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EXTRACTION, PARTIAL PURIFICATION AND KINETICS CHARACTERIZATION OF PROTEASE ENZYMES FROM STERCULIA FOETIDA

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ABSTRACT

Protein catalysts called enzymes speed up the pace at which chemical processes approach equilibrium. This study presents a comprehensive comparative analysis of enzymes extracted from *(sterculia foetida)* java olive, a plant species known for its diverse bioactive compounds. The isolation of this enzyme was done from a fruit seed (S. foetida). After the fruit seed was crushed, and the extract was extracted using mortar and pestle, an enzyme assays were used to determine the presence of protease enzymes. The enzymes of interest were precipitated using ammonium sulphate solutions (60%). After crude extract has been produced and followed by the partial purification by dialysis method. The partially purified seed samples were subjected to Lowry's technique for protein estimation and enzyme activity. To find the enzyme peak activity, a series of biochemical characterization, the maximum activity of protease at optimum pH and temperature were determined respectively. For various substrate concentrations, including gelatin, casein and skimmed milk, the apparent Vmax and Km values were calculated. Furthermore, (Native PAGE) was employed for the separation and visualization of proteins within the enzyme extracts. The kinetic properties of protease were determined by Michealis – Menten Kinetics, Lineweaver burk Kinetics was studied.

Keywords: *Sterculia foetida*, Protease, Ammonium sulphate solution, Lowry's method, Dialysis, enzyme assays, Biochemical characterization – Optimum PH and Temperature, Native-PAGE

INTRODUCTION

An enzyme for hydrolysing proteins is called proteinase. It has broad application value in the areas of food, medicine, and detergents and boasts a high sensitivity to biological components. The term enzyme was coined by Kuhne in 1878 from the Greek word, Due to their specific nature, enzymes can distinguish between substances with similar structural similarities. They can also catalyse reactions over a wide range of temperatures (0o C-110o C) and in the pH range of 2–14 according to their specific nature. This study reveals the presence of enzyme extracted from the fruit seed *Sterculia foetida* (java olive). The isolation of this enzyme was done from a fruit *Sterculia foetida* which is also commonly called as wild almond, java olive, peon, poon tree etc.

Sterculia foetida:

The spreading tree, *Sterculia foetida* Linn. (family: Sterculiaceae), also referred to as Java olive or wild almond, has an eye-catching umbrella shape. In India it is known as Jangli badam (Hindi), Gorapu badam (Tamil). It was first described in the year 1753 by Carolus Linnaeus. Historically, the leaves and seeds have held significant therapeutic value because to the presence of 53% steric acid, a component that has demonstrated intriguing pharmacological applications, including possible anti-inflammatory, anti-diabetic, and anti-obesity effects. The tree produce edible seeds that are 11.4% protein and 30–36% fat.

Historically, bark and leaves have been used as a diuretic, aperient, and to cure rheumatism, obesity, gonorrhea, dropsy, and other skin conditions. Flavonoids and fatty acids abound in the plant. It is a tall stately tree, deciduous in the cold season and produces more or less whorled, horizontal branches. Leaves are crowded at the ends of branches and digitate; leaflets 7–9, coriaceous and glabrous beneath. Inflorescence racemose panicles, 15–30 cm long. The flowers are red and yellow or dull purple, having an offensive smell. The follicles are boatshaped, woody, bright red when ripe. The seeds are black, 10–15 in each follicle, not winged. The family Sterculiaceae is well known for its many benefits in the fields of commerce, medicine, and agriculture. *S. quadrifida* R. Br and *S. foetida* Linn are Sterculiaceae plants that are used medicinally and prized for their ornamental and fruit-bearing qualities.

Medicinal properties:

Sterculia Foetida has been used traditionally as a medicine for its wound healing, antimicrobial, antidiabetic, antioxidant, antimicrobial and anti-inflammatory properties. The seeds of *Sterculia foetida* are known for their laxative properties. They contain mucilage, a gel-forming fiber that can help with constipation. The seeds have been traditionally used to address digestive issues. However, scientific studies on its medicinal properties are limited and its potential health benefits are not medically proven. The leaves of this plant are used as herbal medicine as aperient, diuretic and as insect repellent.

Benefits of sterculia foetida:

This tree attracts a lot of pollinators. The smell of the flowers and rich nectar within attracts bees to the point where they may just start building their hive on *Sterculia foetida*. The fruit of the tree yields an oily nut. You can eat this nut raw or roasted. It tastes a lot like peanuts. The nut also yields an oil that tastes sweet and bland, similar to olive oil. It has many of the same health benefits as olive oil. This tree is grown world-wide because of its commercial use. While the many parts

of the tree are used in traditional medicine, its wood is used as timbre.

PROTEASE ENZYME:

Proteases are a broad class of enzymes that catalyze the hydrolysis of peptide bonds in proteins and polypeptides. They are also referred to as proteinases or proteolytic enzymes. Proteolyticenzymes are more than just isolated catalytic tools that find substrates to hydrolyze.enzymes are more than just isolated catalytic tools that find substrates to hydrolyze. Therefore, a wide range of specialized functional modules or domains are linked by many proteases to their catalytic domains, which confer substrate specificity, direct cellular localization, alter kinetic properties, and alter sensitivity to endogenous inhibitors. These domains are not catalytic; they include autoinhibitory prodomains that stop premature activation, archetypal sorting signals that guide these enzymes to their correct location, and ancillary domains that help homotypic or heterotypic contacts with other proteins, substrates, receptors, or inhibitors. Properties like substrate specificity, catalytic mechanism and active site, stability profile, optimal pH and temperature, and others vary between them. Proteases with high activity and stability in high alkaline range and high temperatures are interesting for bioengineering and biotechnological applications. The isolation of this enzyme was done from a fruit seed *Sterculia foetida* and extracted with 1x PBS buffer and enzyme assays were used to determine the presence of protease. The kinetic parameters controlling the rates of enzyme catalyzed reaction are initial substrate concentration, pH, and reaction temperature. The enzyme peak activity, a series of biochemical characterization, the maximum activity of optimum pH and temperature was determined respectively.

Protein purification from a solution is frequently achieved through the use of ammonium sulfate precipitation. Proteins' exposed polar and ionic groups allow them to form hydrogen bonds with water molecules in solution. Ammonium sulfate and other small, highly charged ions compete with the proteins to bind to the water molecules when added in high concentrations. Precipitation occurs as a result of the protein losing its water molecules and becoming less soluble. The number and location of polar groups, the molecular weight of the protein, the pH of the solution, and the temperature at which the precipitation occurs are important variables that influence the concentration at which a specific protein will precipitate. In order to precipitate desired proteins, ammonium sulfate is typically added as a saturated solution or added directly as a solid.

This phenomenon, known as "salting-in," occurs when more salt is added generally to reduce the solubility of proteins at low salt concentrations (<0.15M), as ions protect the protein molecules from the charges of other molecules. This occurs as a result of the proteins and dissolved salt competing for few available water molecules, which raises the water's surface tension and forces the proteins to fold more tightly. This method was followed by the partial purification of dialysis method.Dialysis is a method of separation that uses passive, selective diffusion through a semi-permeable membrane to help extract unwanted, small compounds from macromolecules in solution. In this procedure, a semipermeable membrane allows the movement of certain molecules based on size. This method can be applied to the removal of buffer, known as desalting, or exchanging buffer molecules or ions from a protein solution. On opposing sides of the membrane are a sample and a buffer solution referred to as the dialysate; typically (200–500

times the volume of the sample). Larger molecules in the sample are trapped on the membrane's sample side while smaller molecules and buffer salts flow through the membrane freely, lowering the concentration of those molecules in the sample. By altering the dialysate buffer, more contaminants can permeate into the dialysate and the small molecules that were previously present in the sample are removed. The concentration-differential across the membrane that drives the dialysis process is created by the difference in composition between the sample and dialysis buffer solutions. A high-volume ratio of buffer to sample aids in preserving the concentration gradient. The results of dialysis are also influenced by the number of dialysate buffer changes and the duration of the procedure. The reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the folinocalyc acid, phosphomolybdic phosphortungstic acid to heteropoly molybdenum blue by the copper-catalyzed oxidation of aromatic acids form the basis of the Lowry method of protein concentration determination [Dunn, 13]. Since the Lowry method is sensitive to pH variations, the assay solution's pH should be kept between 10 and 10.5. Low protein concentrations do not affect the Lowry technique's sensitivity. Protein concentrations range from 0.10 to 2 mg per milliliter according to Dunn [1992] and from 0.005 to 0.10 mg per milliliter according to Price [1996]. The limited pH range that the Lowry method is accurate within is its main drawback. However, we will be using very small volumes of sample, which will have little or no effect on pH of the reaction mixture. In this work to find the total Protein content from crude and ammonium sulphate precipitation and the OD value was determined.

Protease activity assays were run at various pH values between 5 and 11. In order to determine the ideal pH for maximum protease activity. The following buffers were used to prepare 0.65% casein for that purpose: 0.05 M citrate-phosphate buffer (pH 5 to 6), Tris HCl buffer (pH 7 to 8), and glycine buffer (pH 9 to 11). Protease activity was measured as previously mentioned after reaction mixtures were incubated for 30 minutes at 37°C. By pre-incubating the enzyme without substrate at various pH values (5 to 11) using various buffers, the impact of pH on protease stability was ascertained. The assay procedure was followed to determine the residual protease activity. The effect of temperature on the protease activity was determined by incubating the reaction mixtures at different temperatures ranging from 30 to 80°C. After incubation, protease activity was assayed. The effect of temperature on the protease stability was determined by pre-incubating the enzyme without substrate at different temperatures (30, 40, 50, 60, 70 and 80°C). The residual protease activity was determined according to the assay procedure. Furthermore, (Native PAGE) was employed for the separation and visualization of proteins within the enzyme extracts.

Native PAGE is one of the most powerful techniques for studying the composition and structure of native proteins, since both the conformation and biological activity of proteins remain intact during this technique. Molecular weight (MW) measurements are one of its infrequent uses; for this purpose, SDS-PAGE is simpler and more accurate than native PAGE. Finding standard proteins with the same shape, partial specific volume, and degree of hydration as the native protein under study can frequently be challenging. With gradient gels for native PAGE, complex protein mixtures can be separated on a single gel and the protein bands are sharpened. This technique file outlines an enhanced protocol for PhastGel TM gradient 8–25 and PhastGel gradient 10–15 native

polyacrylamide gel electrophoresis (native PAGE) using PhastGel native buffer strips. Commercially available proteins and unrefined protein extracts have been used to optimize the process. As a result, it is broadly applicable and provides a useful foundation for creating techniques for particular applications.

Sterculia foetida

Sterculia foetida is a deciduous wild plant and is mainly distributed in the tropical and sub-tropical regions between the 30°N and 35°S latitudes. It grows as a large and straight tree, about 40 m height and 100–120 cm diameter, with a lifespan of at least 100 years (Silitonga et al., 2013). A huge number of petioles, 125–230 mm in length, grow on *Sterculia foetida* trees, with palmately compound blades containing 7–9 leaflets growing at the end of the petioles (Worldagroforestry.org, 2013). The leaves of *Sterculia foetid*a were collected from the trees available in the local area near Pune in bulk quantity.

Sterculia foetida L. is a species of tree native to regions of East Africa north of Australia and cultivated in many countries such as India and Sri Lanka. The exudate has a color that varies from light yellow to amber, to obtain the polysaccharide it is necessary a purification process to eliminate impurities, such as tree bark, inorganic salts, and nucleic acids, divided into stages of dissolution, centrifugation, precipitation in ethanol and drying. The plant specimen was authenticated by matching with the voucher specimen AHMA:581 available with the Agharkar Herbarium of Maharashtra Association at Agharkar Research Institute, Pune, India. These shade-dried leaves (1100 g) were extracted with ethyl alcohol. The crude extract was obtained as a dark coloured viscous residue (35g, 3.18%). This total crude extract was subjected to pharmacological screening (A.M. Mujumdar et al. Published on: Jan 2011)

Sterculia foetida plants are commonly known as bastard poon tree, java olive tree, wild almond tree, and skunk tree. Green *Sterculia foetida* fruit shell fiber has been extracted and used in the present research work, employing the opinion to utilize such waste fruit (Teli and Pandit, 2018a). *Sterculia foetida* is a tropical tree, while nature has given plenty of renewable and eco-friendly resources.

PLANT COLLECTION AND EXTRACTION PROCESS

The seeds of *Sterculia foetida* were collected from Barkup, Kutubdia, Cox's Bazar, Bangladesh in May 2019 and were identified by Professor Shaikh Bokhtear Uddin, Department of Botany, University of Chittagong, Chittagong, Bangladesh. A voucher sample was deposited within the herbarium for additional reference (DACB: 35459. The methanol extract of S. foetida (MESF) was prepared by macerating ground dried seeds (800 g) in methanol (2 L) with random stirring for 15 days. The solution was evaporated to dryness in a rotary evaporator obtaining a 20 g extract.(Najmul Alam et al. Published: 3 June 2021).

The seeds of *S. foetida* are fit for human consumption, representing a good source of fats (30%–36%) and proteins (11.4%). The leaves and bark are often used as diaphoretic, diuretic, and aperient agents and have been applied to the treatment of rheumatism, obesity, gonorrhea, edema, and skin disease. Based on an evaluation of the literature, this plant has been found to containing various phytoconstituents, including alkaloids, flavonoids, terpenoids, phenols, and steroids. Additionally, *S. foetida* ethanolic seed extract have antioxidant and anticancer activity. (Naureen

Banu et al. Published: 2021 Jun 3).

EXTRACTION USING BUFFER

Extraction of antimicrobial peptides was done by grinding fresh 1gm leaves of each plant in 3.3 ml of 1 Molar Tris-HCl (pH 7.5) and 0.5 Molar NaCl. The sample buffer mixture was incubated for 12 hours at 4C. After that, samples were centrifuged at 12,000 rpm for 20 minutes in a centrifuge at 4C. After centrifugation, the resulting supernatant was analysed for protein concentration determination.31 Extraction of antibacterial protein/peptide by phosphate buffer saline Fresh 0.3 g leaves of each plant sample were ground in a pre-chilled mortar pestle to make a fine powder in 4.5 ml of PBS. Samples were then passed through the freeze thaw cycle 3 times at the interval of about 12 hours. After that the tubes were centrifuged at 10000 rpm for 10 minutes. Collected the supernatant into a separate tube and stored at 4 degrees for quantification. (Ume Habiba et al. 2010).

AMMONIUM SULPHATE PRECIPITATION

Ammonium sulphate, (NH4)2SO4, is often used for salting out because of its high solubility, which allows for solutions of very high ionic strength, low price, and availability of pure material. Additionally, NH4 and SO4 2 are at the ends of their respective Hofmeister series and have been shown to stabilize protein structure (Burgess, 2009). Some proteins follow a reversal of Using ion exchange chromatography to purify a recombinantly expressed protein, Gel filtration chromatography (Size exclusion chromatography) of proteins, Use and Application of Hydrophobic Interaction Chromatography for Protein Purification and Hydroxyapatite Chromatography: Purification Strategies for Recombinant Proteins); thus, if the protein is naturally expressed in the host cell, cloned in the absence of tags, or has an unexposed tag, salting out is an ideal choice for purification. This method may also be applied to purify a protein of unknown sequence. Since the sample is in a high concentration of (NH4)2SO4 at the end of the experiment, hydrophobic interaction chromatography (HIC) may be used immediately to further purify the sample. (Krisna C Duong-Ly et al).

The mechanism of salting-out is based on preferential solvation due to exclusion of the cosolvent (salt) from the layer of water closely associated with the surface of the protein (hydration layer). The hydration layer, typically 0.3 to 0.4 g water per gram protein (Rupley et al., 1983), plays a critical role in maintaining solubility and the correctly folded native conformation. There are three main protein-water interactions: ion hydration between charged side chains (e.g., Asp, Glu, Lys), hydrogen bonding between polar groups and water (e.g., Ser, Thr, Tyr, and the main chain of all residues), and hydrophobic hydration (Val, Ile, Leu, Phe). In hydrophobic hydration, the configurational freedom of water molecules is reduced in the proximity of a polar residues. This ordering of water molecules results in a loss of entropy and is thus energetically unfavorable. When salt is added to the solution, the surface tension of the water increases, resulting in increased hydrophobic interaction between protein and water. The protein responds to this situation by decreasing its surface area in an attempt to minimize contact with the solvent—as manifested by folding (the folded conformation is more compact than the unfolded one) and then self-association leading to precipitation. Both folding and precipitation free up bound water, increasing the entropy of the system and making these processes energetically favorable. Timasheff and his colleagues

provide a detailed discussion of these complex effects.

BIOLOGICAL IMPORTANCE FOR PROTEASE

Proteases are essential to the existence of all kinds of living organisms, and many of the reasons are fairly obvious. For example, living organisms are composed largely of proteins, and commonly need to obtain the amino acid units from which they are synthesized by breaking down preexisting protein molecules. It also follows that the processes of growth and remodeling of cells, as well as the tissues of multicellular organisms, require breakdown of old protein molecules in concert with the synthesis of new ones. In addition to the gross hydrolysis of proteins to amino acids, there are myriad forms of limited proteolysis that are no less vital. For instance, many newly synthesized protein molecules require proteolytic processing to convert them to their biologically active forms. Viruses are commonly dependent upon proteases to segment their polyproteins. In both prokaryotic and eukaryotic cells, secreted proteins require proteolytic removal of the signal peptide in the secretory pathway. Many proteases are themselves synthesized as proenzymes that are catalytically inactive, and are subsequently activated proteolytically at a biologically appropriate time and place. This proteolytic "switching" of the activity of proteins represents an important mechanism of biological regulation. Unlike regulation through reversible binding to receptors, this process is irreversible, and being catalytic it requires only trace amounts of the effector molecule and can therefore provide a large amplification factor. however, there are many others. For instance, the proteolytic destruction of proteins and polypeptides with signaling functions serves to keep the biological signals local in time and space. (János András Mótyán.et al. 2013).

LOWRY'S METHOD FOR PROTEIN ESTIMATION

This method involves the pretreatment of protein with alkaline copper sulphate in the presence of tartrate followed by the addition of Folin's phenol reagent. In a dipeptide copper complex, the copper atom is held in a coplanar tridentate chelate involving the free amino nitrogen, the peptide bond nitrogen and the free carboxyl group. The additional presence of a side-chain nitrogen, as in asparagine and histidine, helps in the formation of a more stable, quadridentate chelate. In a tripeptide copper complex, the quadridentate chelate can be formed with the two available peptide bonds without the participation of a functional group. The chelated protein then reduces the Folin's reagent, a mixture of phosphomolybdic-tungstic acids (Creighton. et al. 1984).

The Folin reagent has a half-life of 8 s at the alkaline pH of the reaction (pH = 10) and therefore rapid electron transfer from the protein is crucial for ultimate color yield. Thus, it is evident that the whole peptide backbone is involved in the color formation when pretreated with copper. Only the chromogenic amino acids such as tyrosine, tryptophan, cysteine and to a lesser extent histidine can contribute to the color yield without copper-pretreatment (Chou & Goldstein 1960). D Amino acids behave in an identical manner to their L-counterparts. Pretreatment with copper does not affect the color formation by tyrosine or tryptophan, but color formation is drastically reduced for cysteine and peptide containing cysteine because the sulfhydryl group is blocked by copper ion. It is interesting to note that penicillin, which contains a dipeptide of cysteine and valine with a blocked amino group, yielded more color than the corresponding free dipeptide; however, when it is cleaved by /? -lactamase to give two amino acids linked through sulfur of cysteine, the color yield is high. Desthiopenicillin did not give any color and penicillamine (PP-dimethyl cysteine)

yielded less color than cysteine confirming the involvement of the sulfhydryl group in color production.

NATIVE PAGE

A biochemical method called Blue Native Page (BN-PAGE) is used to analyze protein complexes, especially those that are embedded in biological membranes. Using mild detergents to solubilize membrane proteins and retain their native conformation during electrophoresis, this technique maintains the native structure of proteins. Protein complexes can be separated by the electrophoresis procedure, which is carried out in a polyacrylamide gel containing Coomassie Brilliant Blue dye. Proteins can be analyzed in their native state using BN-PAGE as opposed to conventional SDS-PAGE. Following electrophoresis, the gel can undergo several investigations that provide information about the stoichiometry, interactions, and composition of membrane protein complexes inside the lipid bilayer. (Wittig, I., Braun, H. P., & Schägger, H., 2006).

The apparent molecular mass of intact protein complexes can be distinguished using a conventional polyacrylamide gel electrophoresis technique that mixes Coomassie Blue dye with mild detergents. This approach has been termed Blue-Native Polyacrylamide Gel-Electrophoresis (BN-PAGE), referring to the blue-colored gel and the gentle way of solubilization yielding native and enzymatically active protein complexes. When examining the respiratory protein complexes of the electron transfer chains of many organisms, such as bacteria, yeasts, mammals, and plants, BN-PAGE has emerged as the preferred technique. It gives information on native interactions between highly hydrophobic protein sets and enables their separation in two dimensions for examination. (Eubel, H., Braun, HP. & Millar, A., 2005)

MATERIALS AND METHODOLOGY

SEED COLLECTION

The fresh edible and healthy seed *Sterculia foetida* was collected from VIM Alloy, Chennai, India. Later it was stored in refrigerator.

CRUDE EXTRACTION

200gm of fresh seed was washed and sliced, were homogenized with 200ml of 1x PBS buffer (pH-7.5) using motor and pestle. The suspension was filtered by muslin cloth and later it was filtered by filter paper, and centrifuged at 8000 rpm for 10 mins. Discarded the pellet and the supernatant was collected, and it was designated as crude enzyme was further used for protein estimation, screening of enzyme activity, ammonium sulphate precipitation and dialysis analysis.

ENZYME ASSAYS

Enzyme assays can be used for a variety of purposes, which include identifying the presence of an enzyme, investigation of specific enzyme kinetics or the activity of inhibition within a sample. In this study to identify the presence of protease enzyme in these seeds. 0.5ml of 1% Gelatin, Casein, and skimmed milk was added in 1ml of enzyme and incubate at 37◦C for 10mins. Later, 5ml of 110mM Trichloro acetic acid was added. Centrifuge the resulting mixture for 8000 rpm for 10 mins. Then, 2ml of supernatant in a test tube were collected. To this test tube, 5ml of (500mM Na_2CO_3) alkaline solution was added. now, 1ml of 25% Folin phenol was added to the solution. As a

result, the colour changes to dark blue indicating the presence of protease. Furthermore, to estimate the protein and protease assay and optimization for further analysis.

AMMONIUM SULPHATE PRECIPITATION

Ammonium sulfate precipitation is a useful technique as an initial step in protein purification because it enables quick, bulk precipitation of cellular proteins. It is also often employed during the later stages of purification to concentrate protein from dilute solution following procedures such as gel filtration.

1. Here we used, 200ml 0f sample was transferred to the conical flask and placed a magnetic stirrer into the flask.
2. While the samples were stirring, the weighed ammonium sulphate powder at the concentration of 60% (120gm) was added slowly respectively.
3. The amount of weighed ammonium sulphate was the same as the amount of supernatant.
4. After the total amount of ammonium sulphate was added to the conical flask and centrifuged at 5000 rpm for 15min.
5. Discarded the supernatant and the pellet was collected.
6. 3ml of PBS buffer was added in each tube and centrifuge for 8 mins at 6000 rpm and the supernatant was removed or discarded using a micropipette from each tube and the pellet was collected and stored in refrigerator for further analysis.

DIALYSIS

Dialysis is a separation technique that facilitates the removal of small, unwanted compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane. Molecules larger than the pores cannot pass through the membrane but small molecules can do so freely. Frequently used buffers for protein samples are phosphate buffer, TRIS, MOPS, and HEPES. Here, Dialysis membrane was pre-treated with 1X PBS (pH 7.5). One end was tied using twine and checked for leakage using buffer. Then the sample was loaded and other end was also tied. The loaded dialysis membrane was soaked in 1X PBS (pH 7.5). This was kept at 4˚C overnight. Then next day transfer the sample to the tube and store at -20˚C for further analysis.

Table: 1 Concentration of Phosphate Buffered Saline.

Phosphate Buffered Saline (pbs)	
NaCl	0.8 g
KCl	0.2 g

KH_2PO_4	0.2 g
Distilled water	1000 mL
pH	7.2

PROTEIN ESTIMATION

- **LOWRY'S REAGENT PREPARATION**

- Solution A -1g Na_2CO_3 in 100 ml 0.1M NaOH (0.1M NaOH -0.4g NaOH in 100ml distilled water)
- Solution B – 0.05g $CuSO_4$ and 0.1g sodium potassium tartrate in 10 ml distilled water
- Lowry reagent - 50 ml sol A + 1ml sol B.
- STD: BSA –Bovine Serum Albumin.

100-500 µl of working standard solution was taken. 100 µl of protein sample was taken in another test tube. Make up to 1 ml of distilled water including blank. 5 ml of Lowry reagent was added in all the tubes. Mix thoroughly, incubate all the tubes at room temperature for 15 minutes. 500 µl of Folin Phenol reagent (1:2) was added and mixed well thoroughly. Incubate for 30 min at dark room and observe blue color and record the OD value at 620nm.

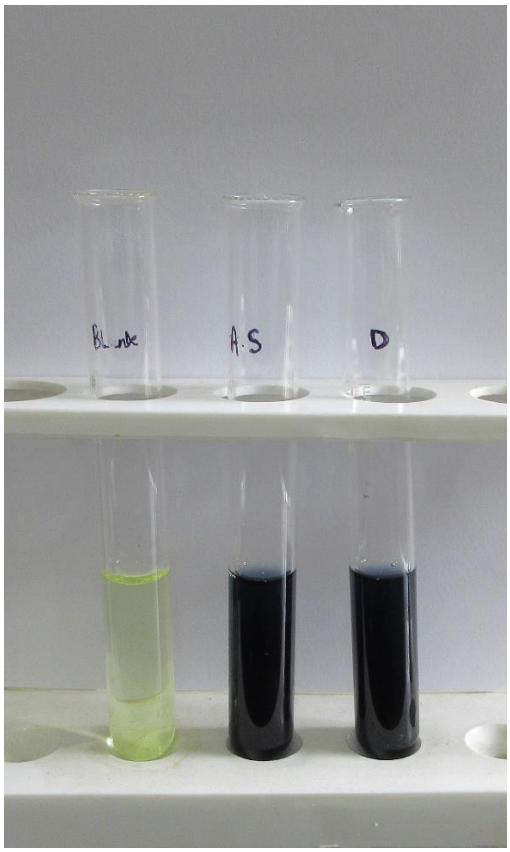


Fig.2. The above picture represented the protein sample observes dark blue color.

PROTEASE ASSAY PROCEDURE

To the sample tubes 2ml of 0.1M (pH 7.5) phosphate buffer, 0.5ml casein and 0.5ml of enzymes was added. To blank 3ml of 5% H_2SO_4 was added. Incubate for 10 minutes at 40ºC in water bath. The enzyme activity was stopped by adding 3ml of 5% H_2SO_4 solution (Sample tubes alone). Incubate for 60 minutes at room temperature. Centrifuge at 10,000 rpm for 10 minutes. Exactly 2ml of supernatant was mixed with 3ml of 2% Na_2CO_3 and 1ml of folin phenol reagent. The blue color was developed and read the OD value at 660nm.

BIOCHEMICAL CHARACTERIZATION FOR PROTEASE PRODUCTION

DETERMINATION OF OPTIMUM pH

2 ml buffer (Tris-HCl) was taken in 6 test tubes separately and their pH was altered to 4, 5, 6, 7, 8, and 9 accordingly. To the buffer, 2ml of substrate (gelatine 1%) followed by 200 µl of purified enzyme was added and allowed to incubate for 15 mins at room temperature.

DETERMINATION OF OPTIMUM TEMPERATURE

2 ml buffer (Tris-HCl) was taken in 6 test tubes separately. 2ml of substrate (gelatine 1%) was added and allowed to incubate for 15 mins at 37˚C, to which 200 µl of purified enzyme was added. The 6 test tubes were later maintained in different temperatures ranging from 20˚C - 70˚C for 15 min.

DETERMINATION OF OPTIMUM SUBSTRATE

2 ml buffer (Tris-HCl) was taken in 3 test tubes separately. 2ml of 3 different substrates (1% casein, gelatin and skimmed milk) were added and allowed to incubate for 15 mins at room temperature. This was further followed by protease assay.

NATIVE PAGE PROCEDURE (Laemmuli, 1970)

A clean notched and rectangular glass plates were assembled and kept in vertical position by placing them in a gel casting stand. The separating gel mixture was prepared and poured into the glass plates uniformly without any leakage. After polymerization of the separating gel, the stacking gel so prepared was poured over it and the comb was inserted gently. This setup was kept for few minutes to allow the gel to polymerize completely. The comb was then carefully removed and wells formed in the gel were rinsed with distilled water completely. 50 µl of isolated protein samples were mixed with 50 µl of sample solublizing buffer (1:1). The protein samples along with standard protein marker were then loaded into the respective wells. The gel was electrophoresed at 50- 100v for 3-4 hours to allow the sample to run completely into the gel. After electrophoresis, the gel was placed in coomassie blue staining solution for 1 hour and washed thoroughly with distilled water. The stained gel was then placed in destaining solution and washed with distilled water and added 20 ml of destaining solution with 20 ml of distilled water and kept in overnight incubation. The proteins fractionated into bands were observed and their molecular weight was determined using standard marker.

RESULTS AND DISCUSSION

CRUDE EXTRACTION

200 grams of java olive seeds were cut into small pieces and ground into paste by adding 200 to 250 ml of 1X PBS (pH 7.5). The paste was then filtered and the extract was further centrifuged. After centrifugation, the supernatant was collected and used for ammonium sulphate precipitation.

Fig.3. Seed sample of *Sterculia foetida.*

Fig.4. Ground seed sample of *Sterculia foetida.*

Fig.5. Representative image of homogenized sample using 1X PBS buffer

Fig.6. Filtered sample of crude *sterculia foetida* extract using 1X PBS buffer.

AMMONIUM SULPHATE PRECIPITATION

Precipitate sample with 60% of ammonium sulphate. Ammonium sulphate was added in small amount while stirring continuously. After complete dissolving incubate over night at 4 °C. Centrifuge at 8,000 rpm for 10 min. Collect the pellet and suspend in Tris-HCl buffer (pH 7.5) and used for purification (dialysis).

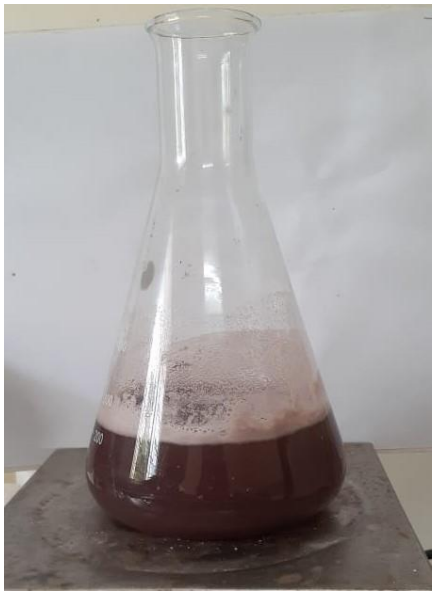


Fig.7. The above picture represented 60% of Ammonium sulphate precipitation sample.

DIALYSIS

The maximum activity and maximum protein were determined by Protease Enzyme assay and Lowrys method of Protein estimation. The dialysed sample was later diluted with 1X PBS buffer and used for Biochemical characterization study.

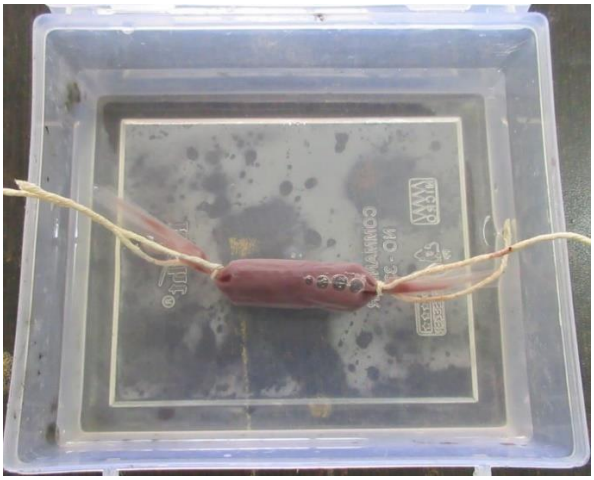


Fig.8. Dialysis of sample treated with 1X PBS.

PROTEIN ESTIMATION USING LOWRY'S METHOD

Table: 2 Determination of total Protein content from dialysed and ammonium sulphate precipitation (crude sample) and the OD value was determined using Lowry's method.

BSA Concentration (µg/ml)	**100 µg**	**200 µg**	**300 µg**	**400 µg**	**500 µg**
OD	0.10	0.20	0.30	0.42	0.53

Sample Concentration (100 µg/ml)	**Dialyzed**	**Ammonium sulphate (crude sample)**
OD	0.95	1.04
Amount (µg/ml)	950	1040

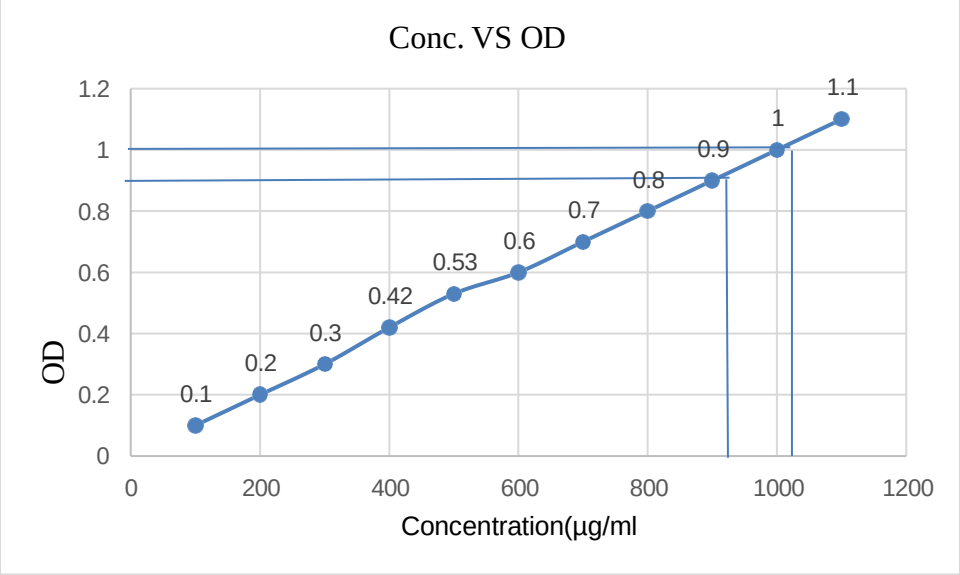


Fig.9. Graphical representation of determination of total protein content using Lowry's method.

The OD values of the crude sample and dialysed sample were determined from colorimeter and was found to be 1.04 nm and 0.95 nm and the graph was plotted OD Vs concentration with the standard values. The graph was found to be linear and from the graph.

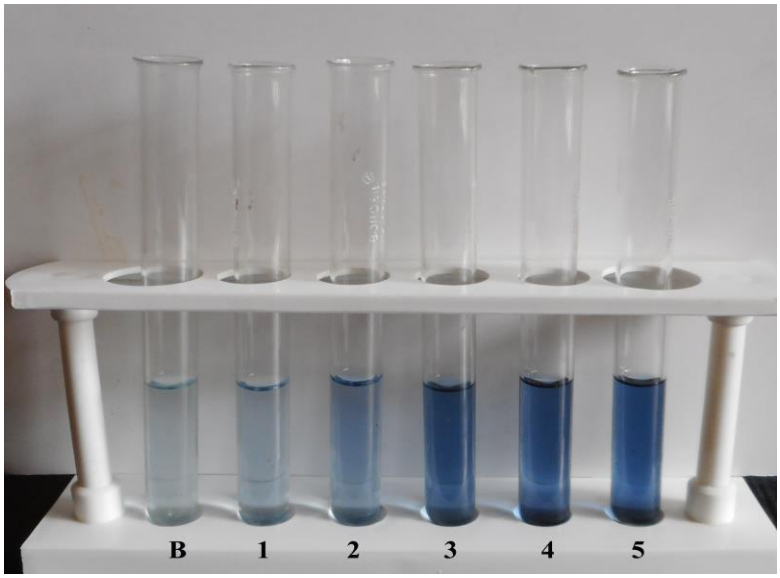


Fig.10. Standard protein samples at different concentration.

PROTEASE ASSAYS

Table 3: Determination of enzyme activity of protease enzyme.

Indication of blue color developed was read at 660nm and the standard (tyrosine) OD value was determined.

Sample Concentration (µg/ml)	**100**	**200**	**300**	**400**	**500**
Standard (Tyrosine) OD	0.28	0.39	0.46	0.53	0.67

Sample Concentration (100 µg/ml)	**Dialyzed**	**Ammonium sulphate crude**
(Enzyme) OD	**0.11**	**0.37**
Amount (µg)	**40**	**180**

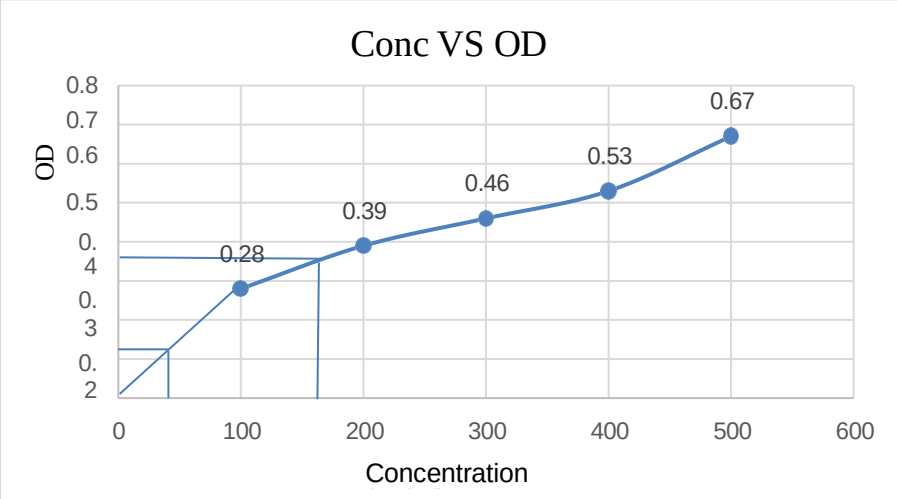


Fig.11. Graphical representation of enzyme activity from protease enzyme.

The graph was plotted OD VS concentration of sample. The OD values of the Enzyme activity of 60% ammonium sulphate crude sample and dialysed sample were determined from colorimeter and was found to be 0.37 nm and 0.11 nm.

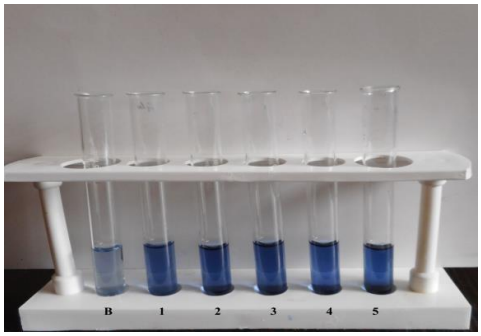


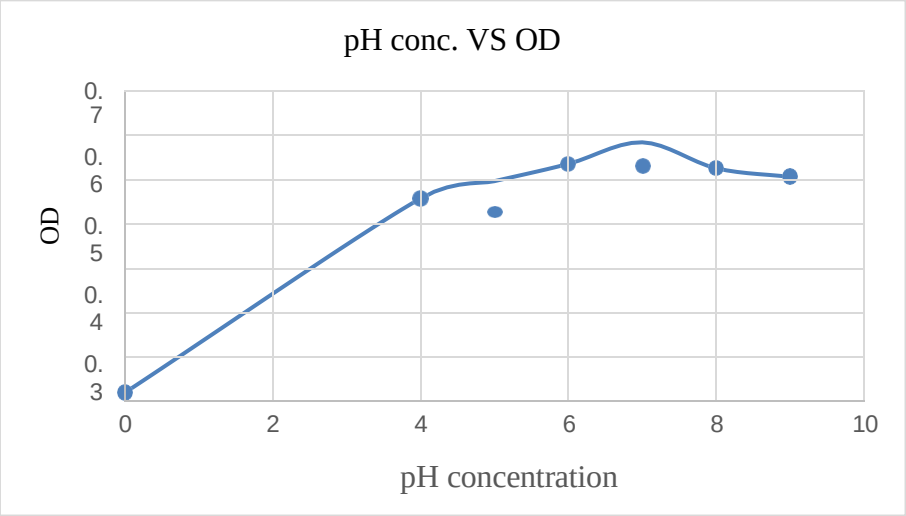
Fig.12. Standard protease assay.

5.4 OPTIMUM pH CONCENTRATION OF PROTEASE:

Table 4: Determination of optimum pH of enzyme activity.

pH and OD Values

4	**5**	**6**	**7**	**8**	**9**
0.45	**0.49**	**0.53**	**0.58**	**0.52**	**0.50**



From the biochemical characterization assays, the enzyme activity was calculated for optimum pH was found to be **7** and OD **0.58nm**.

Fig.13. Graphical representation of optimum pH of enzyme protease.

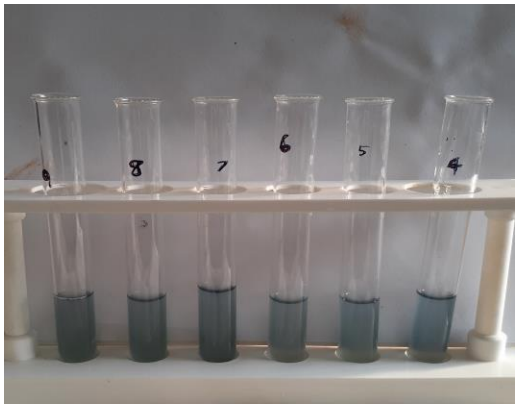


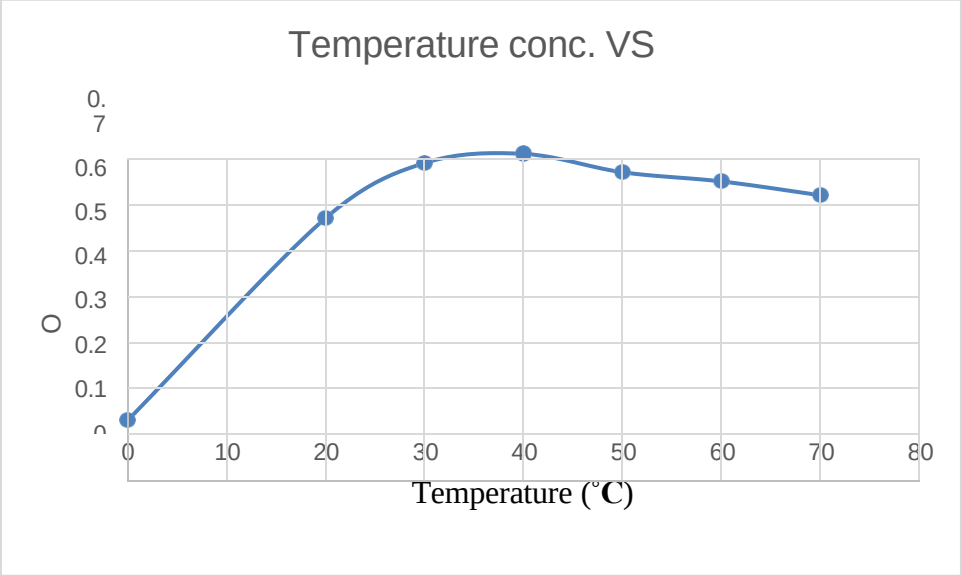
Fig.15. pHconcentration of samples

OPTIMUM TEMPERATURE CONCENTRATION OF PROTEASE:

Table: 5 Determination of optimum temperature of enzyme activity.

TEMPERATURE and OD values

20˚C	30˚C	**40˚C**	50˚C	60˚C	70˚C
0.44	0.56	**0.58**	0.54	0.52	0.49



From the biochemical characterization assays, the enzyme activity was calculated for optimum temperature was found to be **40˚C** and OD **0.58nm**.

Fig.15. graphical representation of optimum temperature of enzyme protease.

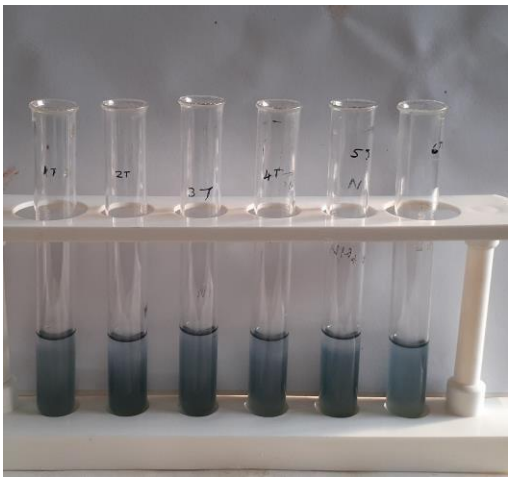


Fig.16. Temperature concentration of samples

OPTIMUM SUBSTRATE CONCENTRATION OF PROTEASE:

Table: 6 Determination of substrate conc. of enzyme activity.

SUBSTRATE

Gelatin	**Skimmed milk**	**Casein**
0.85	**0.11**	**0.42**

Gelatin, skimmed milk and casein are used as the main substrate to determine the protease activity. The maximum activity of substrate concentration of protease was found to be 0.85 in gelatin.

Fig.17. The above picture represented substrate concentration of sample.

NATIVE- PAGE

The proteins fractionated into bands were observed and their molecular weight was determined using standard marker. (Medium range protein).

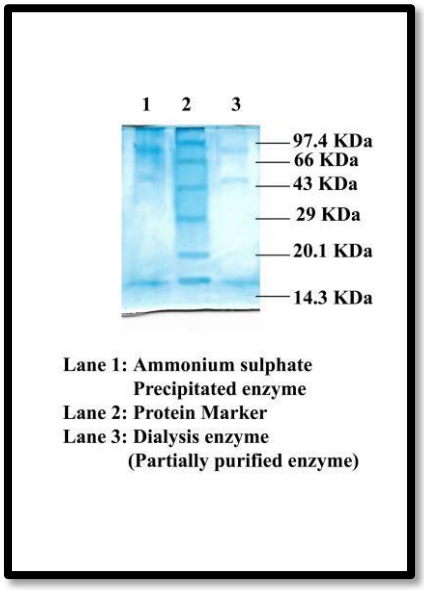


Fig.18. Native-PAGE of protease enzyme

SUMMARY AND CONCLUSION

In conclusion, this study delves into the catalytic prowess of enzymes extracted from the (*sterculia foetida*) java olive, specifically focusing on protease enzymes. The meticulous process of isolation involved crushing fruit seeds, extracting through mortar and pestle, and subsequent precipitation using ammonium sulphate solution. The resulting crude extract underwent partial purification via dialysis, and Lowry's technique was employed for protein estimation and enzyme activity determination. Biochemical characterizations revealed crucial insights into the enzymatic behavior, with optimal pH and temperature conditions identified for maximum protease activity. The study encompassed a thorough analysis of substrate concentrations, including gelatin, casein, and skimmed milk, yielding calculated apparent Vmax and Km values. Native PAGE was instrumental in separating and visualizing proteins within the enzyme extracts, providing a comprehensive understanding of the enzyme composition. The exploration of kinetic properties through Michealis-Menten Kinetics and Lineweaver Burk Kinetics enhanced our comprehension of the protease's catalytic efficiency. This research not only contributes to the broader understanding of enzyme functionality but also sheds light on the unique bioactive compounds present in (*sterculia foetida*) java olive. The methodology and findings presented herein pave the way for future studies in enzymology and plant biochemistry, offering a valuable resource for researchers in these domains

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