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Isolation and purification of peroxidase from *Macrotyloma uniflorum* and its application.

Aparna K Dhavade*, and Deepak Khairnar.

Department of Biotechnology, Dr. D. Y. Patil, ACS College, Pimpri, Pune -4110018, Maharashtra, India.

ABSTRACT

Peroxidase enzymes are a versatile group of oxidoreductases that catalyse the oxidation of various substrates using hydrogen peroxide as an electron acceptor. These enzymes are widely distributed in nature, found in plants, animals, and microorganisms. Their ability to catalyse oxidative reactions makes them valuable in numerous biotechnological applications. Therefore, this investigation contains germination, isolation, and purification from horse-gram seeds and biochemical studies of peroxidase. The isolation begins with the homogenization of the plant source followed by a series of purification including centrifugation, ammonium sulphate precipitation, dialysis, and chromatographic techniques like ion-exchange chromatography and gel filtration chromatography. The resulting enzyme showed 41.5 purification fold and same had used for further biochemical studies. The enzyme is optimized at parameters such as pH, temperature, incubation time, and substrate concentration. Variation in these parameters help in finding the highest activity of the enzyme at a specific parameter and further use it all together at that given parameter. On 5th day germinated seeds of horse-gram showed optimized highest activity of peroxidase enzyme at pH 7 and temperature 37 ^{0}C with 30 min incubation time. Vmax and Km values were also calculated of enzyme. Due to the oxidation property of peroxidase enzyme, it could be used in preparation of face mask.

Keywords: Horse-gram, peroxidase, purification, face mask ect.

Corresponding author

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INTRODUCTION

Horse gram is a pulse crop that can be grown in adverse conditions, while providing a quantifiable amount yield. It is a highly under-utilized crop being rich in nutrition and providing a rich source of Proteins, Minerals and Nutrients. Along with its nutritive contents, there are certain amount of various Bio-active compounds present in the plant such as phytic acid, phenols, enzymes, fibers, etc. It has provided insight to be used as a source for isolation of peroxidase enzyme, initially formed during the germination of horse gram legumes.

Horse gram (*Macrotyloma uniflorum*) as mostly known for its nutritional value, is also a valuable source for isolation of peroxidase enzyme. This enzyme has seen in certain conditions in the growth of legumes during germination. The enzyme as in, is seen for its production in the legume showing signs of providing stability during stress conditions. It is seen that horse gram enhances antioxidant enzyme activity such as superoxide dismutase, catalase, and glutathione peroxidase while not significantly affecting inflammatory mediators. The enzyme can be seen as insight to provide information between the production of the enzyme in the legume and oxidative stress during germination (1).

Peroxidase Enzyme

Peroxidase are enzyme whose basic function is to oxidize hydrogen donors of peroxides. The enzyme works most specifically on hydrogen peroxide (2). But can also oxidize a variety of hydrogen donors such as polyphenols. The reactions catalyzed by the enzyme are**:**

$$AH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} A + 2H_2O$$

$$2AH + H_2O_2 \xrightarrow{\text{peroxidase}} AA + 2H_2O$$

The substrate (AH_2, AH) is oxidized while hydrogen peroxide is reduced to water. Peroxidases are glycoproteins with a hematin compound as a cofactor. The hematin cofactor refers to iron (heme) group. The iron (heme) group is present at the active site of the enzyme. The heme group plays an important role in the catalytic cycle as it alters the oxidation state of the enzyme, allowing it to regulate the transfer of electrons from the substrate to the hydrogen peroxide. The molecular weight of the enzyme ranges from 30-50 kDa. The enzyme activity is known to be optimum between the pH 4 to 7. The Enzymes Thermostability varies for its isoenzymes and show variation in its enzyme activity at different temperatures. The Enzyme as well as its isoenzymes show activity from 30 °C to 70 °C.

Structure & Function

Peroxidases are a group of enzymes that does the oxidation of substrate by hydrogen peroxide or other organic peroxides (Benzoyl peroxide, Methyl ethyl ketone peroxide). Most peroxidases are ferric heme protein; one notable exception being the glutathione peroxidase, which is a selenium-containing enzyme. They are present in virtually all living species. (3)

Most peroxidases can oxidize a variety of substrates of widely different structures, varying from halide ions to hydro-quinones to aromatic azo compounds. A bonding site for the substrate to be oxidized is therefore virtually absent in most of these enzymes. In fact, simple molecules consisting of 8-12 amino acids and a covalently linked heme moiety, with a histidine residue forming the proximal ligand, have considerable peroxidase activity. These compounds are known as micro-peroxidases (3).

Mechanism of Peroxidase

The heme protein in the enzyme peroxidase plays a crucial role in the oxidation of enzyme by peroxides which leads to formation of the ferryl (Fe^4) heme moiety which indicates the transfer of electrons from the either of the porphyrin ring or an amino acid residue of the protein. The complex compound formed is referred as Compound I. It reacts with a substrate and cause a one-electron oxidation of the substrate. This causes the conversion of enzyme into Compound II. When the compound II is formed, the heme is present in the ferryl (Fe^4) form, the structure of parphyrin ring or amino acids residue is restored. Further in the reaction when the second electron is transferred, the enzyme goes back to its native ferryl state. (3)

Plants Based Peroxidase (Class III Peroxidase)

Plant based peroxidases (also called Class III Peroxidases) are the most used enzymes for biotechnological studies and applications. These enzymes are actively present in plants like Horseradish and Soyabean. (4) They are present in the extracellular matrix of these plant's Cells. Peroxidase enzyme as defence mechanism using guaiacol or guaiacol peroxidase against Bacterial Parasites. (5)

Horseradish peroxidase (HRP) is the most used peroxidase enzyme for biochemical and immunological test. It is present in the roots of the Horseradish plant (*Armoracia rusticana*). HRP is a metalloenzyme which carries the role of catalysing oxidation reactions. It is an antibody conjugate and therefore, use in tests like ELISA and other Immunological assays. (4)

General Applications of Peroxidase Enzymes

Peroxidase enzymes, with their ability to catalyse redox reactions involving hydrogen peroxide (H_2O_2), have found a remarkable range of applications across various fields. Here is a glimpse into their diverse uses:

Industrial Biotechnology (6)

- Wastewater treatment: Peroxidases efficiently degrade a wide range of pollutants in industrial wastewater, including phenolic compounds, dyes, pesticides, and pharmaceuticals.
- Textile Industry: They are used in textile bleaching (replacing harsh chemicals) and for denim fading, providing an environmentally friendlier alternative.
- Paper Industry: Peroxidases aid in lignin degradation during pulp bleaching, reducing the use of chlorine-based bleaching agents.
- Food Industry: They are used for removing unwanted flavours or colours, improving food texture, and developing biosensors for food quality control.

Biosensing and Diagnostics (7)

- Clinical Diagnostics: Peroxidase-based assays are widely used for detecting glucose, cholesterol, and other analytes in blood and urine samples.
- Highly Sensitive Detection: Immobilizing peroxidase on nanoparticles like gold or magnetic nanoparticles can significantly amplify the signal in biosensors. This leads to highly sensitive detection of various analytes, including pollutants, toxins, and disease biomarkers.
- Environmental Monitoring: They are incorporated into biosensors for detecting pollutants, toxins, and pathogens in water, soil, and air.
- Immunoassays: Peroxidases serve as reporter enzymes in ELISA (enzyme-linked immunosorbent assay) and other immunoassays, enabling the detection of specific antibodies or antigens.

Bioremediation (8)

- Pollutant removal: Peroxidases can break down a variety of environmental pollutants, including polycyclic aromatic hydrocarbons, pesticides, and industrial solvents.
- Efficient pollutant removal: Immobilized peroxidase on magnetic nanoparticles can efficiently remove organic pollutants like phenolic compounds and dyes from wastewater. The nanoparticles increase the enzyme's surface area, leading to better contact with pollutants and faster degradation.
- Efficient pollutant degradation: Nanoparticle-immobilized peroxidase can be used for in situ bioremediation of contaminated soil and water. The nanoparticles can penetrate soil pores or bind to pollutants, bringing the enzyme into proximity with the contaminants for efficient degradation.
- Soil remediation: They are used to remediate contaminated soils by degrading organic pollutants and improving soil health.

Organic synthesis (8)

- Fine chemical synthesis: Peroxidases catalyse selective oxidation reactions, making them valuable tools for synthesizing pharmaceuticals, flavours, fragrances, and other fine chemicals.
- Polymer Synthesis: They are used in the synthesis of conducting polymers and other bio-based materials.
- Biofuel Production: Peroxidases are being explored for their potential in lignin degradation for biofuel production from lignocellulosic biomass. Immobilized peroxidase on nanoparticles can enhance the breakdown of lignin, making it easier to convert biomass into biofuels.
- Hair Dye Industry: They are used in some hair dyes as an alternative to ammonia, providing a less damaging colouring process.

Materials

Guaiacol, Hydrogen peroxide, Ammonium sulfate salt purchased from Hi Media Pvt Ltd.

Methodology

Optimization of enzyme activity during seed germination

Horse-gram seeds were kept for germination for 10 days. On after every day 10g of germinated seeds were collected and extracted peroxidase enzyme as mentioned above. Enzyme activity and protein were checked after extraction to check the highest activity formed at the time of seed germination from 1st to the 8th day of germination.

Extraction

Horse-gram seeds were germinated for different days and seedlings were extracted with saline (100g/ 400ml) at 4°C for 4 h. Next day the homogenate was filtered through cheese cloth and centrifuged at 4°C for 15 min. Filtrate was subjected to ammonium sulphate precipitation (0 to 30%) at 4 °C. On next day, homogenate centrifuged and residue was discarded and supernatant kept for 30 to 70 % ammonium sulphate precipitation and pellet were collected, dissolved in distilled water, and dialyzed finally against phosphate buffer (pH 7.0, 10 mm). The dialyzed protein solution (Fraction A) was used to purify the peroxidase enzyme by ion exchange chromatography using UnoS pre-equilibrated with above phosphate buffer and checked its enzyme activity and protein concentration using U.V. spectrophotometer.

Peroxidase enzyme assay (9)

The enzymatic assays are used to measure the rate of enzyme activity. The enzymatic assay for the peroxidase uses guaiacol as a chromogenic substrate which gives color when it is oxidized with the help of hydrogen peroxide (H_2O_2)

$$2H_2O_2 \xrightarrow{\text{peroxidase}} 2H_2O + O_2$$

The oxygen molecule released because of peroxidase enzyme oxidizes guaiacol hence giving a brown color in impact to presence of hydrogen peroxide as well as its respective enzyme.

Volume of total mixture is assembled to 2 ml total volume for each test tube. 0.2 ml of enzyme was added with 0.1 ml of guaiacol and diluted to a total of 1.6 ml with buffer ph 7. The tubes are incubated at a temperature of 37 °C for 30 min and the optical density is checked under a spectrophotometer at 410 nm while 0.1 ml of hydrogen peroxide is to be added in the test tubes at the time of taking readings of the enzyme activity (i.e., making a total volume of 2 ml). For respective blanks of the chemicals and enzyme used in the assay, the substrates and enzyme are not added and the volume is totaled up with the help of the buffer. The phosphate buffer is used as a control in taking of readings of the enzymatic activity.

Ion exchange chromatography of peroxidase enzyme on UnoS Sphere (10)

The slurry of UnoS (10 ml) was stirred with 1 M NaCl (2-3 column volumes) and then it was equilibrated with phosphate buffer (pH 7.0, 10 mm). A glass column (L= 20 cm, Φ= 2 cm) with capacity of

40 ml was packed with UnoS matrix in phosphate buffer (pH 7.0, 10 mm). Above Fraction A obtained was loaded on the column (12 ml, 10 mg/ml) followed by washing of the column with equilibrating buffer till protein absorbance at 280 nm was ≤ 0.02. The adsorbed proteins were eluted with a discontinuous gradient of sodium chloride (0.1, 0.2, 0.5 and 1 M) in the same buffer. Fractions of 5ml each were collected at a flow rate of 15 ml/h and monitored for their protein content by taking the absorbance at 280 nm and for peroxidase activity. Enzyme rich fractions were pooled, lyophilized and subjected to further purification by gel filtration on a Sephadex G-100 column. The peroxidase was obtained after gel filtration was used for further studies.

Optimization of Enzyme

Determination of optimum temperature

The temperature optima of the peroxidase were determined as follows; The enzyme (0.2 ml) was pre-incubated with guaicol substrate (0.1 ml) in a total reaction volume of 2 ml adjusted with phosphate buffer at 30, 40, 50, 60 and 70 °C for 30 min. The residual peroxidase activity at each temperature was determined by enzyme assay as mentioned above. Corresponding controls were also run simultaneously at the respective temperatures.

Temperature stability

The thermal stability of the peroxidase was determined by incubating the enzyme (0.2 ml) in phosphate buffer (1.6 ml, pH 7.0 and 50 mm) at a temperature range of 30-70 °C for 1 h. The above mixture was pre-incubated with 0.1 ml of guaicol for appropriate time interval and determined temperature stability by performing enzyme activity as mentioned above.

Determination of optimum pH

The optimum pH of the peroxidase was determined as follows; the peroxidase (0.2 ml) was incubated with different buffers (pH 2, 3, 4, 5, 6, 7, 8 and 9; 50 mm) in a total reaction volume of 2 ml at 37 °C for 30 min. The residual enzyme activity at each pH were determined by performing enzyme assay as described earlier. Corresponding controls were also run simultaneously at the respective pH.

pH stability

The pH stability of the peroxidase was determined as follows; the enzyme (0.2 ml) was incubated in 1.6 ml of appropriate buffers (pH 2, 3, 4, 5, 6, 7, 8 and 9; 50 mm) at 37 °C for 12 h. At the end of the incubation period pH was adjusted to 7.0 using acid or alkali. 0.2 ml of the above mixture was pre-incubated with 0.1 ml guaicol in phosphate buffer (pH 7.0, 50 mm) for appropriate time intervals at 37 °C in a total reaction volume of 2.0 ml and the enzyme activity was determined by the as described above.

Kinetic Studies

Determination of K_m and V_{max}

Lineweaver–Burk plots were drawn for the peroxidase. The rate of enzyme was determined at different (0.1 to 1 mm/ml) in a total reaction volume of 2 ml containing phosphate buffer (pH 7.0, 50 mm) and guaicol. The rate of peroxidase was also determined at different guaicol concentrations after pre-incubation with peroxidase in phosphate buffer (pH 7.0, 50 mm) in a total volume of 2 ml. A double reciprocal plot was drawn according to the method of Lineweaver and Burk. The K_m and V_{max} determined were used in the following formula (11).

In the peroxidase assay two substrates were used, one was guaiacol and other was hydrogen peroxide, where hydrogen peroxide was our main substrate which binds to catalytic site of the peroxidase enzyme and catalyses a reaction where water and oxygen gas is released. guaiacol acts as a chromophore, which gets oxidized in the reaction and resulting in giving a reddish-brown colour in the assay. To optimize and see the highest activity of the Enzyme for both substrates, they are optimized at a varying concentration to see the highest activity generated by the enzyme-substrate reaction.

Applications

Peroxidase enzymes, with their ability to catalyse redox reactions involving hydrogen peroxide (H_2O_2), have found a remarkable range of applications across various fields.

Oxidative Face Mask

Peroxidase enzyme can be utilized as a catalase enzyme to break down hydrogen peroxide (H_2O_2), releasing oxygen that revitalizes the skin. This helps in control oxidative stress and reduce irritation on the skin. Using the purified enzyme the Oxidative face mask was made using following ingredients to provide additional properties to the face mask cream.

Ingredients

1. Catalase Enzyme (Peroxidase): (0.1-1%) the active ingredient to break down hydrogen peroxide.
2. Hydrogen peroxide solution (1-3%) – Oxidation source.
3. Aloe vera gel (10-20%): Soothing and hydrating agent.
4. Glycerin (5-10%): Moisturizer and humectant
5. Green Tea Extract (0.5-1%): Antioxidant to reduce oxidative stress.
6. Purified water: As needed to adjust consistency.
7. Oils (optional, 0.1-0.5%): to add fragrance.

Preparation

1. Mix aloe vera gel, glycerin in a sterile container.
2. Add the enzyme and gently to maintain enzyme activity.
3. Add antioxidant and essential oil in the mixture
4. Add hydrogen peroxide just before application on the skin, this ensures oxygen release on contact with skin.

RESULTS

Seed germination of horse gram (*Macrotyloma uniflorum)* seeds

The process of seed germination starts from the first formation of radicle of the plant in the span of 1-2 days, to formation plumule in the span of 3-5 days, to formation of the first leaf along with root hair network around the roots in the span of 6-8 days. Throughout the process of seed germination, peroxidase enzyme is formed in the *Macrotyloma uniflorum* seeds. One of the challenges to determine the highest enzymatic activity formed at the interval of the growth of the seeds during seed germination (12). Therefore, the seeds were kept for germination (Fig 1) and the enzyme is to schedule to be extracted at the interval of each day following an enzymatic assay and protein concentration to check the highest activity formed at the time of seed germination from 1st to the 10th day of germination.

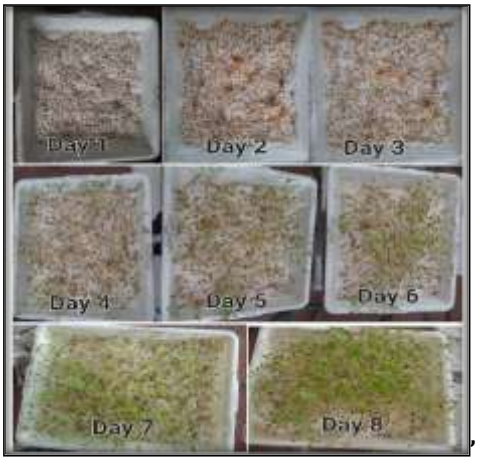

Figure 1: Seed Germination of *Macrotyloma uniflorum*

During the seed germination (1 to 10 days) after each day seedlings of horse gram was collected and extracted the peroxidase. Highest peroxidase activity was observed on 5th day of germination (Fig 2) and same day seedlings were used further for extraction, isolation, and purification.

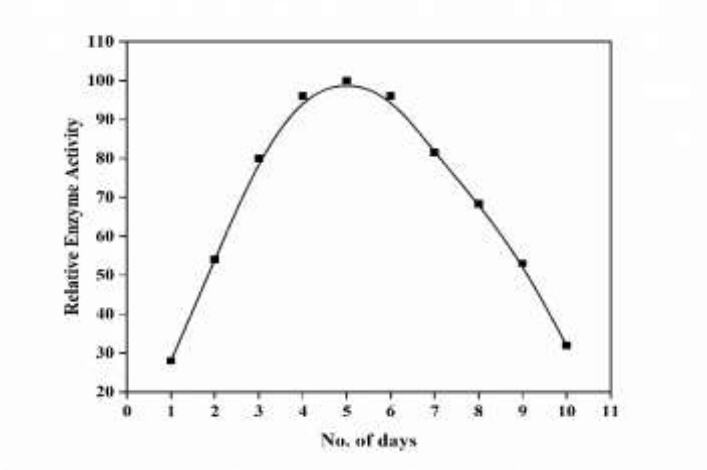

Figure 2: Seed germination

Ammonium Sulphate Precipitation and Dialysis: Ammonium sulphate precipitation was done at 0-30% saturation and from 30-70%. Both the samples were kept for dialysis. This range showed more activity compared to 0-30% saturated sample. Therefore, 30-70% saturated sample was further used in the further purification process.

Ion-exchange chromatography: Ion-exchange chromatography was successfully done for the purification of the enzyme following increasing salt concentration in the elution (0.1M, 0.2M, 0.5M, of NaCl in 10 mm phosphate buffer) and desired protein was eluted in the 0.1 M NaCl elution (Fig 3). The graph was plotted to show the variable values where the protein concentration was highest, as well as the enzyme activity was checked to determine the peaks.

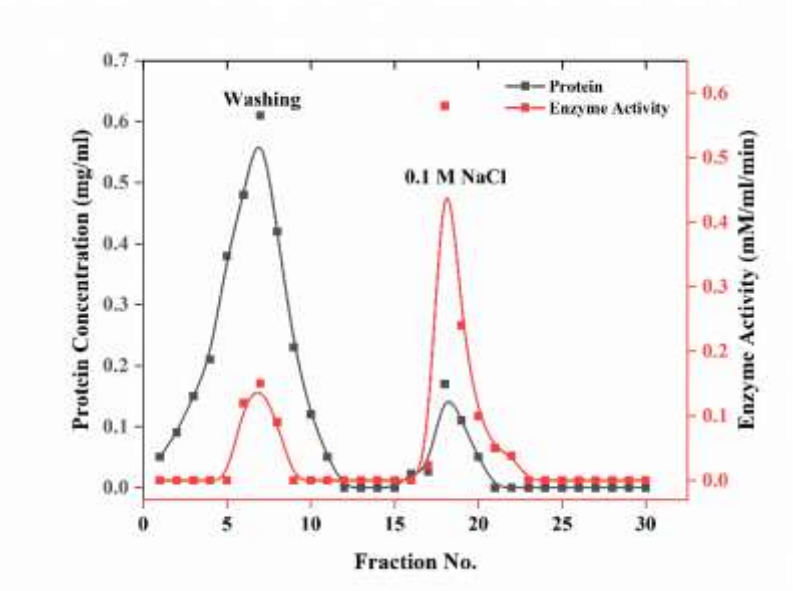

Figure 3: Ion Exchange Chromatography

Graph were plotted of the following observation and samples showing peak in the graph were taken for further purification process.

Gel Filtration Chromatography: Gel filtration chromatography was performed and the sample was further purified and eluted. The Protein estimation and enzyme activity was given as the following graph:

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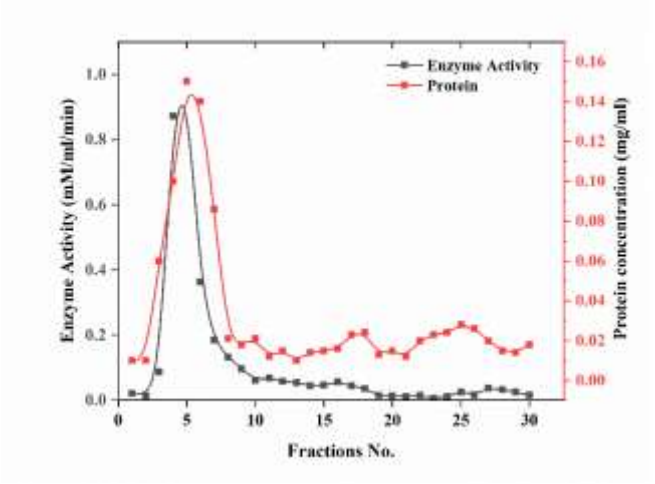

Figure 4: Gel Filtration Chromatography

The fractions 4, 5 and 6 (Fig 4) showed highest enzyme activity and used further for optimization of the enzyme.

Table 1: Summary of purification

Purification Step	Volume ml	Protein mg/ml	Activity U/ml/min	Specific Activity	Purification Fold
Crude protein Extract	400	264	2.53	0.0095	1
Fraction A	55	94	1.1	0.011	1.15
Ion Exchange Chromatography	10	6.8	0.84	0.12	12.6
Gel Filtration	5	1.3	0.51	0.39	41.5

Temperature optimization: Temperature optimization of the enzyme was determined and highest activity observed at the temperature 40°C (Fig 5A). Therefore, determining the optimum temperature required by the enzyme to use to its fullest efficiency. Peroxidase were shown stability in the range of temperature 30-80 °C (Fig 5B).

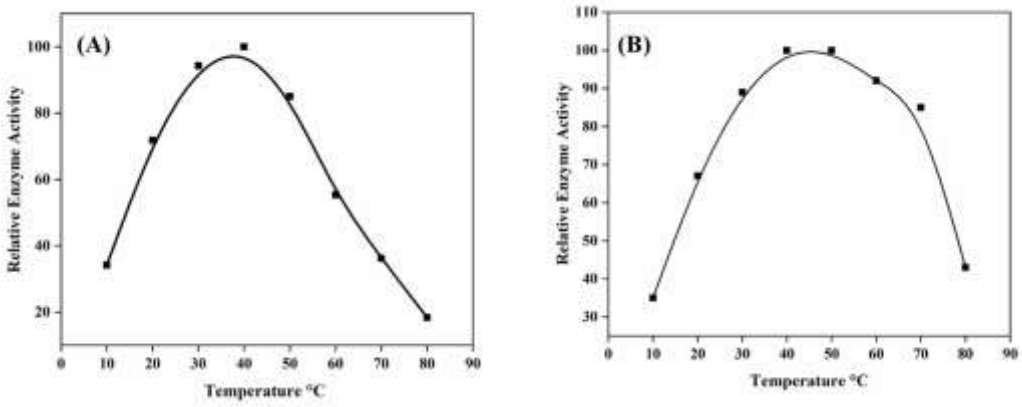

Figure 5: (A) Optimum Temperature (B) Temperature stability

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Optimum pH: The pH was optimized at specific pH ranging from pH 4 to 9 with the help of various buffers (Tris HCl Buffer, Acetate Buffer, Phosphate Buffer). The enzyme activity was successfully measured using enzyme assay and measured at absorbance of 410 nm. It was found that the highest enzyme activity was observed at pH 6 (Fig 6A).

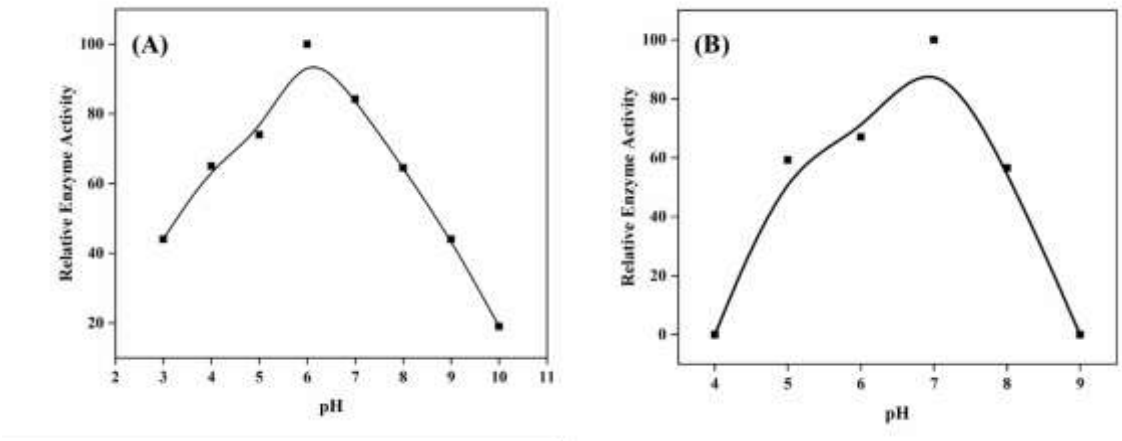

Figure 6: (A) Optimum pH (B) pH stability

pH Stability: The enzyme was kept for stabilization at specific pH overnight (i.e. in buffers ranging from pH 4 to 9) and the enzyme activity was checked the next day. The result determined at lower pH as well as at higher pH enzyme activity was seen low or none to less. The enzyme activity at pH 4 as well as at pH 9 were lowest, indicating enzyme have been denatured at this specific pH values. The enzyme showed more stability at pH 5 to 8 (Fig 6B), showing the highest enzyme activity.

Incubation time optimization: Incubation time of the enzyme assay also played an important role to optimize the enzyme activity. Specific temperature determined by previous temperature optimization helps in optimizing the incubation time. Incubation of enzyme helps in activation of the enzyme and determine the amount of incubation required by the enzyme to activate and conclude a reaction. The optimization of incubation time determined that the enzyme activity was highest at 30 min (Fig 7). Therefore, the enzyme required the amount of optimum incubation time required by the enzyme to active.

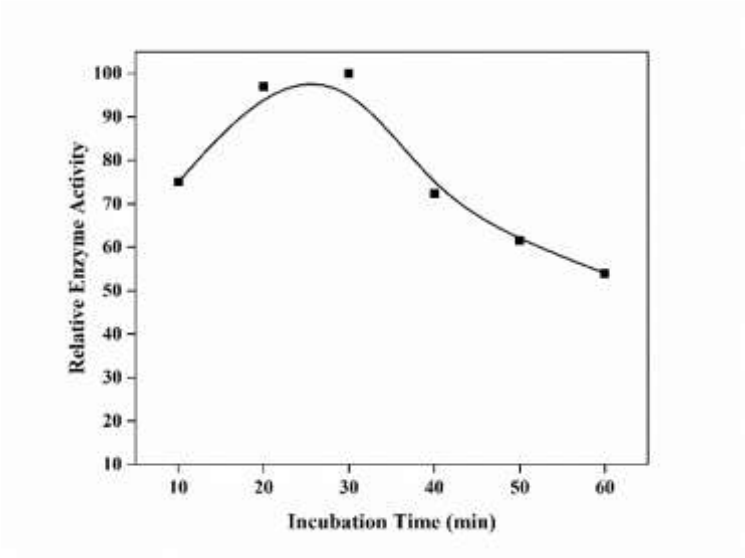

Figure 7: Optimization of incubation time

Substrate Concentration Optimization: The influence of varying substrate concentration on peroxidase activity was studied using both substrates (hydrogen peroxide and guaicol). Lineweaver Burk plot of 1/V vs 1/[S] was used to determine the Km and Vmax. As seen in figure, a Km of hygrogen peroxide 3.3 mg/ mL and Vmax = 0.08 mM/ ml/ min as well as Km of guaicol 4.53 Mm/ml and 0.23 mM/ ml/min were obtained.

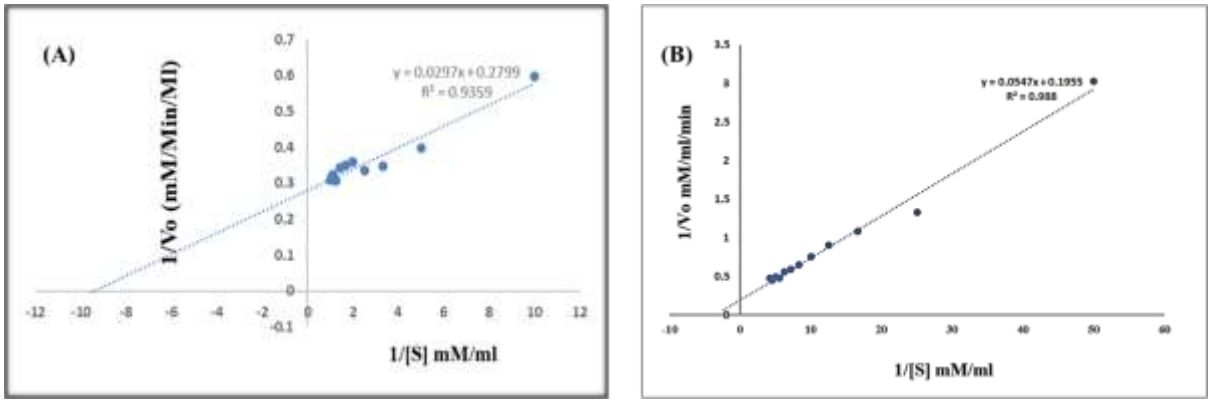

Figure 8: Lineweaver Burk plot

Guaiacol which acted as a chromophore in the enzyme assay showed decrease in its activity as the concentration of guaiacol added increased, this may due to substrate inhibition where excessive substrate binds to the non-active site of enzyme reducing the enzyme activity or due to competitive inhibition between the both substrates. The highest enzyme activity was seen between sample volumes 0.1-0.3ml (concentration of 1-3 mm). Showing the optimal amount of chromophore to be present for the enzyme assay, while increasing concentration show inhibition in the enzyme activity.

Hydrogen peroxides the main substrate that catalyses the reaction show continuous increasing enzyme activity as the concentration of the substrate increases. The highest activity was seen at substrate 8 mm showcasing the highest enzyme activity. The rate of reaction can be calculated using Michaelis-Menten equation.

Application

Peroxidase enzymes, with their ability to catalyse redox reactions involving hydrogen peroxide (H_2O_2), have found a remarkable range of applications across various fields. Using the reliable latent knowledge and research, Peroxidase enzyme can be used to catalyse the reaction to release oxygen molecules by conduction of the redox reaction of hydrogen Peroxide, breaking the compound and releasing oxygen. The oxygen molecules released are susceptible and can be used to remove certain impurities on the skin by providing oxidation to impurities. This helps in control oxidative stress and reduce irritation on the skin. The hydrogen peroxide used in the process when broken down using the enzyme allows the release of oxygen, can be used to reduce inflammation on skin to acne, or to dry out pimples (13).

The provision of using hydrogen peroxide along with the enzyme comes with certain drawbacks, as the chemical supplement can also cause certain skin irritation and certain level of cell damage, therefore to be used in lower concentration (>3%).

Used along other products such as Aloe vera gel, glycerin, Antioxidants such as Ascorbic acid, Bentonite clay, and certain oils to add fragrance. Each with their own objective, the cream can be made to be utilized as applied to the skin providing a new application of peroxidase enzyme.

The use of the certain products in the cream can be described as follow and their work:

- Catalase enzyme (Peroxidase): (0.1-1%) the active ingredient to break down hydrogen peroxide.
- Hydrogen peroxide solution (1-3%) – Oxidation source, reduce inflammation on skin.
- Aloe vera gel (10-20%): Soothing and hydrating agent.
- Glycerin (5-10%): Moisturizer and humectant
- Ascorbic Acid (0.5-1%): Antioxidant to reduce oxidative stress.
- Purified water: As needed to adjust consistency.
- Oils (optional, 0.1-0.5%): to add fragrance.
- Bentonite Clay: Absorbs Impurities on the skin

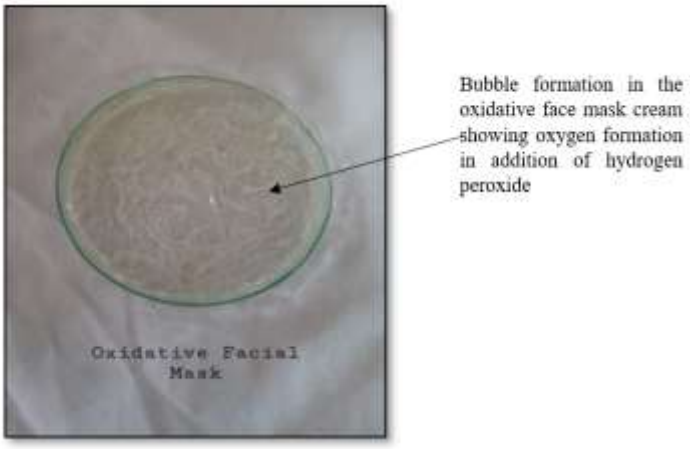

Figure 9: Oxidative face mask cream

CONCLUSION

Isolation and purification of peroxidase enzyme from germinated seeds of *Macrotyloma uniflorum*. Highest activity of peroxidase shown on 5th day of germination. Horse-gram seedlings were extracted and partial peroxidase was isolated in range of 30-70 % ammonium sulphate. This Fraction A was purified using UnoS ion exchange and gel filtration chromatography. Physiochemical parameters of purified peroxidase were carried out and it has shown highest enzyme activity at pH 6, optimum temperature 40 ^{0}C. This enzyme stable in pH range from pH 5 to 8 and temperature stability from 30 to 70 ^{0}C with the optimized incubation time was 30 min. Purified peroxidase was used in preparation of facial mask to recover free radicle formation from face.

REFERENCES

[1] Vrinda Rajagopal , Chithra K Pushpan , Helen Antony , Comparative effect of horse gram and black gram on inflammatory mediators and antioxidant status, J Food Drug Anal, 2017 Oct;25(4):845-853, doi: 10.1016/j.jfda.2016.08.010

[2] Rajkumar R, Yaakob Z, Takriff MS, et al. Optimization of medium composition to produce peroxidase by *Bacillus* sp. *Der Pharma Chemica.* 2013; 5:167–174.

[3] Monika Soudi, Marcel Zamocky , Christa Jakopitsch, Paul G Furtmüller, Christian Obinger, Molecular Evolution, Structure, and Function of Peroxidasins, Chem Biodivers 2012 Sep 13;9(9):1776–1793. doi: 10.1002/cbdv.201100438.

[4] Pandey VP, Awasthi M, Singh S, et al. A comprehensive review on function and application of plant peroxidases. *Biochem Anal Biochem.* 2017; 6:1.

[5] Nenad Bakalovic, Filippo Passardi, Vassilios Ioannidis , Claudia Cosio, Claude Penel, Laurent Falqu et, Christophe Dunand. Christophe Dunand, in Phytochemistry, 2006.

[6] Sepideh Motamed, Farnoosh Ghaemmaghami, Iran Alemzadeh, Turnip (Brassica rapa) Peroxidase: Purification and Characterization, November 2009, Industrial & Engineering Chemistry Research 48(23), DOI:10.1021/ie801997e.

[7] Valero, E., González-Sánchez, M., & Pérez-Prior, M T. (2014). Removal of organic pollutants from industrial wastewater by treatment with oxidoreductase enzymes. Springer Nature, 317-339.

[8] Muhammad Qamar Khan, Jahangir Khan, Muhammad Abbas Haider Alvi, Hifza Nawaz, Muhammad Fahad & Muhammad Umar, Nanomaterial-based sensors for microbe detection: a review, Discover Nano , Volume 19, article number 120, (2024).

[9] Thikra Hasan Mathkor*, 'Hathama Razooki Hasan and Zainab Maki Daham, Screening And Extraction Of Peroxidase Enzyme From Different Plant Wastes, Biochem. Cell. Arch. Vol. 19, Supplement 1, pp. 2739-2746, 2019, DOI:10.35124/bca.2019.19.S1.2739.

[10] Zibaee, A., Bandani, A R., & Malagoli, D. (2011, January 1). Purification and characterization of phenoloxidase from the hemocytes of Eurygaster integriceps (Hemiptera: Scutelleridae). Elsevier BV, 158(1), 117-123.

[11] Powers J R., Cuibertson J D., (1982). In vitro effect of bean amylase inhibitor on insect amylases. *J Food Protection* 45: 655-657.

[12] Different Modes of Hydrogen Peroxide Action During Seed Germination, Łukasz Wojtyla [1,*], Katarzyna Lechowska [1], Szymon Kubala [1,†], Małgorzata Garnczarska, Front Plant Sci. 2016 Feb 4;7:66. doi: 10.3389/fpls.2016.00066.
[13] Use of enzymes in cosmetics: proposed enzymatic peel procedure, Sara Diogo Gonçalves, Cos ACTIVE J. 2021;1:29–35