A

PROJECT REPORT ON

"Investigation and Characterization of Phosphatase Enzyme Activity in Mung Bean (V. radiata) & Urad (V. mongo)"

SUBMITTED TO

VIVEKANAD COLLEGE, KOLHAPUR

(EMPOWERED AUTONOMOUS)

DEPARTMENT OF BIOTECHNOLOGY

SUBMITTED BY

Shri. Chetan Balkrishna Patil

FOR THE PARTIAL FULFILMENT OF BACHELOR OF SCIENCE IN

BIOTECHNOLOGY

For the year

2024-2025

UNDER THE GUIDANCE OF,

Prof. Mr. S. G. Kulkarni

Department of Biotechnology

"Dissemination of Education for Knowledge, Science and Culture"

- Dr. Bapuji Salunkhe

SHRI SWAMI VIVEKANAND SHIKSHAN SANSTHA'S

VIVEKANAND COLLEGE, KOLHAPUR

(EMPOWERED AUTONOMOUS)

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

This is to certify that_____

Exam no _____has satisfactorily carried out the required Project Work prescribed by the BoS Department of Biotechnology, Vivekanand College, Kolhapur (Empowered Autonomous) for B.Sc. - Part- III Semester VI, this Report represents his/her Bonafide work in the year 20____-20____

Place:

Date:

Teacher In-charge **Examiner** **Head**

ACKNOWLEDGEMENT

I acknowledge my deep sense of gratitude towards assistant professor Mr. S.G. Kulkarni Head of the department Biotechnology for being a great source of inspiration.

I am thankful to my project guide assistant professor Mr. S.G. Kulkarni for their abled guidance and extreme cooperation conferred on me during the Entire completion of the project.

I thank him for helping me to collect all the information required through the project and to study as well as to" "Investigation and Characterization of Phosphatase Enzyme Activity in Mung Bean (V. radiata) & Urad (V. mungo)"

I am grateful to all the teachers for valuable suggestion during my project work.

I also thank that not teaching staff members and friends who helped me carry out the Project satisfactorily.

Record my since you thanks to Vivekanand College, Kolhapur for allowing me to carry My project works successfully in the college labs.

Shri. Chetan Balkrishna Patil

Declaration

I hereby declare that the project work entitled "Investigation and Characterization of Phosphatase Enzyme Activity in Mung Bean (V. radiata) & Urad (V. mongo)"is Submitted to Vivekanand College, Kolhapur for the award off degree of Bachelor of Science, Biotechnology This is the result of Bonafide work carried out by me under the Guidance of assistant professor Mr. S.G. Kulkarni.

I further declare that the results presented here are not the basis for reward of any other Degree

Place:

Date:

Shri. Chetan Balkrishna Patil

Content

Investigation and Characterization of Phosphatase Enzyme Activity in *Vigna radiata* (Mung) & *Vigna mungo* (Urad)

➢ ABSTRACT

This study investigates and characterizes phosphatase activity in mung beans (Vigna radiata) and urad beans (Vigna mungo). The research focuses on the extraction, estimation, and environmental effects on phosphatase enzymes. Phosphatase activity was confirmed using an artificial substrate, para-nitrophenyl phosphate (PNPP), which produced a color change upon reaction. Enzyme purification was conducted using ammonium sulfate precipitation, with post-purification results showing increased enzymatic activity.

The study also examined the effects of temperature and pH on enzyme efficiency. Optimal phosphatase activity was observed at 30°C and pH 6.6 for both mung and urad beans. Protein content was measured using the Biuret method, indicating a higher protein concentration in urad beans compared to mung beans.

The findings highlight the significance of phosphatases in biochemical processes, with potential applications in molecular biology, environmental science, clinical diagnostics, and industrial processes. The study concludes that both acidic and alkaline phosphatases are present in the tested legumes, reinforcing their role in nutrient recycling and biological regulation.

Keywords: Phosphatase, Acid & Alkaline Phosphatase, Black gram, green gram, PNPP

➢ INTRODUCTION

The history of the advances of the science is the story of marvelous discoveries & invention. These inventions have revolutionalised our life & the best way to predict the future is to invent it. Enzyme is a biocatalyst which accelerates biological reactions; However, the concept of biocatalyst is very wide. It includes pure enzyme, crude cell extract, viable plant cells, viable animal cells, viable microbial cells & intact non-viable microbial cells. Source of enzymes used in commerce is plant and animal cells. The sources of enzymes are micro-organisms, higher plants & animals. Out of these sources, plant enzymes also account for applications in industries & medicine.

Now a days, number of enzymes are under study of enzyme therapy and number of enzyme preparation have an important application in research & industries. Miller (1976), Prescott (1987), Tripathi (1999) had sited a good account of application of enzyme in industries, research & medicine. Enzyme therapy is also used in treatment of cancer, genetic disorders, clothing therapy.

Phosphate esters are widely distributed in any organism. Nucleic acids, metabolic intermediates like glucose-6-phosphate, energy rich substrates (AMP, Creatine phosphate) are some obvious examples. While many metabolic intermediates are activated through the transfer of phosphate groups (e.g. by kinases) it is equally important that phosphate esters can also be rapidly broken
down. The hydrolytic removal of phosphoesters is catalyzed by phosphatase. Many phosphatases are highly substrate specific, like those enzymes involved in signal transduction.

A number of phosphatases, however, cleave virtually any phosphate ester. Such unspecific enzymes function mainly in the catabolic breakdown of metabolites or nutrients.

Depending on the pH at which such phosphatase has optimal activity. We distinguish between acidic phosphatase (also called acid phosphatase) & alkaline phosphatase. Phosphate is an essential component of a number of biologically important molecules, including DNA, RNA, Phospholipids of cell membranes, ATP and many metabolites' phosphatases are key enzymes in liberating & recycling the phosphate that is necessary for many fundamental biological processes.

A range of research interests focus on phosphatases. They have for e.g., been implicated in such diverse roles as possible allergens in part of the protein coat of pollen grains & as markers of abnormal prostate gland function.

- **ENZYMES**

Cells function largely because of the action of enzymes. Life is a dynamic process that involves constant changes in chemical composition These changes are regulated by catalytic reactions, which are regulated by enzymes. At one time, the cell was actually conceived of as a sac of enzymes. It was believed that if we knew all of the reactions and their rates of action, we could define the cell, and indeed life itself. Few biologists continue to think of this as a simple task, but we know that life as we know could not exist without the function of enzymes. Ideally, we would examine enzymes within an intact cell, but this is difficult to control Consequently, enzymes are studied in vitro after extraction from cells.

Since all enzymes are proteins, and proteins are differentially soluble in salt solutions, enzyme extraction procedures often begin with salt (typically, ammonium sulfate) precipitation. On the simplest level, proteins can he divided into albumins and globulins on the basis of their solubility in dilute salts. Albumins are considered to be soluble while globulins are insoluble. Solubility is relative, however, and as the salt concentration is increased, most proteins will precipitate.

Thus, if we homogenize a tissue in a solution that retains the enzyme in its soluble state, the enzyme can be subsequently separated from all insoluble proteins by centrifugation or filtration. The enzyme will be impure, since it will be in solution with many other proteins. If aliquots of a concentrated ammonium sulfate solution are then added serially, individual proteins will begin to precipitate according to their solubility. By careful manipulation of the salt concentrations, we can produce fractions which contain purer solutions of enzymes, or at least are enriched for a given enzyme Fortunately, absolute purity of an enzyme extract is seldom required, for when it is, the fractions must be subjected to further procedures designed for purification (such as electrophoresis and/or column chromatography)

In order to determine the effectiveness of the purification, each step at the extraction procedure must be monitored for enzyme activity. That monitoring can be accomplished in many ways, but usually involves a measurement of the decrease in substrate, or the increase in product specific to the enzyme.

Specific Activity -

This brings us to a definition for enzyme activity. **specific activity** is defined in terms of enzyme units per mg enzyme protein. An enzyme unit is the amount of substrate converted to product per unit time under specific reaction conditions for pH and temperature.

As generally accepted, an enzyme unit is defined as that which catalyzes the transformation of 1 micromole of substrate per minute at 30°C and optimal chemical environment (pH and Substrate concentration). Specific Activity relates the enzyme units to the amount of protein in the sample.

While it is relatively easy to measure the protein content of a cell fraction, there may be a variable relationship between the protein content and a specific enzyme function. Remember that the initial extraction of an enzyme is accomplished by differential salt precipitation. Many proteins will precipitate together due to their solubility, but have no other common characteristics.

To determine both protein content and enzyme activity requires two different procedures. We can measure the amount of protein, or we can kinetically measure the enzyme activity. Combining the two will give us the specific activity.

- **PHOSPHATASES**

A **phosphatase** is an enzyme that removes a phosphate group from its substrate by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group. (dephosphorylation). This action is directly opposite to that of phosphorylases and kinases,

which attach phosphate groups to their substrates by using energetic molecules like ATP. Phosphorylated compounds are widely distributed in living systems. They serve as storage forms for energy (ATP and phosphocreatine), as allosteric effectors of certain enzymes (e.g. fructose-1-6-bisphosphatase), as a second messenger (e.g. cAMP, cGMP, inositol phosphates)

In general, these enzymes catalyze the hydrolysis of phosphate monoesters as follows

Alkaline phosphatase (ALP) (EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. The optimal pH for the activity of the E. coli enzyme is 8.0 while the bovine enzyme optimum pH is slightly higher at 8.5

It is sometimes used synonymously as **basic phosphatase**

Acid phosphatase (EC 3.1.3.2) is a phosphatase, a type of enzyme, used to free attached phosphate groups from other molecules during digestion. It is basically a phosphomonoesters. It is stored in lysosomes and functions when these fuse with endosomes, which are acidified while they function; therefore, it has an acid pH optimum. Acid phosphatase catalyzes the following reaction at an optimal pH below 7:

Orthophosphoric monoester + H_2O→ alcohol + H_3PO_4

Acid phosphatase is the name given to a generic enzyme activity at hydrolyzes a phosphate group from a wide variety of natural and artificial substrates. The enzyme activity is present in most eukaryotic cells, here it is important in recycling phosphate residues.

The substrate for an acid phosphatase reaction must contain a phosphate functional group attached to some other molecule through a chemical bond involving the phosphate and a hydroxyl (-OH or alcohol) functional group. The products of the reaction are the free phosphate group and the rest of the molecule with a free-OH group.

Acid phosphatase does not much care what the rest of the substrate molecule is, as long as it has a phosphate group attached. We take advantage of this lack of specificity in the laboratory when we assay acid phosphatase using an artificial substrate that is never found in cells. The artificial substrate is called para-nitro-phenyl-phosphate or PNPP. When acid phosphatase acts upon PNPP the products are a free phosphate group and a substance called para-nitro-phenol or PNP, which is an alcohol.

Phosphatase enzymes are also used by soil microorganisms to access organically bound phosphate nutrients. An assay on the rates of activity of these enzymes may be used to ascertain biological demand for phosphates in the soil. Different forms of acid phosphatase are found in different organs, and their serum levels are used as a diagnostic for disease in the corresponding organs. For example, elevated prostatic acid phosphatase levels may indicate the presence of prostate cancer.

- **SELECTION OF SOURCE**

  Mung bean also known as green bean, mung, mongo, moong, moong dal (in Bengali), mash bean, munggo or mongo, green gram, golden gram, and green soy, is the seed of Vigna radiata which is native to Bangladesh, India, and Pakistan. The split bean is known as moong dal, which is green with the husk, and yellow when dehusked. The beans are small, ovoid in shape, and green in color. The English word "mung" derives from the Hindi moong.

  The mung bean is one of many species recently moved from the genus Phaseolus to Vigna and is still often seen cited as Phaseolus aureus or Phaseolus radiatus. These variations of nomenclature have been used regarding the same plant species.

  Mung beans are mainly cultivated in China, Thailand, Philippines, Indonesia, Burma, Bangladesh and India, but also in hot and dry regions of South Europe and Southern

USA. Mung beans are tropical (or sub-tropical) crops, and require warm temperatures (optimally round 30- 35°C). Loamy soil is best for pusap cultivation.

Mung beans

Scientific classification

- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Magnoliopsida
- Order: Fabales
- Family: Fabaceae
- Genus: Vigna
- Species: V. radiata

Urad (Vigna mongo), also referred to as urad dal, urad dal, urd bean, urd, urid, black matpe bean, black gram: white lentil (Vigna mungo), is a bean grown in southern Asia. It is largely used to make dal from the whole or split, dehusked seeds. It, along with the mung bean, was placed in Phaseolus but has been transferred to Vigna. It was at one point considered to belong to the same species as the mung bean.

Black gram originated in India where it has been in cultivation from ancient times and is one of the most highly prized pulses of India. It has also been introduced to other tropical areas mainly by Indian immigrants.

It is an erect, sub-erect or trailing, densely hairy annual herb. The tap root produces a branched root system with smooth, rounded nodules. The pods are narrow, cylindrical and up to 6 cm long. The bean is boiled and eaten whole or after splitting into dal, prepared like this it has an unusual mucilaginous texture. Ground into flour or paste, it is also extensively used in culinary preparation like dosa, idli, vada, and papadum. When used this way, the white lentils are usually used. It is very nutritious and is recommended for diabetics, as are other pulses.

Urad

Scientific classification

- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Magnoliopsida
- Order: Fabales
- Family: Fabaceae
- Subfamily: Faboideae
- Genus: Vigna
- Species: V. mungo

➢ Review of Literature

Acid phosphatase is widely distributed in plants and animals. It can be purified and characterised from tubers, seeds, roots, leaves and seedlings. Historically, those were negelated by researchers less studied than protein kinase, but recently there has been more interest in both proteins, i.e. genetic characterization and regulation. It catalyses hydrolysis of phosphate monoesters in acidic condition with pH 4-7. Few members of AP family are metallohydrolase having two heterovalent metal ions in catalytic centre. Phosphatases are broadly classified on the basis of their specificity for tyrosine or serine/threonine amino acid substrates. Although serine/threonine phosphatases were originally classified based on amino acid sequence homology within their catalytic subunits. The phosphatases are presently grouped according to biochemical characteristics, sensitivity to endogenous inhibitor proteins and substrate specifically.

Now a days, Isolation and purification of enzyme is very easy because various advanced techniques are available. But according to the Elizabeth et al (2011) purification of acid phosphatase is difficult due to its role as storage protein. They used ammonium sulfate precipitation method for purification and fully purified via gel excision and heat denaturation yielding 29kDa protein.

Similarly, Asaduzzaman et al (2011) used some advanced technique for purification and characterization of acid phosphatase. They used germinating black gram seedlings as a source and purification is carried out by ion exchange chromatography, SDS-polyaccrylamide slab gel electrophoresis. They studied the metabolic processes of germination and maturation of plants & observed that it continuously expressed in seeds during germination. The enzyme which was estimated to be 25kDa. It showed maximum activity at pH 5.

During the last several years, only purification and characterization is carried out but in recent years Asha Anand, Pramod Kumar Srivastava have focused more on the biochemical properties, structure, catalytic mechanism and application of acid phosphatase. It plays an important role in agriculture and clinical fields. In agriculture phosphatase acts as a potent agent for sustainable agriculture practices and diagnostic marker in bone metabolic disorders related to clinical field. In the current studies it plays a significant role in prostate cancer therapies as a therapeutic target. During cancer expression level of secretory acid phosphatase is observed to be higher whereas cellular prostatic level is lower. By using most specific immunogenic candidates such as prostatic acid phosphatase and prostate specific antigen seems to be the most promising approach in vaccines development for prostate cancer.

David L. Brautigan in the year 2012 gives the combinational nature and complexity of serine/threonine phosphatases for a better understanding of their roles in signalling network and in the development of new, highly specific drugs. But recent development shows that regulation and specificity are achieved through assembly of multisubunit holoenzymes, transient phosphorylation and the action of inhibitor proteins still not widely appreciated, these are hundreds of discrete protein serine/threonine phosphatases are available to counteract protein kinases, offering potential therapeutic targets.

As we discuss earlier that plants are the source of phosphatase enzyme but can also be isolated from free living microorganisms and the isolation is carried out by Fitriatin et al. They identify phosphate producing bacteria from sanggabuana forest and check their capability to hydrolyse organic phosphate. This work was supported by Indonesian centre for biodiversity and Biotechnology(ICBB).

In Present study attempt was made for identification and isolation of phosphatase enzyme from the germinated mung seeds & urad seeds. Also, the presence of enzyme is detected by reacting the enzyme with substrate PNPP and pale-yellow colour was observed indicates the presence of enzyme. Isolated enzyme characterised by using SDS-PAGE method.

➢ MATERIAL & METHODS

1) Materials

- Black gram & Green gram Beans
- Electrophoresis Equipment
- Power supply
- Gel casting unit
- Running chamber

2) Chemicals

- Std M/15 of KH_2PO_4 = 20µg/ml
- Molybdate reagent
- Color reagent ANSA
- Substrate PNPP
- Enzyme
- Buffer
- Polyacrylamide Gel Preparation Acrylamide and Bis-acrylamide
    - Tris-HCl buffer
    - Sodium dodecyl sulfate (SDS)
    - Ammonium persulfate (APS) and TEMED (catalysts for gel polymerization)
- SDS-loading buffer (Laemmli buffer) containing β-mercaptoethanol or DTT
- Electrophoresis Buffer
    - Tris-Glycine-SDS buffer
    - Molecular Weight Markers
    - Protein ladder (pre-stained or unstained)
- Staining and Destaining Solutions
    - Coomassie Brilliant Blue stain
    - Destaining solution (Methanol/Acetic acid solution)

3) Enzyme Isolation

- **Growth of seedlings**

  Organic mung & urad bean seeds were obtained from a local food cooperative. The seeds were soaked overnight in water, drained, spread out on paper towels, and covered with aluminium foil to ensure darkness for germination. The seeds were allowed to germinate at room temperature or at 37°C for an average of four days.

- **Extraction and homogenization**

  Remove the long seedlings of mung beans and urad. Place the pieces in mortar or a blender along with buffer. The Final mixture after grinding is known as homogenate. Filter the homogenate through 2 layer of muslin cloth. The liquid which passes through the cloth is known as the filtrate. Collect the filtrate Clarify the filtrate by centrifugation, at high speed for 10 minutes to produce a tight pellet. At the end of the centrifuge run, a clear liquid portion over the pellet known as supernatant is taken in a clean tube. Supernatant is used as an "enzyme"

Flow chart for enzyme extraction

Seedlings

↓

Blending

↓

Filtration

↓

Centrifugation of the filtrate at high speed for 10 minutes

↓

Discard the pellet and collect the supernatant

↓

Use as an enzyme source

- **Investigating the presence of phosphatase in the source**

For investigating the presence of phosphatase, the seedlings of mung beans & urad beans were taken. The source was crushed and centrifuged.

The supernatant was taken as an enzyme source. For this phosphatase, we take advantage of the broad substrate specificity and use an artificial substrate that changes its color after hydrolytic removal of the phosphate group.

Enzyme from each 3 source was reacted with PNPP an artificial substrate and was incubated at 37°c. Pale yellow color was observed in each 3test tube. By this way investigation was carried out for its initial presence.

- **Estimation of inorganic phosphate by Fiske- Subbarow method**

Inorganic phosphate reacts with ammonium molybdate in an acid solution to form phosphomolybdic acid. A reducing agent such as ANSA is added which reduces molybdium to give a blue color but doesn't affect uncombined molybdic acid. It is very sensitive method. The concentration of inorganic phosphate is directly proportional to intensity of color

1) Pipette out 0.0, 0.4, 0.8, 1.2,1.6 and 2.0 ml of std KH_2PO_4, solution in test tubes.
2) Adjust volume to 3.6 ml by D/W in each test tube
3) Add 1ml of ammonium molybdate to each test tube.
4) Add 0.4 ml ANSA.
5) Take readings within 10 mins.
6) Measure intensity of color at 660nm
7) Plot graph of O.D. at 660nm Vs concentration of KH_2PO_4
8) Pipette out 1 ml of sample solution and adjust volume 3.6 ml by D/W and repeat above procedure.
9) From this valve calculate unknown conc. Of KH_2PO_4 from std graph

- **Protein assay by Biuret method**

This method is general for compound having a peptide bond. Alkaline, $CuSO_4$ reacts with compound containing 2 or more peptide bond to give violet or pinkish color compound of cupric ions with unshared electron pairs of peptide nitrogen and oxygen of water.

- Protein Purification

  As the ionic strength in increased further, the solubility of protein begins to decrease. At sufficiently high ionic strength a protein may be almost completely precipitated (from solution, an effect called salting out the physiochemical basis of salting out is rather complex one factor is that the high concentration of salt may remove water of hydrasuon from protein molecule, thus reducing its solubility. Ammonium sulphate is preferred for salting out proteins because it is soluble in water that vary high ionic strengths can be attained the enzyme was concentrated using 50% ammonium sulphate

Flow Chart

25 ml of sample+7.3 gm of ammonium sulphate
↓
Dissolve properly and keep it in ice bath for 24 hrs.
↓
Centrifuge it at 3000 rpm for 10mins
↓
Supernatant was removed
↓
Pellet was suspended in 2 ml buffer
↓
Check for enzyme activity

- **SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis)**

1. Gel Preparation
   - · Prepare a resolving gel (separating gel) and pour it into the gel casting plates.
   - · Overlay with water to remove air bubbles and allow polymerization.
   - · Prepare a stacking gel, pour it on top of the resolving gel, and insert a comb to create sample wells.
   - · Let the gel polymerize.
2. Sample Preparation
   - · Mix enzyme samples with SDS-loading buffer.
   - · Heat the samples at 95°C for 5 minutes to ensure complete denaturation.
3. Loading and Electrophoresis
   - · Place the gel in the electrophoresis chamber and fill it with Tris-Glycine-SDS buffer.
   - · Load the protein marker and enzyme samples into separate wells.
   - · Run the gel at 80-100V for stacking and 120-150V for resolving until the dye front reaches the bottom.
4. Staining and Visualization
   - · Soak the gel in Coomassie Brilliant Blue stain for 1 hour.
   - · Destain using Methanol/Acetic acid solution until clear bands appear.
5. Analysis
   - · Compare the bands with the molecular weight marker to estimate the size of the phosphatase enzyme.

4) Enzyme Activity

The enzyme is mostly affected by different conditions and factors. We kept the enzyme at different conditions or factors such as Temperature, pH, Time interval and Substrate concentration to get the optimum enzyme activity

- **Effect of temperature on rate of enzyme activity**

  The enzyme phosphatase hydrolyses p-nitrophenol phosphate. The inorganic phosphate that is released reacts with ammonium molybdate to form phosphomolybdate, which is then reduced by amino naphthol sulphonic acid (ANSA) to form a deep blue-colored product, which is measured at 660 nm. The intensity of color formed is directly proportional to the amount of phosphate in the sample.

  1) Take clean test tubes and label them with10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C
  2) Add 0.5 ml of substrate and 0.5 ml enzyme in each of test tube.
  3) Incubate the tubes at various temperature for 10 mins
  4) Take out 1 ml from reaction mixture and react with 1 ml ammonium molybdate and then with 0.4 ml ANSA reagent.
  5) Note down the absorbance at 660 nm

- **Effect of pH on rate of enzyme activity**

  The enzyme acid phosphatase hydrolyses disodium phenyl orthophosphate to phenol & disodium orthophosphate. The inorganic phosphate that is released reacts with ammonium molybdate to form phosphomolybdate, which is then reduced by 1-ammino-2naphthol-4-sulphonic acid (ANSA) to form a deep blue-colored product, which is measured at680 nm. The intensity of color formed is directly proportional to the amount of phosphate in the sample. Phosphate buffer pH range 3.0-9.0 is used to determine the optimum pH of acid phosphatase.

  1) Take clean tubes test and label them with as 5.8, 6.2, 6.6, 7.0, 7.4, 7.8
  2) Add 0.5 ml of substrate and 0.5 ml enzyme in each of test tube
  3) Incubate the tubes at various pH for 10 mins
  4) Take out 1 ml from reaction mixture and react with I ml ammonium molybdate and then with 0.4 ml ANSA reagent.
  5) Note down the absorbance at 660 nm

- **Effect of time on rate of enzyme activity**

  The enzyme phosphatase hydrolyzes para-nitrophenyl phosphate (PNPP), releasing inorganic phosphate and para-nitrophenol. The inorganic phosphate reacts with ammonium molybdate to form phosphomolybdate, which is then reduced by amino naphthol sulphonic acid (ANSA) to produce a deep blue-colored product measurable at 660 nm. The intensity of the color is directly proportional to the enzyme activity. Measuring the enzyme activity over time helps determine how reaction progress and enzyme stability change with time**.**

  1. Label test tubes according to different time intervals (e.g., 3, 6, 9, 12, and 15 minutes).
  2. Pipette 1 ml of the substrate (PNPP) into each test tube.
  3. Add 0.5 ml of enzyme solution to each test tube.
  4. Incubate the reaction mixtures at a fixed optimal temperature (e.g., 30°C) for the respective time intervals.
  5. At the end of each incubation period, withdraw 1 ml from each reaction mixture and add 1 ml of ammonium molybdate.
  6. Add 0.4 ml of ANSA reagent to each tube to develop the blue color.
  7. Measure the absorbance at 660 nm using a spectrophotometer.
  8. Plot a graph of absorbance (O.D. at 660 nm) versus time to observe the effect of time on enzyme activity.

- **Effect of Substrate concentration on rate of enzyme activity**

Km (Michaelis constant) represents the substrate concentration at which the reaction rate reaches half of its maximum velocity. The exact Km value for a phosphatase enzyme can vary depending on factors like the specific substrate used, pH, temperature, and the source of the enzyme. Km is typically determined using Michaelis-Menten kinetics and Lineweaver-Burk plots. The Km value of acid phosphatase from mung bean seedlings (Vigna radiata) is typically around 0.27mM and urad (black gram) seedlings is typically around 0.49 when using 4-nitrophenyl phosphate as a substrate.

A low Km value means the enzyme binds to the substrate readily at relatively low concentrations, while a high Km value indicates a weaker affinity, requiring a higher substrate concentration to reach half the maximum rate.

1. Preparation of Enzyme Extract
   - Mung bean or urad seedlings are homogenized in buffer and centrifuged.
   - The supernatant is collected as the enzyme source.
2. Preparation of Substrate Solutions
   - Prepare different concentrations of PNPP (e.g., 0.2, 0.4, 0.6, 0.8, 1.0 mM).
3. Reaction Setup
   - Take 0.5 mL of enzyme extract in each test tube.
   - Add 1 mL of substrate solution (PNPP) at different concentrations.
   - Adjust the final volume with buffer to 3.9mL.
   - Incubate at 30°C for 10 minutes.
4. Stopping the Reaction
   - Add 1 mL ammonium molybdate.
   - Add 0.4 mL ANSA reagent.
   - Read absorbance at 660 nm within 10 minutes.
5. Plotting and Analysis
   - Measure the absorbance values and plot substrate concentration vs. reaction velocity.

➢ Result and Discussion

- Initially before starting the project, it is necessary to detect the presence of enzyme so to investigate it the seedlings were grind into paste and was centrifuged. The supernatant was taken as enzyme source.
- Enzyme was reacted with PNPP. Pale color was observed, this confirmed the presence of enzyme phosphatase
- Enzyme assay was done and inorganic phosphate was determined by Fiske-Subbarow method. Protein content was determined by Biuret method. It was found that the protein content was high in urad than mung beans. (Table No.1)
- According to Asaduzzaman AK, Rahman HM, Yeasmin T, the highest activity of acid phosphatase from black gram was found after 70% (NH4)2SO4 saturation. But in case of green gram (Hailey Elizabeth Lightle, Sarah Anne Fosco, Rachel Nicole Giles, Jordyn Marie Meekma,
- Nicole Marie Moore, Megan Vawn Palacio and Suzanne Elisabet Williams) the highest activity of acid phosphatase found after 30% (NH4)2SO4 saturation & we also got the same results. It was noted that enzyme showed maximum activity after purification. Enzyme activity was higher for Moong (Table No.2)
- Rao etal (2019) determined the phosphatase activity with optimum temperature 35°c for Urad and 30°c for mung. But we determined the optimum temperature for mung and urad was 30°c (Table No.4)
- Ferreira etal 1998, Duff etal 1989, Hass etal 1991, Gonneyty etal 2006, determined the optimum pH for Urad was range from 5-6 pH i.e. acid phosphatase. We were kept enzymes at different experimental pH conditions to get the optimum pH of the phosphate's enzymes. The optimum pH enzyme obtained for Mung urad seedlings was 6.6 i.e. acidic phosphatase. (Table No.5)
- Singh etal (2020) detected the optimum time for enzyme activity is 20 min for mung and 30 min for urad. We observed the optimum time for enzyme activity 6 min at which it shows high activity under optimum temp. & pH. (Table No.6)
- The increase in absorbance over time indicates the progressive release of phosphate due to enzyme action. The reaction may reach a plateau, showing that the enzyme activity has slowed or stabilized due to substrate depletion or enzyme denaturation. (Table No.7)

- **Estimation by Fiske-Subbarow method**

Sr. No	Standard solution (ml)	Conc. Of Std sol. (ug)	D/W	Ammonium Molybdate (ml)	ANSA (ml)		OD.at 660nm
1	0.0	0	3.6	1	0. 4	Take	0.00
2	0.4	8	3.2	1	0. 4	Reading	0.35
3	0.8	16	2.8	1	0. 4	Exactly	0.39
4	1.2	24	2.4	1	0. 4	At	0.53
5	1.6	32	2.0	1	0. 4	10 min	0.56
6	2.0	40	1.6	1	0. 4		0.80
7	0.5 ml substrate	0.5 ml Sample A		1	0.4		0.28
8	0.5 ml substrate	0.5 ml Sample B		1	0.4		0.32

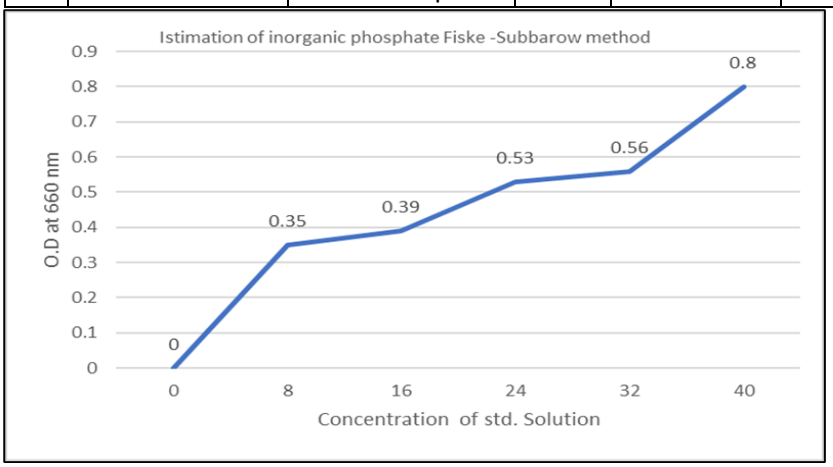


Table No. 1

- **Biuret method**

1) Sample A =Enzyme from mung seedlings
2) Sample B = Enzyme from urad beans

Sr	Enzyme	D/W	Biuret Reagent		O.D 530nm
1	1ml Enzyme A	4ml	5ml	Incubate for 10 min	0.10
2	1ml Enzyme B	4ml	5ml		0.12

- **Protein Purification**

Substrate	Enzyme		Ammonium molybdate	ANSA		O.D at 660nm (Before Purification)	O.D at 660nm (After Purification)
1 ml	1 ml (moong)	Wait for 5 min	1 ml	0.4 ml	Take reading after 10 min	0.56	1.34
1 ml	1 ml (Urad)		1 ml	0.4 ml		0.88	1.26

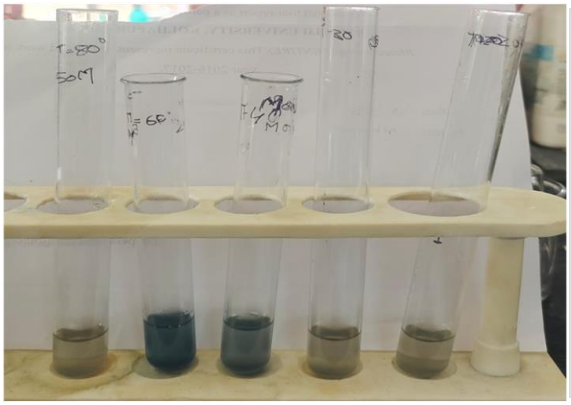
Table No .2

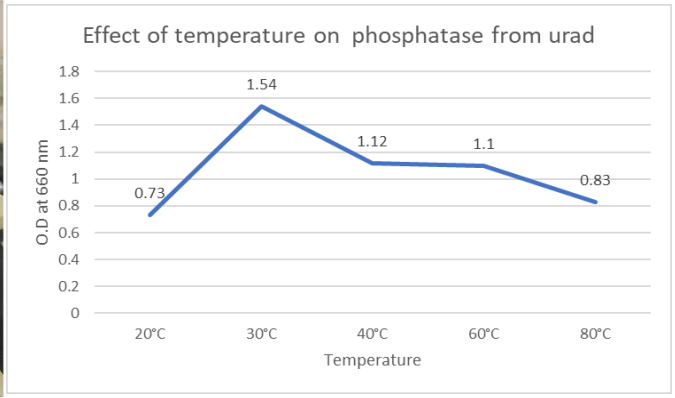
- **SDS-PAGE**

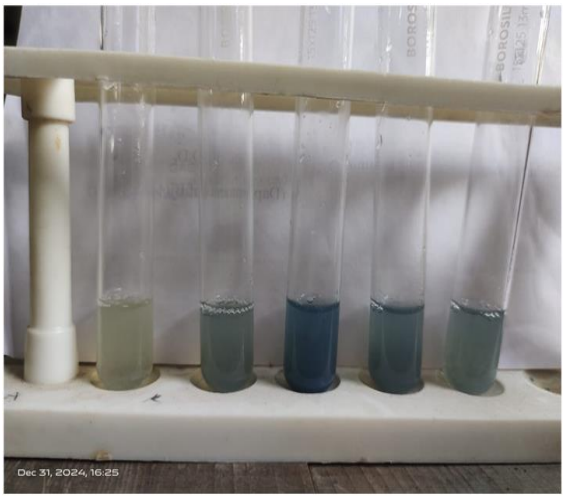
Table No. 3

- **Effect of Temperature**

Sr. No	Substrate (ml)	Enzyme (ml)	Temperature		Ammonium Molybdate	ANSA	Take	O.D at 660 nm (Mung)	O. D at 600 nm (Urad)
1	1 ml	0.5 ml	20	Incubate	1 ml	0.4 ml	Reading	0.39	0.73
2	1 ml	0.5 ml	30		1 ml	0.4 ml		0.65	1.54
3	1 ml	0.5 ml	40	for	1 ml	0.4 ml	after	0.44	1.12
4	1 ml	0.5 ml	60	10 min	1 ml	0.4 ml	10	0.42	1.10
5	1 ml	0.5 ml	80		1 ml	0.4 ml	min	0.21	0.83







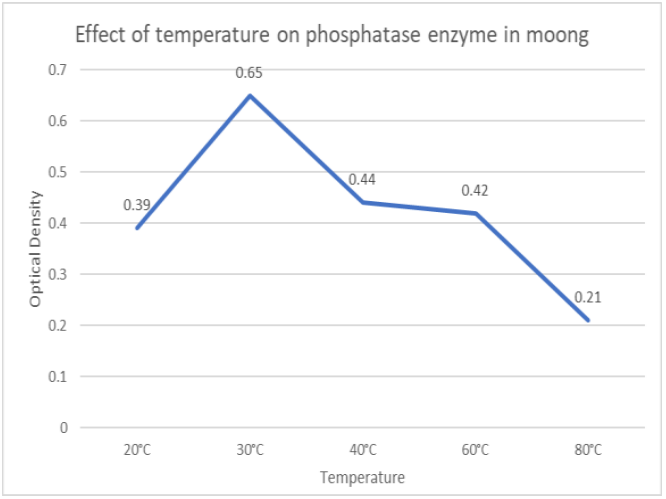
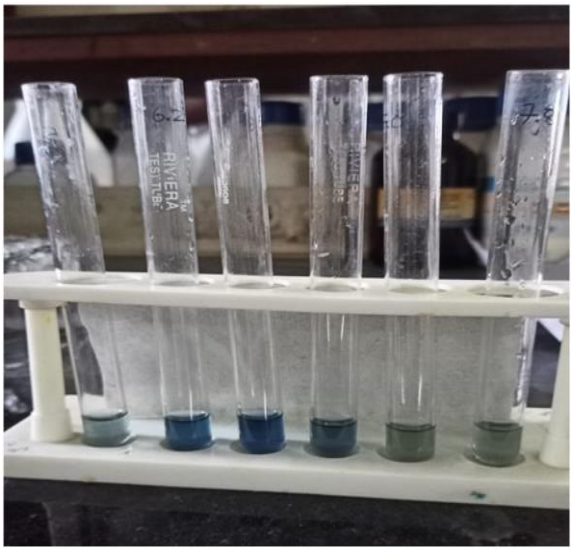
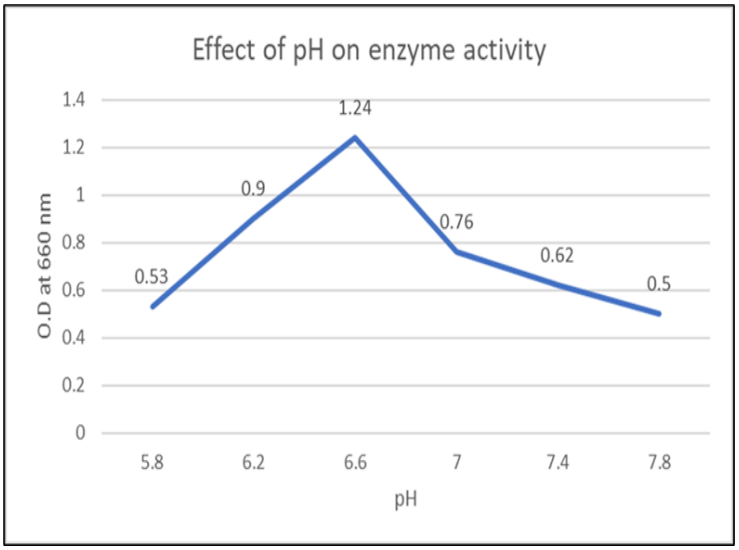


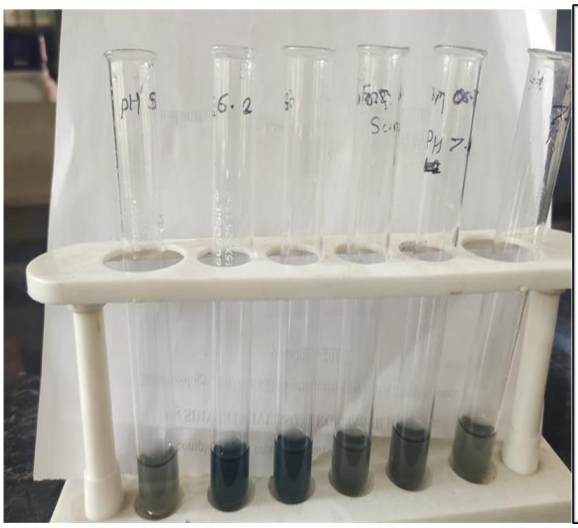
Table No .4

- **Effect of pH**

Sr. no	Volume of substrate (ml)	Volume of enzyme	pH		Ammonium molybdate (ml)	ANSA (ml)		O. D at 660nm (Mung)	O.D at 660nm (Urad)
1	1 ml	0.5 ml	5.8		1 ml	0.4 ml		0.53	0.50
2	1 ml	0.5 ml	6.2		1 ml	0.4 ml		0.90	1.00
3	1 ml	0.5 ml	6.6		1 ml	0.4 ml		1.24	1.18
4	1 ml	0.5 ml	7.0		1 ml	0.4 ml		0.76	0.95
5	1 ml	0.5 ml	7.4		1 ml	0.4 ml		0.62	0.93
6	1 ml	0.5 ml	7.8		1 ml	0.4 ml		0.50	0.86







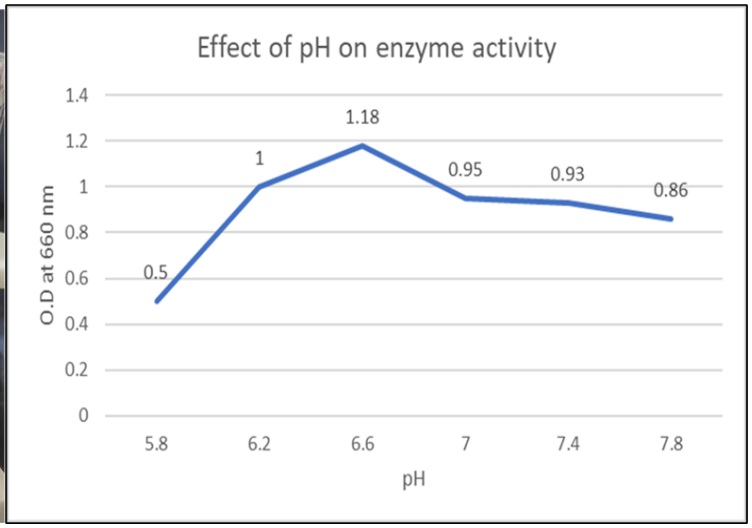
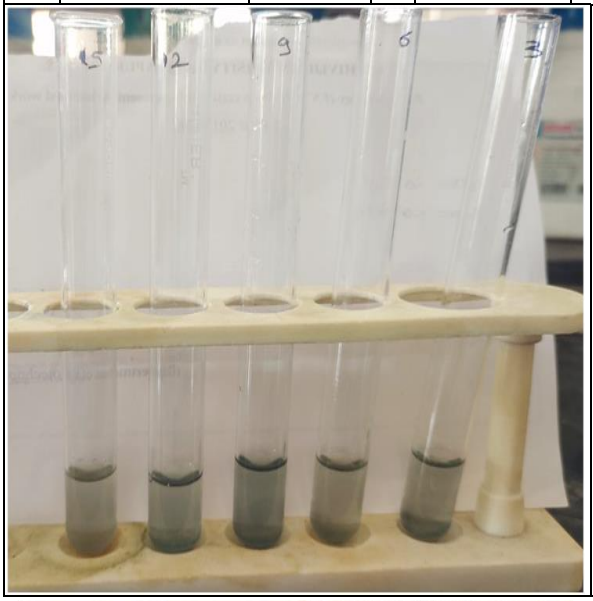
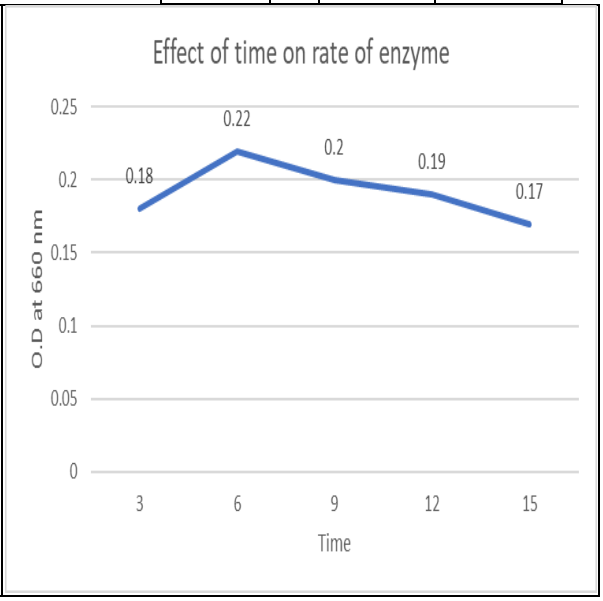


Table No. 5

- **Effect of Time**

Sr	Volume of substrate(ml)	Volume of enzyme		Incubation time	Ammonium molybdate (ml)	ANSA (ml)		O.D at 660nm (Mung)	O. D at 660nm (Urad)
1	1ml	0.5ml		3	1ml	0.4ml		0.18	0.93
2	1ml	0.5ml		6	1ml	0.4ml		0.22	1.78
3	1ml	0.5ml		9	1ml	0.4ml		0.20	1.30
4	1ml	0.5ml		12	1ml	0.4ml		0.19	1.25
5	1ml	0.5ml		15	1ml	0.4ml		0.17	1.20





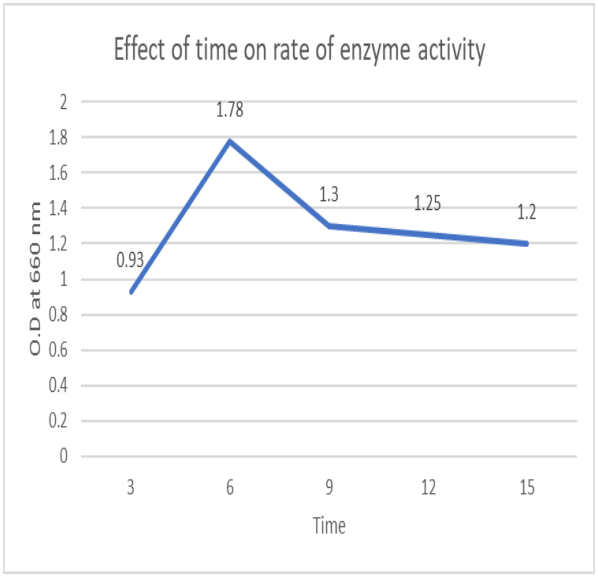
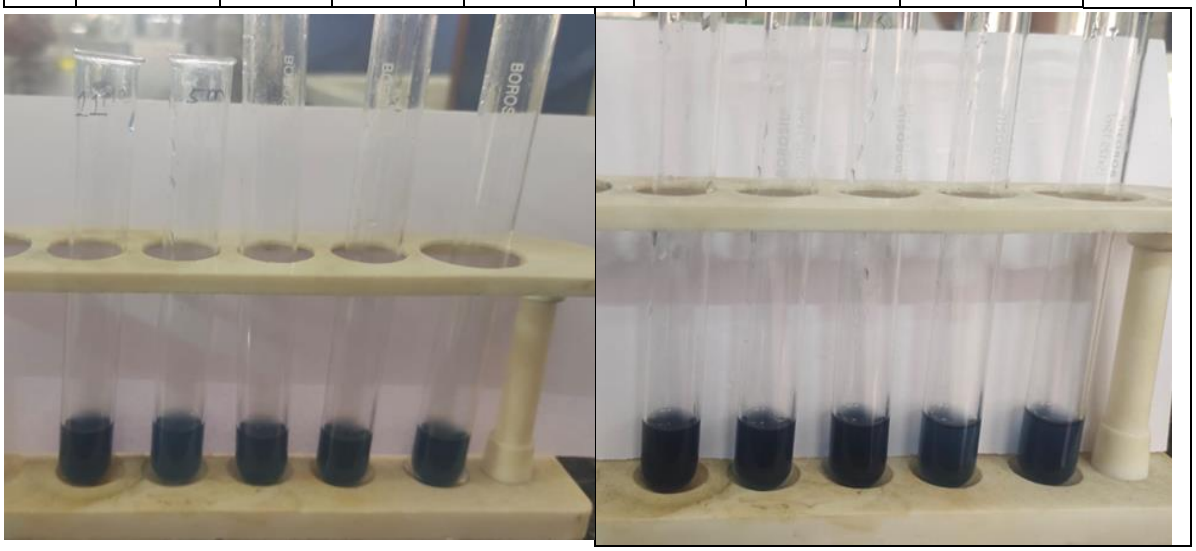


Table No. 6

- **Effect of Substrate Concentration**

Sr No	Substrate	Buffer	Enzyme	Ammonium Molybdate	ANSA	O.D at 660 nm(mung)	O.D at 660 nm (Urad)
1	0.0	2	0.5	1	0.4	0.0	0.0
2	0.1	1.9	0.5	1	0.4	0.37	0.69
3	0.2	1.8	0.5	1	0.4	0.40	0.70
4	0.3	1.7	0.5	1	0.4	0.42	0.82
5	0.4	1.6	0.5	1	0.4	0.44	0.85
6	0.5	1.5	0.5	1	0.4	0.48	0.90
7	0.6	1.4	0.5	1	0.4	0.55	0.92
8	0.7	1.3	0.5	1	0.4	0.60	0.96
9	0.8	1.2	0.5	1	0.4	0.62	1.05
10	0.9	1.1	0.5	1	0.4	0.78	1.06
11	1.0	1.0	0.5	1	0.4	0.78	1.06



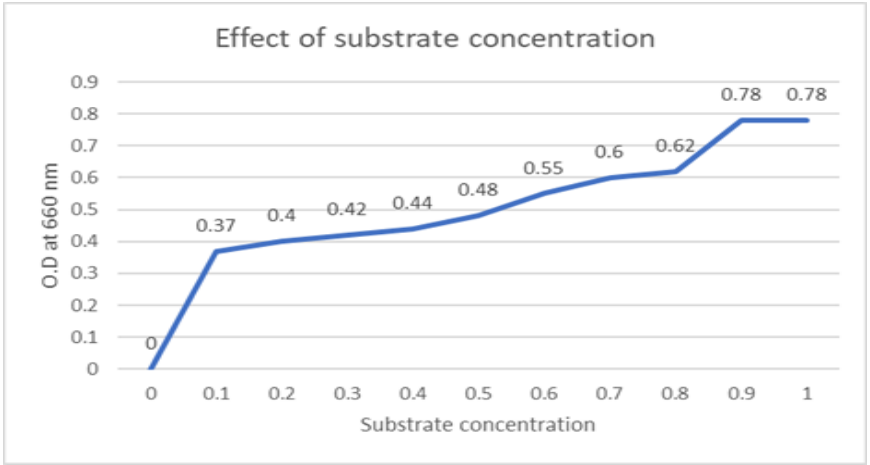
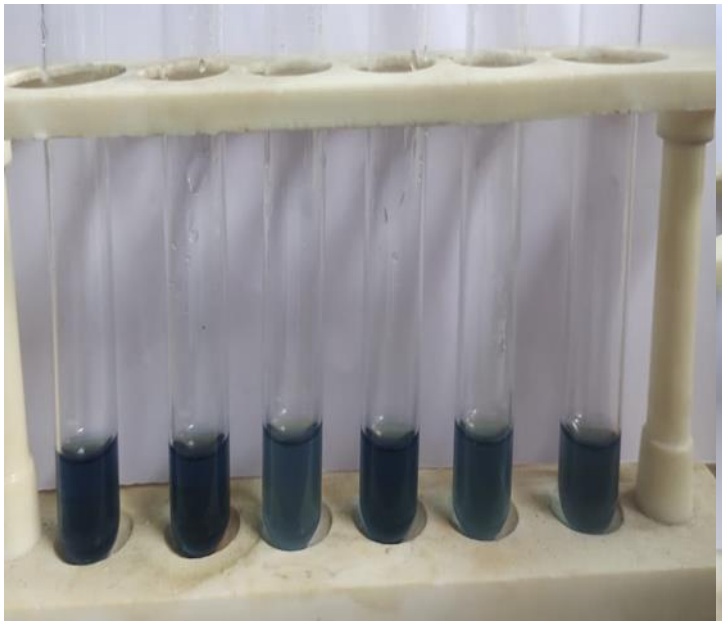
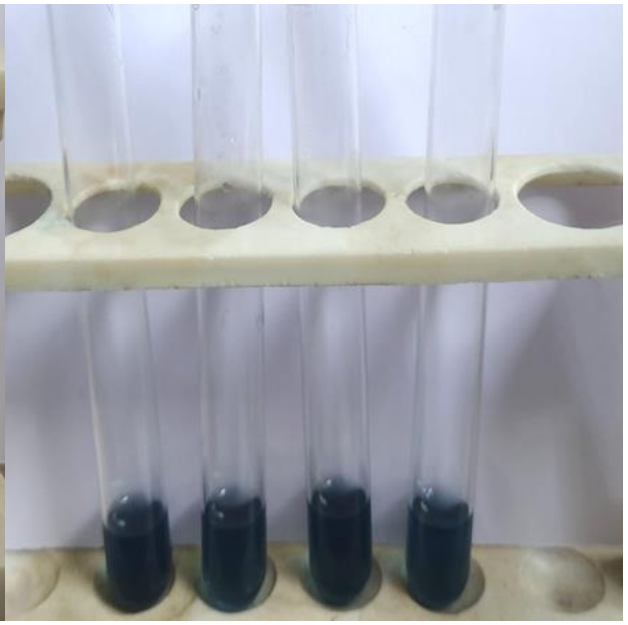


Table No .7





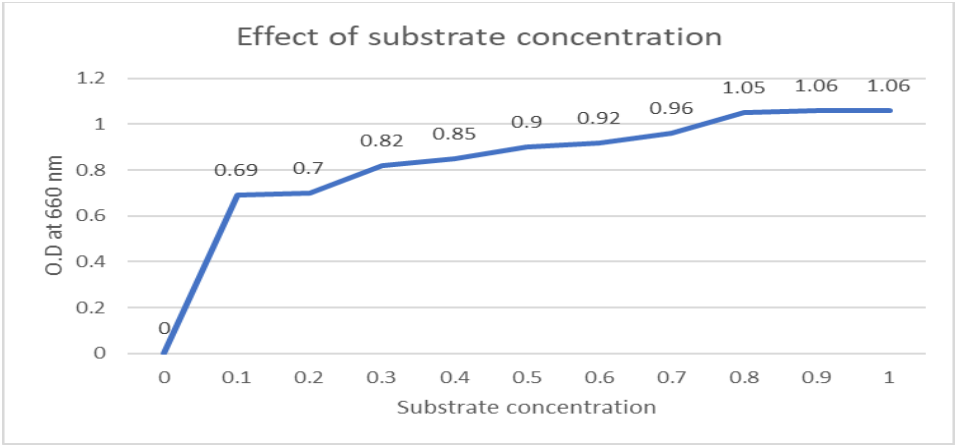

Effect of substrate concentration
O.D at 660 nm
1.2
1
0.8
0.6
0.4
0.2
0
0.69
0.7
0.82
0.85
0.9
0.92
0.96
1.05
1.06
1.06
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Substrate concentration

Table No.7

➢ Conclusion

- A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolyzing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group.
- Acid phosphatase is a phosphatase, a type of enzyme, used to free attached phosphate groups from other molecules during digestion
- These enzymes exhibit excellent performance on both animals and plants proteins. Thus, acidic phosphatase was detected in potato and alkaline in mung beans and urad seedlings.

➢ Reference

• Almeida VM, Marana SR (2019). Optimum temperature may be a misleading parameter in enzyme characterization and application. PLoS ONE 14(2):e0212977.

• Anand A, Srivastava PK (2012). A molecular description of acid phosphatase. Applied Biochemistry and Biotechnology 167(8):2174-2197.

• Anand A, Srivastava PK (2014). Isolation and enzymatic properties of a nonspecific acid phosphatase from Vigna aconitifolia seeds. Biotechnology and Applied Biochemistry 61(2):145-152.

• Asaduzzaman AK, Rahman HM, Yeasmin T (2011). Purification and characterization of acid phosphatase from a germinating black gram (Vigna mungo L.) seedling. Archives of Biological Sciences 63(3):747-756.

• Baldwin JC, Karthikeyan AS, Raghothama KG (2001). LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant Physiology 125(2):728-737.

• Bargaz A, Ghoulam C, Devron JJ (2013). Specific expression and activity of acid phosphatases in common bean nodules. Plant Signaling and Behavior 8(8):e25022.

• Bheri M, Mahiwal S, Sanyal SK, Pandey GK (2021). Plant protein phosphatases: What do we know about their mechanism of action? The FEBS Journal 288(3):756-785.

• Brautigan DL (2013). Protein Ser/Thr phosphatases - the ugly ducklings of cell signalling. The FEBS Journal 280(2):324-345.

• Collet JF, Stroobant V, Pirard M, Delpierre G, van Schaftingen E (1998). A new class of phosphotransferases phosphorylated on an aspartate residue in an amino-terminal DXDX(T/V) motif. Journal of Biological Chemistry 273(23):14107-14112.