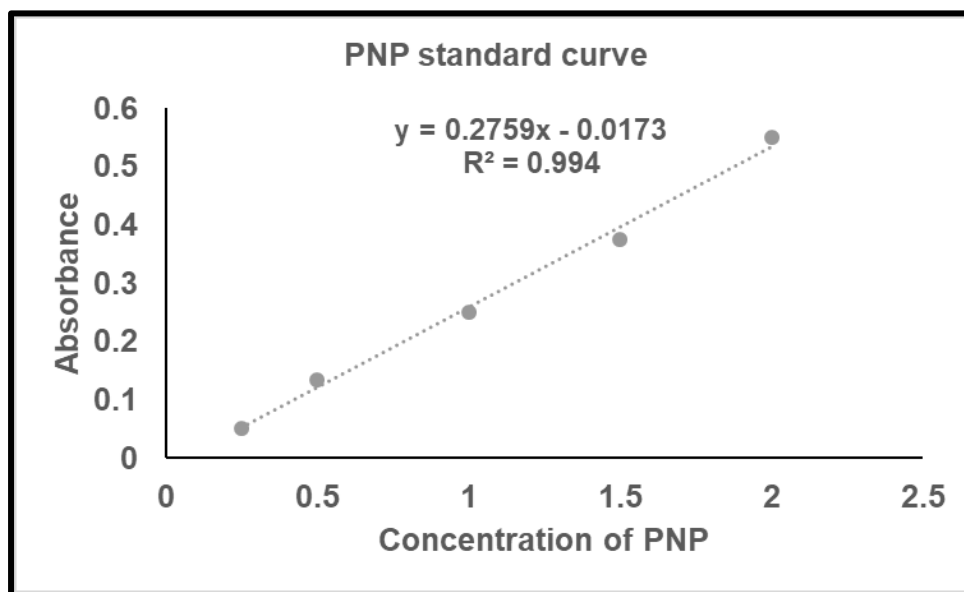


Figure1: Standard curve using PNP

2. Estimation of protein using biuret reagent:

BSA (standard protein) which was used for estimation of protein using biuret reagent. Different concentrations of BSA were prepared. Linear graph with equation $y = 0.8299x + 0.0627$ and $R^2 = 0.9913$

Sr.no	DDW(µl)	BSA(µl)	Conc.(mg/ml)	Biuret reagent(ml)	Incubate for 15min	OD at 540nm
Blank	1ml	0	0	1.5ml	37°C	0
S1	0	1ml	1mg/ml	1.5ml	37°C	0.859
S2	250µl	750µl	0.75mg/ml	1.5ml	37°C	0.716
S3	500µl	500µl	0.5mg/ml	1.5ml	37°C	0.504
S4	750µl	250µl	0.25mg/ml	1.5ml	37°C	0.267
S5	900µl	100µl	0.1mg/ml	1.5ml	37°C	0.125

Table 2: BSA dilution table for estimation of protein

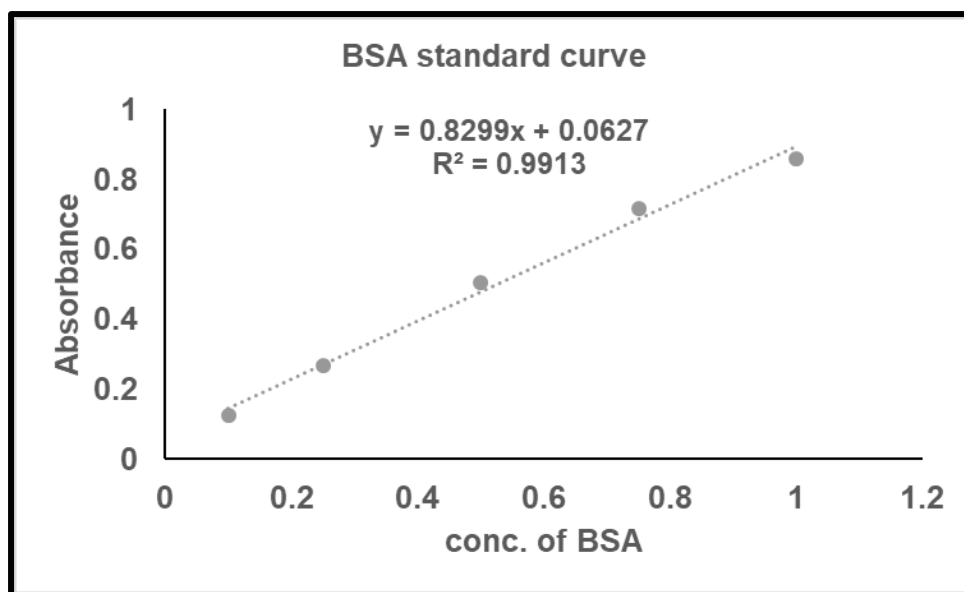


Figure2: BSA standard curve

3. Detection of concentration of protein in test sample using BSA standard equation:

Using standard equation of BSA, the unknown concentration of protein in the test sample was obtained by taking O.D. Further, the concentration of each test sample was obtained i.e. in maize 0.8544mg/ml, in kidney bean 1.244mg/ml, 1.784mg/ml.

Test	Volume of test sample(ml)	Biuret reagent(ml)	Incubate for 15min	OD at 540nm	Concentration obtained(mg/ml)
Maize	1ml	1.5ml	37°C	0.468	0.8544mg/ml
Kidney bean	1ml	1.5ml	37°C	0.343	1.244mg/ml
Black gram	1ml	1.5ml	37°C	0.460	1.784mg/ml

Table 3: Detection of concentration in test sample

4. Detection of concentration of enzyme extracts using PNPP as a substrate:

PNPP was used as a substrate which converts PNPP to PNP and Pi. In the same way, concentration of acid phosphatase was obtained by taking O.D. at 405nm. Out of three sample, kidney bean shows its highest activity.

Test	Conc. Of sample (mg/ml)	OD at 405nm
Blank	0	0
Maize	0.8544mg/ml	0.253
Kidney bean	1.244mg/ml	0.697
Black gram	1.784mg/ml	0.364

Table 4: Estimation of protein concentration

5. Detection of enzyme activity at different incubation period:

A. Maize(0.1mg/ml)

Different incubation period i.e 0min, 5min, 10min, 15min and 30min with concentration of 0.1mg/ml was taken corresponding to which activity of acid phosphatase was measured at 405nm. Out of these, maximum activity was observed at 30min with O.D. of 0.05

Incubation time(min)	OD (0.1mg/ml)
0min	0.052
5min	0.0525
10min	0.0475
15min	0.0575
30min	0.059

Table 5(A): Detection of enzyme activity at different incubation period in maize(0.1mg/ml)

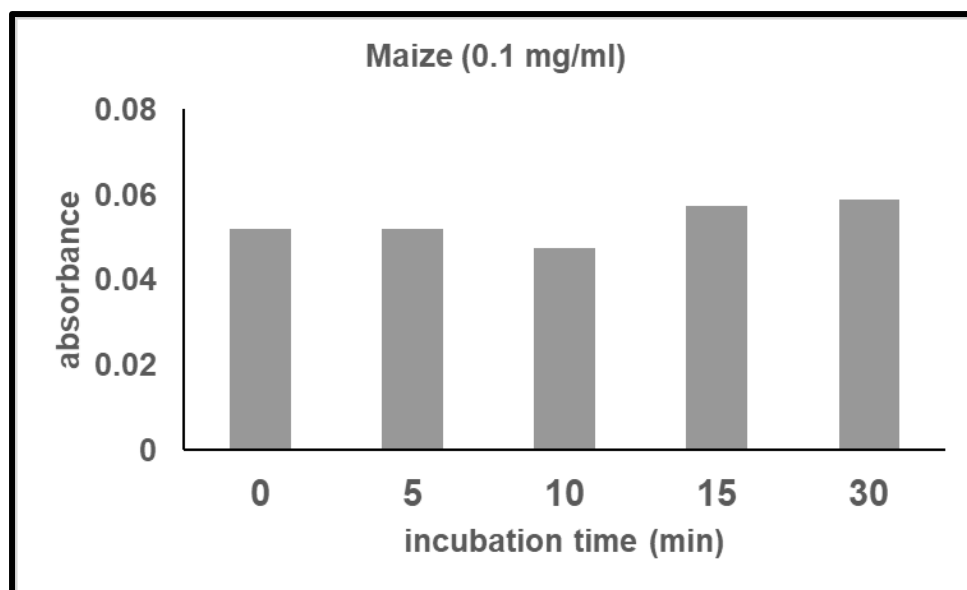


Figure 3: Graph showing absorbance at different incubation time in maize

B. Kidney bean(0.1mg/ml)

Different incubation period i.e 0min, 5min, 10min, 15min and 30min with concentration of 0.1mg/ml was taken corresponding to which activity of acid phosphatase was measured at 405nm. Out of these, maximum activity was observed at 30min with O.D. of 0.709.

Incubation time(min)	OD (0.1mg/ml)
0min	0.449
5min	0.462
10min	0.477
15min	0.532
30min	0.709

Table 5(B): Detection of enzyme activity at different incubation periods in kidney bean(0.1mg/ml)

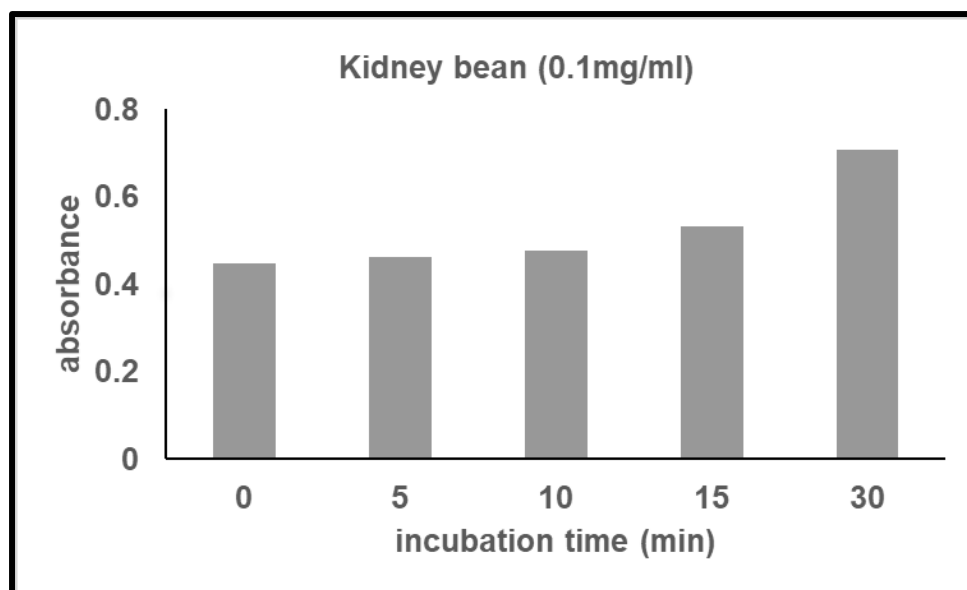


Figure 4: Graph showing absorbance at different incubation period in kidney bean

C. Black gram(0.1mg/ml)

Different incubation period i.e. 0min, 5min, 10min, 15min and 30min with concentration of 0.1mg/ml was taken corresponding to which activity of acid phosphatase was measured at 405nm. Out of these, maximum activity was observed at 30min with O.D. of 0.410.

Incubation time(min)	OD (0.1mg/ml)
0min	0.389
5min	0.383
10min	0.395
15min	0.391
30min	0.410

Table 5(C): Detection of enzyme activity at different incubation period in black gram(0.1mg/ml)

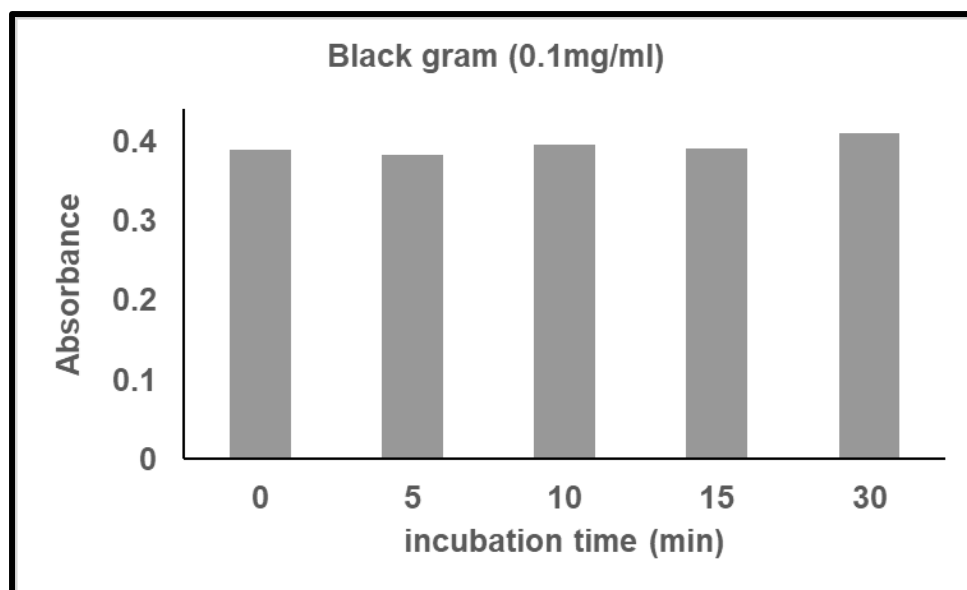


Figure 5: Graph showing absorbance at different incubation period in black gram

D. Comparative analysis of acid phosphatase at different incubation period in maize, kidney bean and black gram(0.1mg/ml)

Comparative analysis in case of three different samples were obtained by plotting a bar graph. Kidney bean was found to show maximum activity of acid phosphatase at incubation time of 30min.

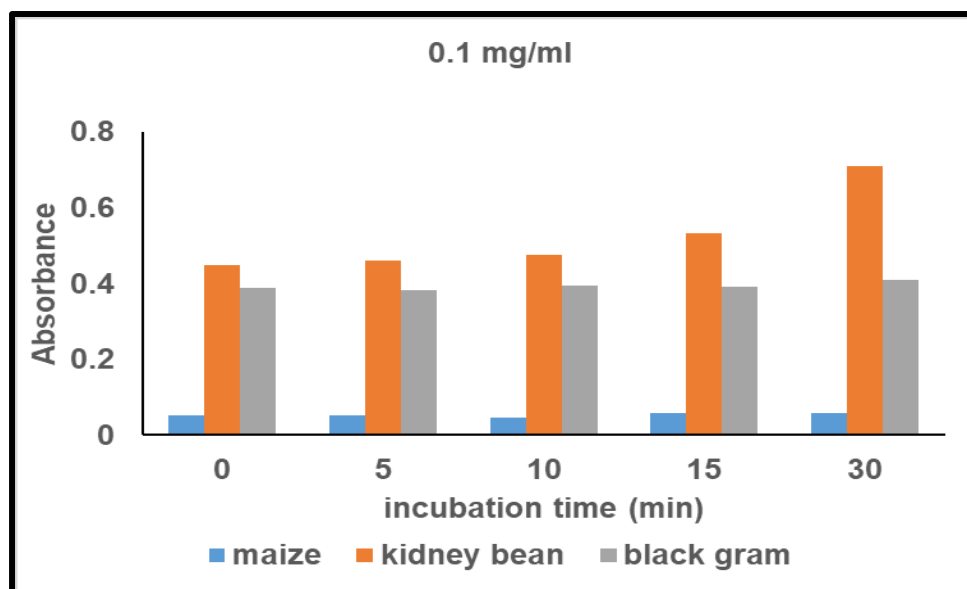


Figure 6. Comparative analysis of different incubation period with concentration 0.1mg/ml

E. Maize (0.2mg/ml)

At different incubation period i.e 0min, 5min, 10min, 15min, 30min with concentration of 0.2mg/ml was observed. 10min of incubation showed maximum activity of acid phosphatases with O.D. 0.031

Incubation time(min)	OD (0.2mg/ml)
0min	0.027
5min	0.025
10min	0.031
15min	0.024
30min	0.026

Table 5(D): Detection of acid phosphatase at different incubation time in maize(0.2mg/ml)

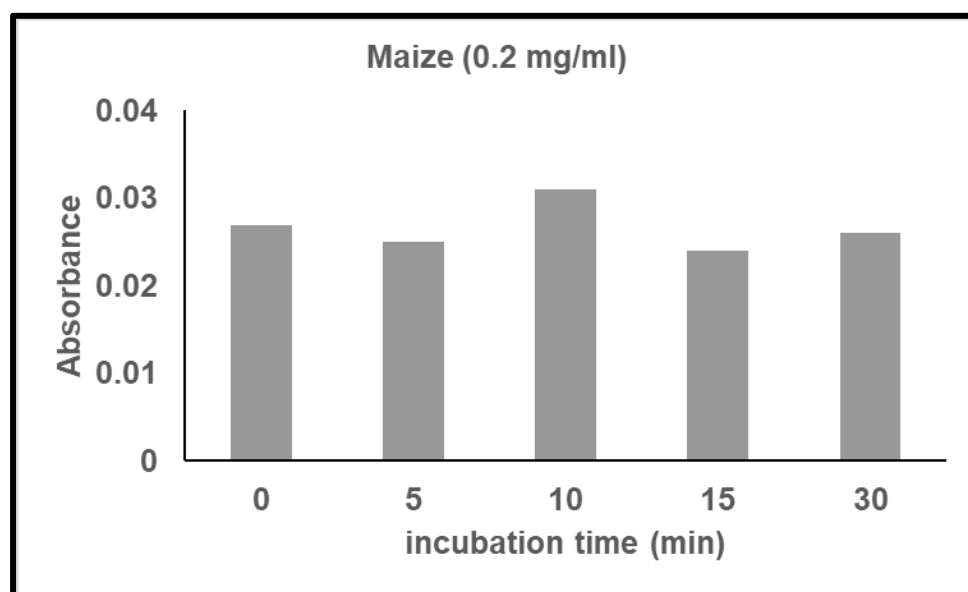


Figure 7: Graph showing absorbance at different incubation period in maize

F. Kidney bean (0.2mg/ml)

Enzyme activity was analysed at different incubation periods i.e 0min, 5min, 10min, 15min, 30min with concentration of 0.2mg/ml. 30min of incubation showed maximum activity of acid phosphatase with O.D 0.101.

Incubation time(min)	OD (0.2mg/ml)
0min	0.0465
5min	0.046
10min	0.054
15min	0.058
30min	0.101

Table 5(E): Detection of acid phosphatase at different incubation period in kidney bean(0.2mg/ml)

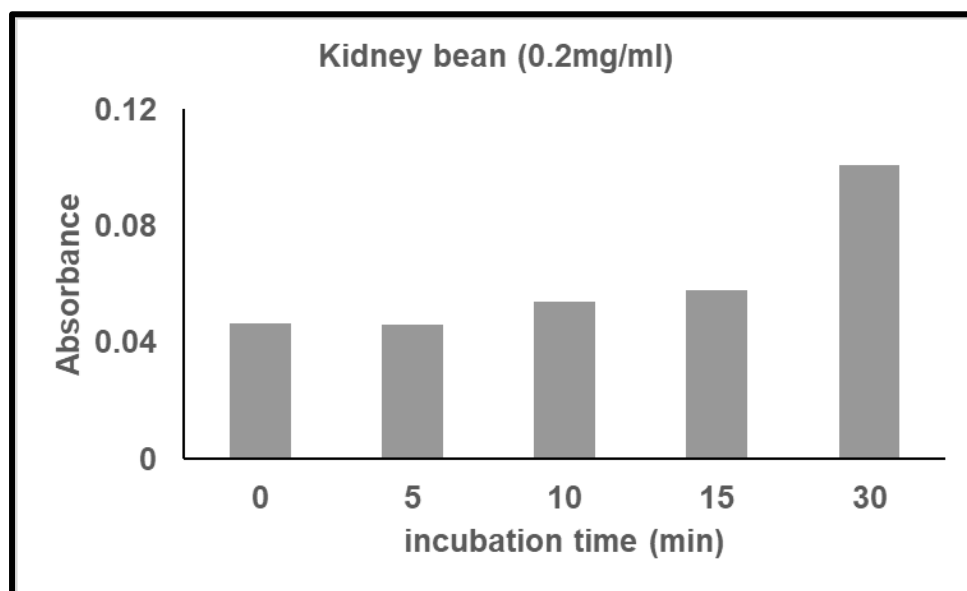


Fig 8: Graph showing absorbance at different incubation period in kidney bean

G. Black gram (0.2mg/ml)

At different incubation period i.e 0min, 5min, 10min, 15min, 30min with concentration of 0.2mg/ml was observed. 30min of incubation showed maximum activity of acid phosphatase with O.D 0.113.

Incubation time(min)	OD (0.2mg/ml)
0min	0.101

5min	0.113
10min	0.102
15min	0.111
30min	0.113

Table 5(F): Detection of acid phosphatase at different incubation period in black gram (0.2mg/ml)

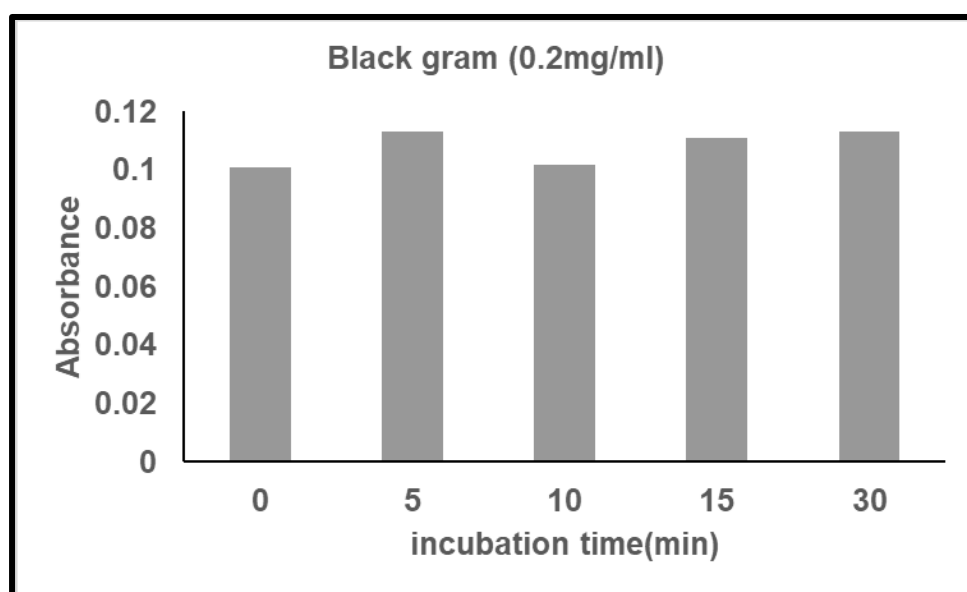


Fig 9: Graph showing absorbance at different incubation period in black gram

H. Comparative analysis of acid phosphatase at different incubation period in maize, kidney bean and black gram(0.2mg/ml)

Comparative analysis in case of three different samples with concentration of 0.2mg/ml were obtained by making graph. The graph showed black gram to have maximum activity of acid phosphatase with incubation time of 30min.

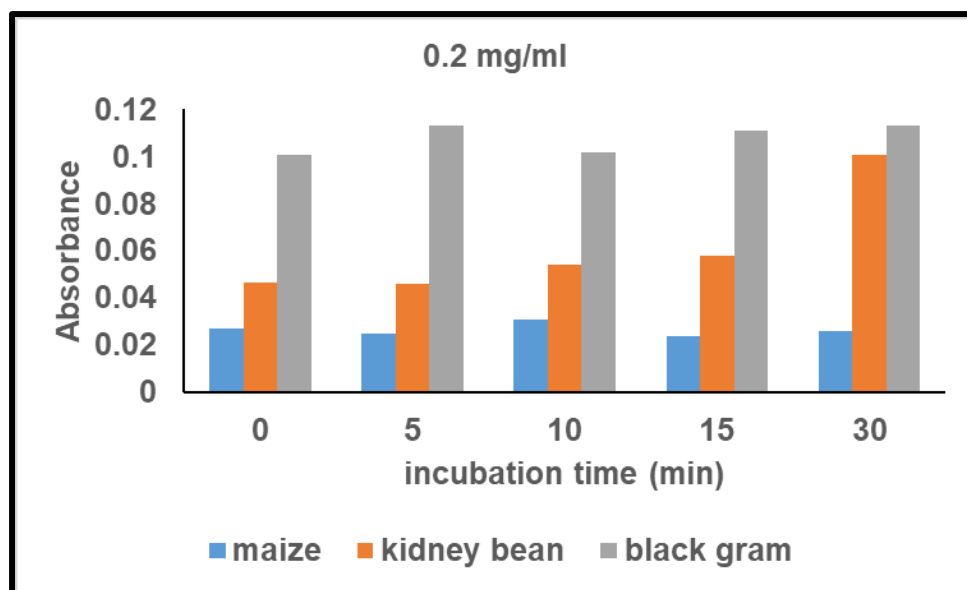


Fig 10: Graph showing absorbance at different incubation period

I. Maize (0.5mg/ml)

At different incubation period i.e 0min, 5min, 10min, 15min, 30min with concentration of 0.5mg/ml was observed. Of 30min of incubation show maximum activity of acid phosphatase with O.D 0.093.

Incubation time(min)	OD (0.5mg/ml)
0min	0.052
5min	0.066
10min	0.066
15min	0.07
30min	0.093

Table 5(G): Detection of acid phosphatase at different incubation period in maize (0.5mg/ml)

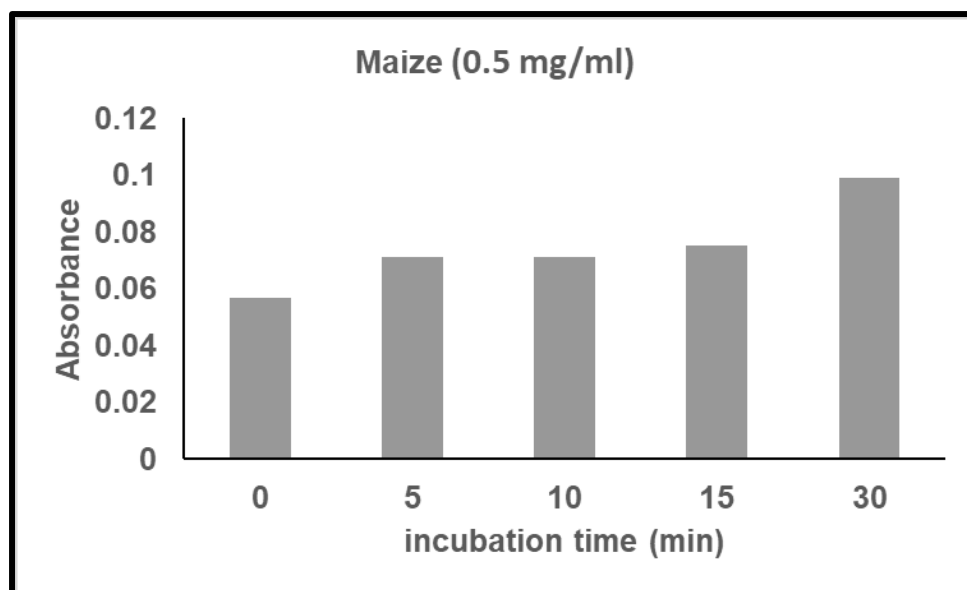


Fig 11: Graph showing absorbance at different incubation period in maize

J. Kidney bean(0.5mg/ml)

At different incubation period i.e 0min, 5min, 10min, 15min, 30min with concentration of 0.5mg/ml was observed. 30min of incubation period showed maximum activity of acid phosphatase with O.D 0.295.

Incubation time(min)	OD (0.5mg/ml)
0min	0.204
5min	0.189
10min	0.190
15min	0.175
30min	0.295

Table 5(H): Detection of acid phosphatase at different incubation period in kidney bean (0.5mg/ml)

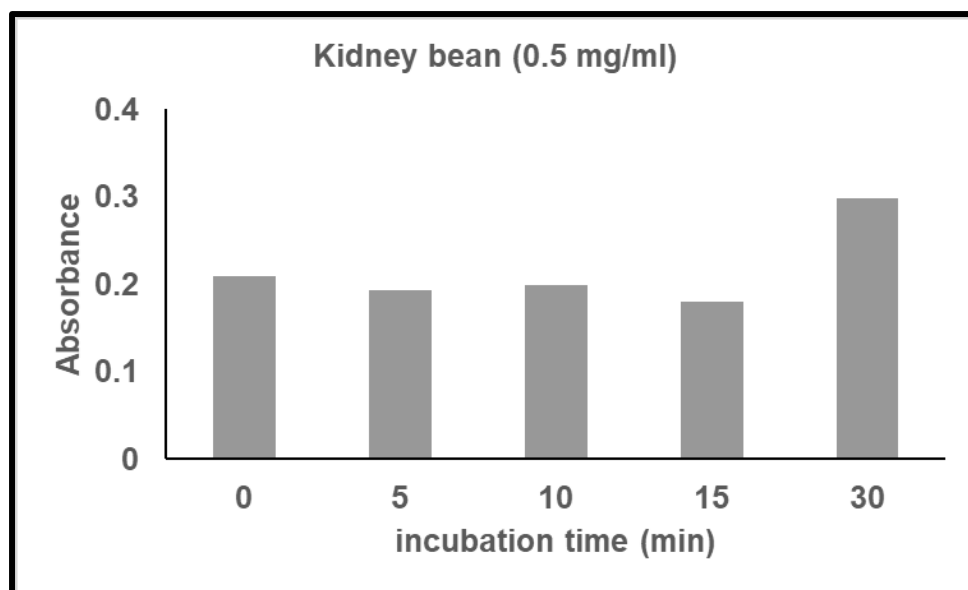


Fig 12: Graph showing absorbance at different incubation period in kidney bean

K. Black gram (0.5mg/ml)

At different incubation period i.e 0min, 5min, 10min, 15min, 30min with concentration of 0.5mg/ml was observed. 15min of incubation show maximum activity of acid phosphatase with O.D 0.468.

Incubation time(min)	OD (0.5mg/ml)
0min	0.218
5min	0.328
10min	0.337
15min	0.468
30min	0.369

Table5(I): Detection of acid phosphatase at different incubation period in black gram(0.5mg/ml)

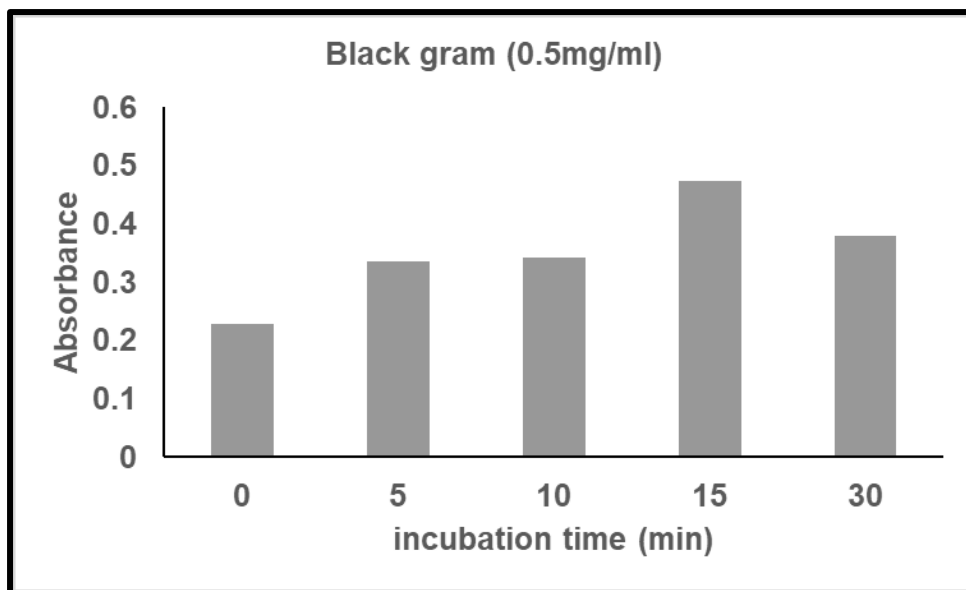


Figure 13: Graph showing absorbance at different incubation period in black gram

L. Comparative analysis of acid phosphatase activity at different incubation period with concentration 0.5mg/ml

Comparative analysis in case of three different samples with concentration of 0.5mg/ml were obtained by making graph. Results showed maximum activity of acid phosphatase in black gram with incubation time of 15min.

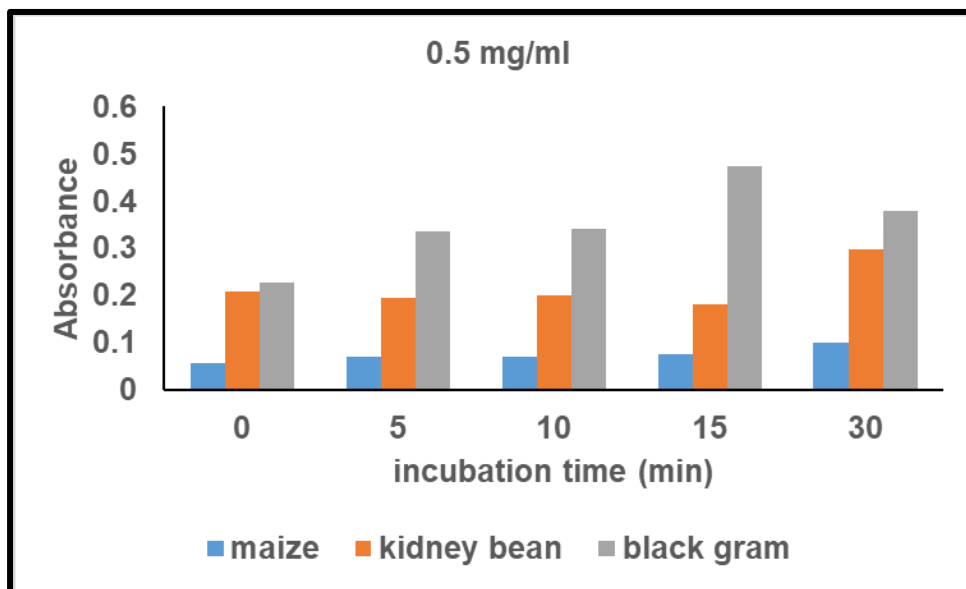


Figure 14: Graph showing absorbance at different incubation period

6. Detection of acid phosphatase at different temperature:
a) Maize (0.5mg/ml)

At different temperature i.e 4°C, 25°C, 37°C and 45°C with concentration of 0.5mg/ml was observed. It shows maximum activity of acid phosphatase at 45°C with O.D. 0.185.

Different temperature	OD (Maize)
4°C	0.139
25°C	0.147
37°C	0.138
45°C	0.185

Table 6(a): Detection of acid phosphatase at different temperature in maize (0.5mg/ml)

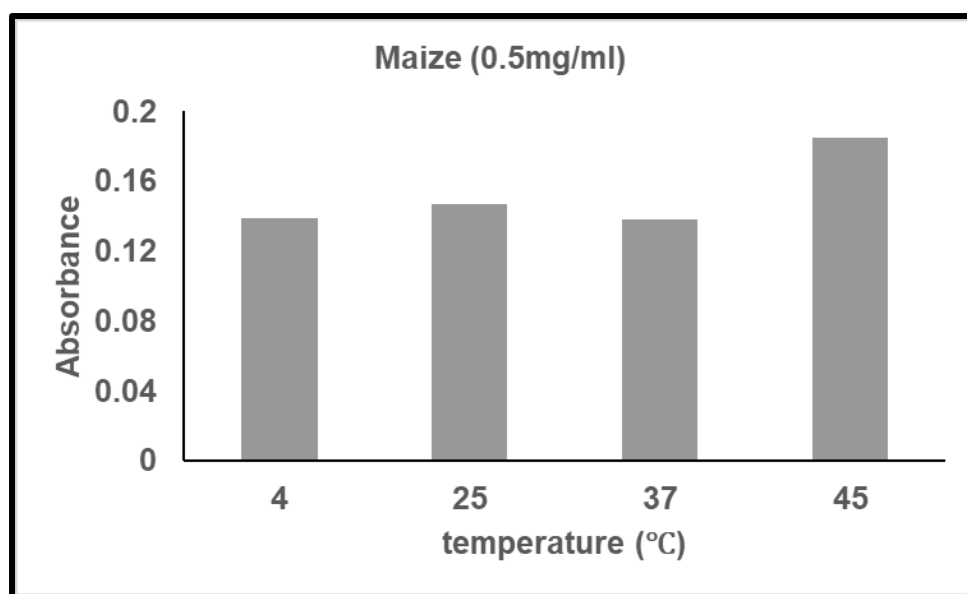


Figure 15: Graph showing absorbance at different temperature in maize

b) Kidney bean (0.5mg/ml)

Activity was analyzed at different temperature i.e 4°C, 25°C, 37°C and 45°C with concentration of 0.5mg/ml. It showed maximum activity of acid phosphatase at 45°C with O.D of 0.262.

Different temperature	OD (kidney bean)
4°C	0.153
25°C	0.186
37°C	0.182

45°C	0.262
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Table 6(b): Detection of acid phosphatase at different temperature in kidney bean(0.5mg/ml)

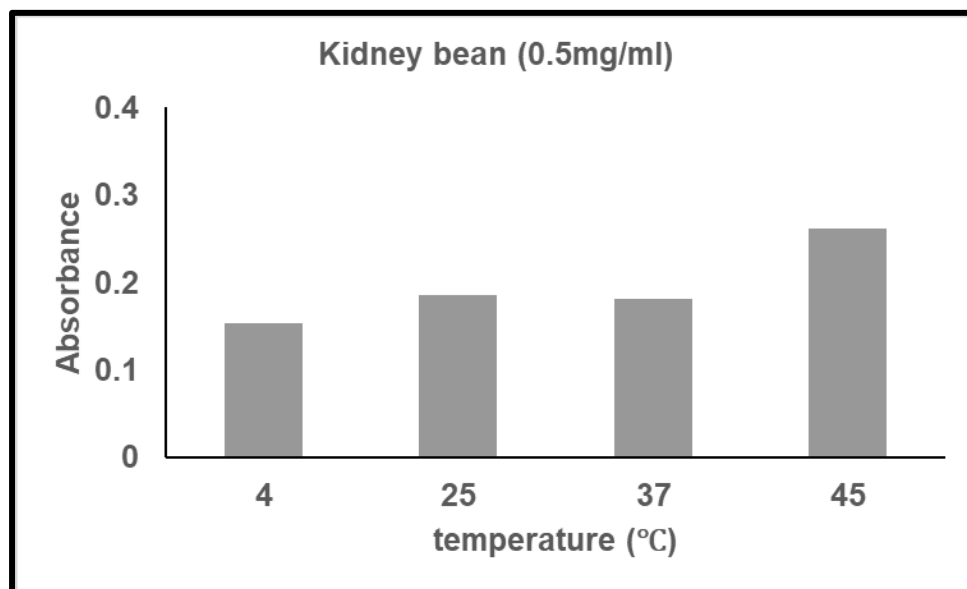


Fig 16: Graph showing absorbance at different temperature in kidney bean

c) Black gram (0.5mg/ml)

At different temperature i.e 4°C, 25°C, 37°C and 45°C with concentration of 0.5mg/ml was observed. It shows maximum activity of acid phosphatase at 4°C with O.D. 0.337.

Different temperature	OD (black gram)
4°C	0.337
25°C	0.234
37°C	0.284
45°C	0.218

Table 6(c): Detection of acid phosphatase at different temperature in black gram(0.5mg/ml)

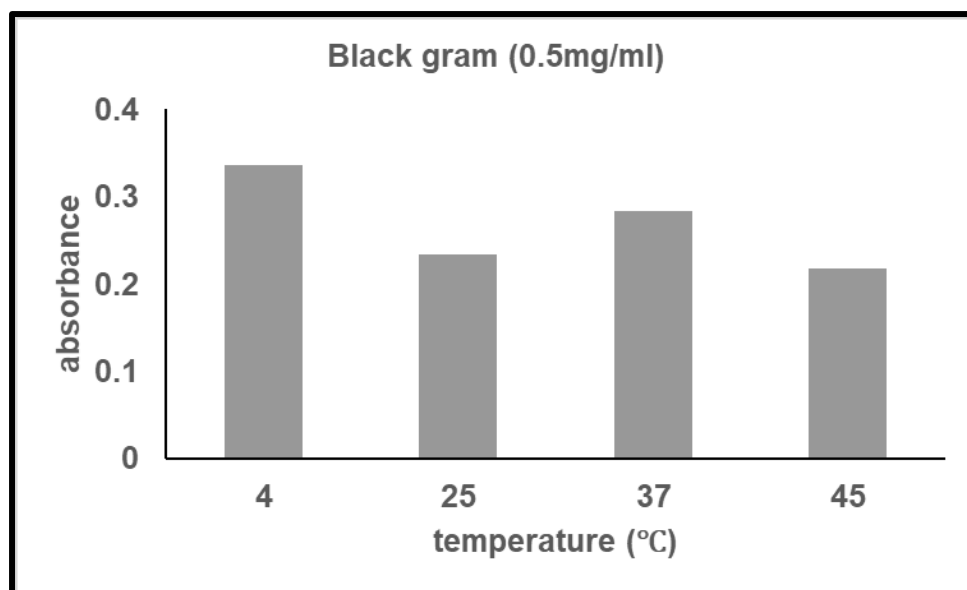


Fig 17: Graph showing absorbance at different temperature in black gram

d) Comparative analysis of acid phosphatase at different temperature in maize, kidney bean and black gram

Comparative analysis in case of three different samples with concentration of 0.5mg/ml were obtained by making graph. black gram showed maximum activity of acid phosphatase with temperature of 4°C.

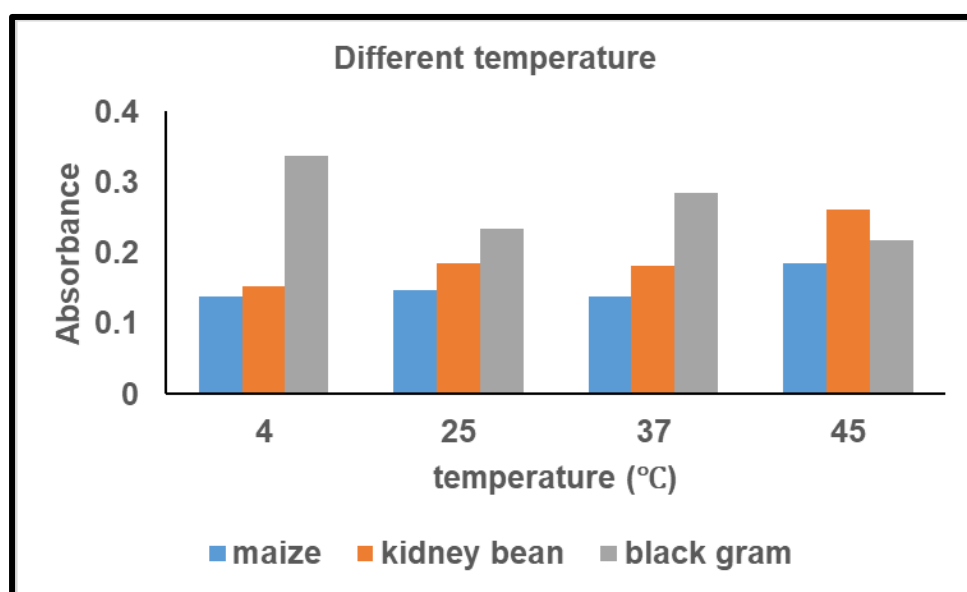


Figure 18: Graph showing comparison of absorbance at different temperature

7. Detection of acid phosphatase at different pH range:

Maize (0.5mg/ml)

At different pH range of 3, 4.6, 5.5 and 8.2 with concentration of 0.5mg/ml was observed. It shows maximum activity of acid phosphatase at 4.6 with O.D 0.175.

Different pH	OD (Maize)
3	0.0615
4.6	0.175
5.5	0.116
8.2	0.0935

Table 7(a): Detection of acid phosphatase at different pH in maize(0.5mg/ml)

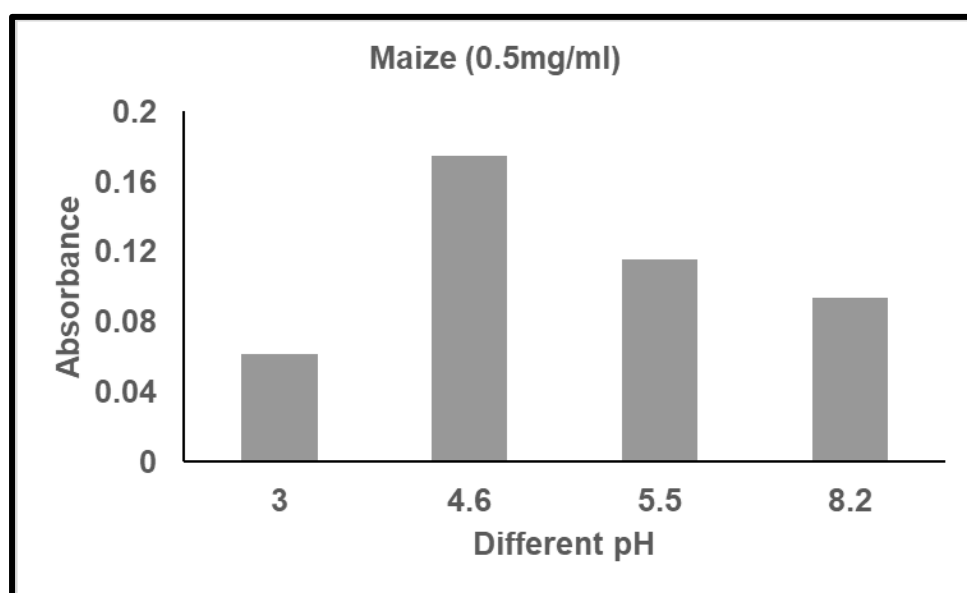


Figure 198: Graph showing absorbance at different pH range in maize

Kidney bean(0.5mg/ml)

At different pH range of 3, 4.6, 5.5 and 8.2 with concentration of 0.5mg/ml was observed. It shows maximum activity of acid phosphatase at 8.2 with O.D 0.0465.

Different pH	OD (kidney bean)
3	0.068
4.6	0.0625
5.5	0.0715
8.2	0.0465

Table 7(b): Detection of acid phosphatase at different pH range in kidney bean(0.5mg/ml)

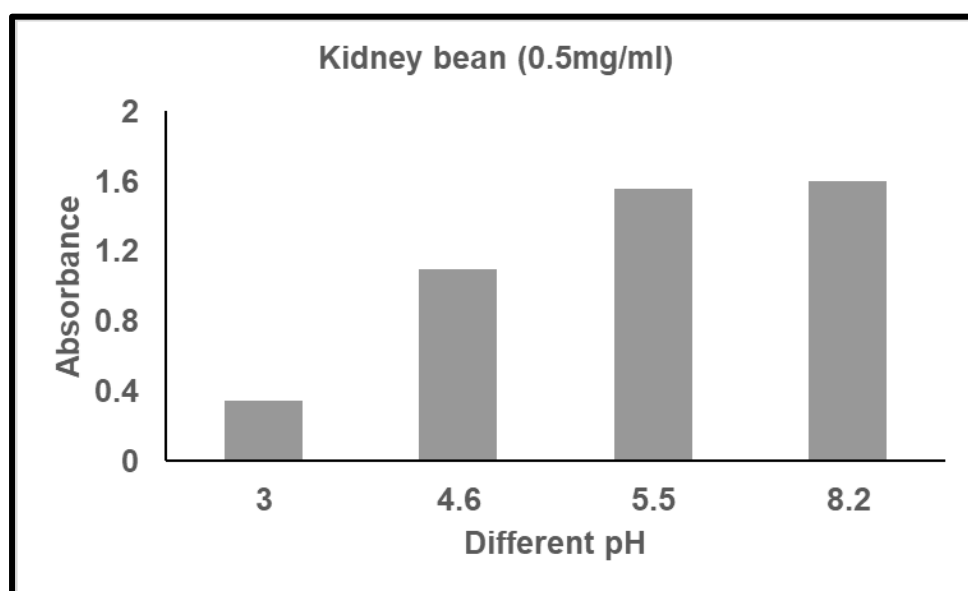


Fig 20: Graph showing absorbance at different pH range in kidney bean

Black gram(0.5mg/ml)

At different pH range of 3, 4.6, 5.5 and 8.2 with concentration of 0.5mg/ml was observed. It shows maximum activity of acid phosphatase at 5.5 with O.D 1.555.

Different pH	OD (black gram)
3	0.347
4.6	1.096

5.5	1.555
8.2	1.60

Table 7(c): Detection of acid phosphatase at different pH in black gram(0.5mg/ml)

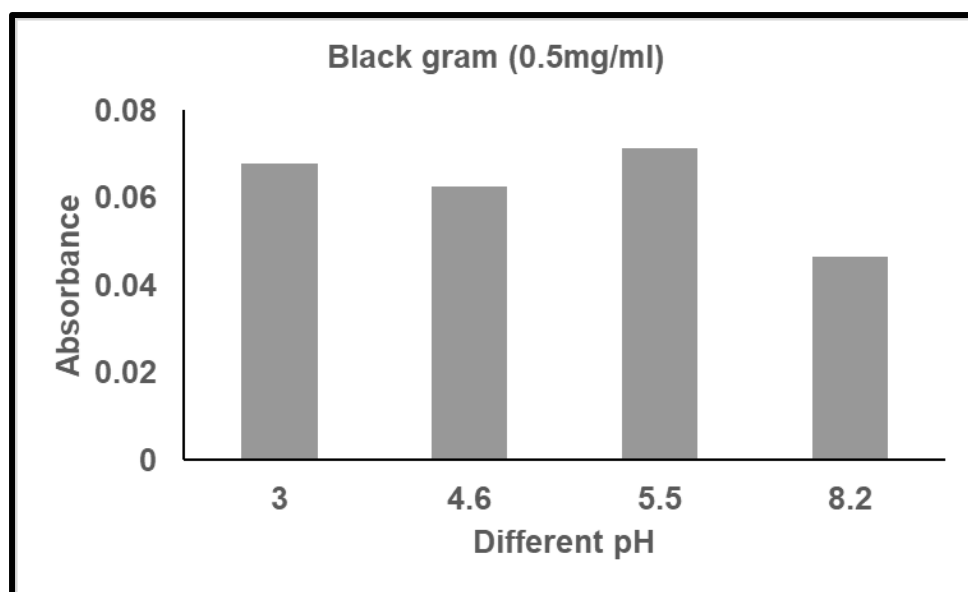


Fig 21: Graph showing absorbance at different pH range in black gram

Comparative analysis of acid phosphatase at different pH in maize, kidney bean and black gram

Comparative analysis in case of three different samples with concentration of 0.5mg/ml were obtained by making graph. Out of which the observation found to be that kidney bean showed maximum activity of acid phosphatase with pH of 8.2.

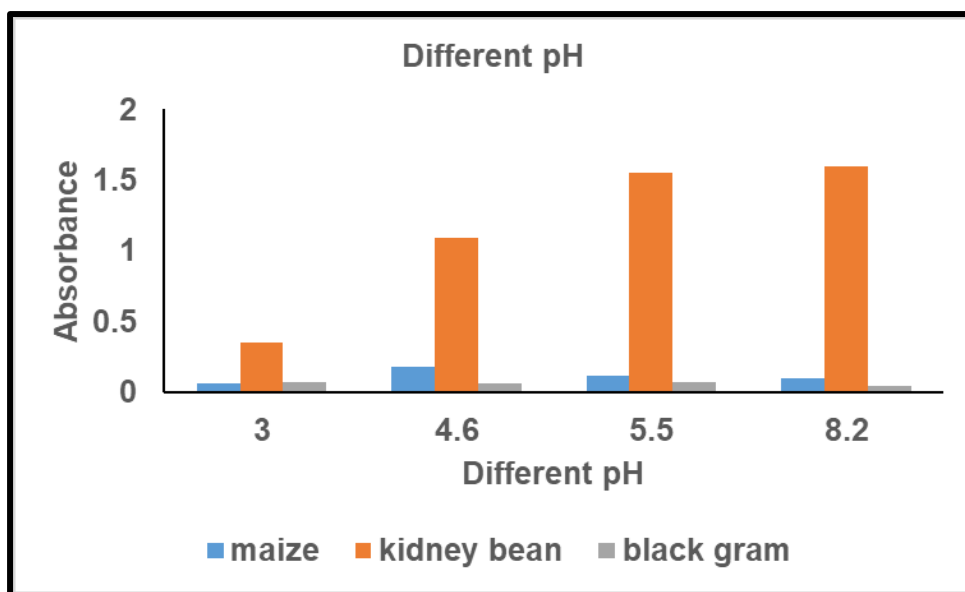


Fig 22: Graph showing comparison of absorbance at different pH range

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