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# RESEARCH ARTICLE

## OPTIMIZING CALLUS CULTURE FOR HIGH-PEROXIDASE PRODUCTION: A CASE STUDY OF *VITEX* WITH UNIQUE ENZYME CHARACTERISTICS

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**Abstract:** Efficient callus induction and proliferation protocols have been successfully established for several plant species known to be potent sources of peroxidases. Phenolic compounds, while inhibitory to callus formation, have been found to stimulate peroxidase production. The level of peroxidase activity in callus cultures varies significantly depending on both the type of plant growth regulator used and the morphological nature of the callus. Among the species studied, *Vitex* peroxidase is particularly unique, as callus cultures exhibit exceptionally high enzyme activity despite minimal or undetectable activity in the intact plant body. N-terminal sequencing of the *Vitex* peroxidase revealed a novel sequence distinct from all previously reported plant peroxidases, which likely accounts for its remarkably high specific activity. These findings highlight the potential of *Vitex* callus cultures as a valuable biotechnological system for peroxidase production.

**Keywords:** Callus culture, Peroxidase, *Vitex*, N-terminal sequencing

### INTRODUCTION

Peroxidases (EC 1.11.1.x) are heme-containing oxidoreductase enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as an electron acceptor. These enzymes play crucial roles in plant growth, lignification, defense responses, wound healing, and stress adaptation. Owing to their broad substrate specificity and catalytic efficiency, peroxidases have gained significant industrial importance in biotechnology, clinical diagnostics, biosensor development, wastewater treatment, bioremediation, food processing, and pharmaceutical applications. Recent studies have highlighted the expanding physiological and biotechnological significance of Class III plant peroxidases, emphasizing the need for novel sources of highly active and stable enzymes (Freitas et al., 2024).

Commercial production of peroxidases is largely dependent on horseradish peroxidase (HRP), which remains the most widely used plant-derived enzyme. However, increasing industrial demand, coupled with the need for enzymes possessing enhanced catalytic activity, stability, and cost-effective production systems, has stimulated the search for alternative plant sources. Plant tissue culture technology offers an attractive platform for enzyme production because it enables controlled growth conditions, independence from seasonal variations, and the possibility of enhancing metabolite and enzyme yields through manipulation of culture conditions. *In vitro* cultures, particularly callus and suspension cultures, often exhibit altered gene expression patterns that can lead to the synthesis of valuable biomolecules absent or minimally expressed in intact plants.

*Vitex negundo* Linn. (Nirgundi) is an important medicinal shrub widely distributed throughout India and other parts of Asia. The plant has been extensively utilized in traditional medicine systems including Ayurveda, Siddha, and Unani for the treatment of inflammatory disorders, respiratory diseases, microbial infections, pain, and various chronic ailments. Modern investigations have revealed that *V. negundo* contains diverse bioactive constituents, including flavonoids, iridoids, lignans, phenolic acids, and terpenoids that contribute to its broad pharmacological activities. Recent reviews have further documented its antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, neuroprotective, and anticancer potential, highlighting its growing importance as a medicinal and biotechnological resource (Dube *et al*., 2024; Review, 2026).

Although considerable attention has been directed toward the phytochemical and pharmacological properties of *V. negundo*, relatively little information is available regarding its potential as a source of industrially important enzymes. An intriguing observation from preliminary investigations is that the intact plant exhibits negligible peroxidase activity, whereas its callus cultures produce substantial quantities of the enzyme. Such

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differential expression may result from activation of stress-responsive genes and metabolic pathways during cellular dedifferentiation and callus formation. The phenomenon provides a unique opportunity to investigate the regulation of peroxidase biosynthesis while simultaneously developing an alternative source of commercially valuable enzymes.

The present study was therefore undertaken to establish efficient protocols for callus induction, proliferation, and maintenance in *Vitex negundo* and to evaluate the influence of culture conditions on peroxidase production. Furthermore, the extracellular peroxidase produced by the callus cultures was purified and characterized with respect to its biochemical properties, molecular weight, optimum reaction conditions, and N-terminal amino acid sequence. The study also aimed to compare the characteristics of the purified enzyme with commercially available horseradish peroxidase and to assess its novelty and potential industrial applicability. The findings provide valuable insights into the unique expression of peroxidase in *V. negundo* callus cultures and support the development of plant tissue culture-based systems for sustainable enzyme production.

## MATERIALS AND METHODS

### Plant Material and Explant Preparation

Healthy plants of *Vitex negundo* Linn. were collected from the vicinity of the Regional Research Laboratory (RRL), Thiruvananthapuram, India. Young leaves were selected as explants because of their high regenerative potential and suitability for callus induction. The explants were washed thoroughly under running tap water, followed by surface sterilization using standard aseptic procedures employed in plant tissue culture studies. Recent advances in medicinal plant biotechnology have demonstrated that leaf explants are highly responsive for in vitro regeneration and metabolite production.

### Callus Induction and Proliferation

Surface-sterilized leaf explants were cultured on Murashige and Skoog (MS) basal medium supplemented with different combinations of 6-benzylaminopurine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The cultures were incubated at 25 ± 2°C under a 16 h photoperiod. Callus induction was assessed after four weeks of culture.

The optimum callus induction response was obtained on MS medium containing 0.5 mg $L^{-1}$ 2,4-D and 0.3 mg $L^{-1}$ BA. The induced callus was compact and yellow in appearance. Similar cytokinin–auxin interactions have been reported to regulate cellular dedifferentiation and biomass production in medicinal plant cultures (Kruglova et al., 2023).

### Establishment of Cell Suspension Cultures

Compact callus aggregates (CCAs) obtained during subculturing were transferred to liquid MS medium and maintained on a rotary shaker at 100–120 rpm. Suspension cultures were periodically sub cultured to ensure continuous proliferation and extracellular enzyme production. Plant cell suspension cultures have been recognized as effective systems for the production of high-value enzymes and secondary metabolites because of their scalability and controlled growth conditions.

### Regeneration Studies

To investigate the effect of organogenesis on peroxidase production, callus cultures were transferred to regeneration medium containing varying concentrations of coconut water (CW). Among the concentrations tested, 1% (v/v) CW produced the highest regeneration frequency. Morphogenic responses were recorded at different stages, and corresponding peroxidase activities were measured. Coconut water is known to contain cytokinin-like compounds such as zeatin and has been widely used to promote shoot organogenesis in plant tissue culture systems.

### Extraction and Peroxidase Assay

Extracellular peroxidase was harvested from suspension culture media by filtration through sterile cheesecloth to remove cellular debris. Enzyme activity was determined spectrophotometrically using ABTS, guaiacol, and o-phenylenediamine (o-PDA) as substrates in the presence of hydrogen peroxide. Peroxidase assays were performed according to standard enzymatic procedures routinely employed for plant peroxidase characterization (Freitas *et al.*, 2024).

One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 µmol of substrate per minute under assay conditions.

### Purification of Peroxidase

The crude enzyme preparation was concentrated using ultrafiltration membranes with a molecular weight cut-off of 10 kDa. The concentrated sample was subsequently lyophilized and subjected to preparative isoelectric focusing using a Rotofor apparatus. Fractions exhibiting maximum enzyme activity were collected and used for further characterization.

Purification strategies involving ultrafiltration and isoelectric focusing are commonly employed for obtaining highly active plant peroxidases suitable for biochemical characterization (Freitas et al., 2024).

### SDS–PAGE Analysis

The purified enzyme was analyzed by SDS–PAGE using a 12% resolving gel and a 5% stacking gel. Electrophoresis was carried out at 200 V and 110 mA, followed by Coomassie Brilliant Blue staining. Molecular weight determination was performed using standard protein markers. SDS–PAGE remains

the most widely adopted technique for evaluating purity and molecular mass of plant-derived enzymes.

**Determination of Optimum pH and Temperature**

The effect of pH on enzyme activity was investigated using appropriate buffer systems over a broad pH range. Similarly, enzyme activity was measured at different temperatures to determine the optimum reaction temperature. The pH and temperature profiles were constructed from relative enzyme activity values.

**Effect of Metal Ions on Enzyme Activity**

The influence of selected metal ions and inhibitors on enzyme activity was studied by incubating the purified enzyme with 1 mM concentrations of KI, $NaN_3$, $MgSO_4$, $ZnSO_4$, $MnSO_4$, $CaCl_2$, $HgCl_2$, and NaCl. Residual activity was measured under standard assay conditions and expressed as a percentage of the untreated control.

**Protein Estimation**

Protein concentration was determined using bovine serum albumin (BSA) as the standard. Specific activity was calculated as enzyme units per milligram of protein.

**N-Terminal Amino Acid Sequencing and Bioinformatic Analysis**

Purified peroxidase was electrophoresed on SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The protein band corresponding to the purified enzyme was excised and subjected to automated Edman degradation using an Applied Biosystems 477A Protein Sequencer.

The obtained amino acid sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) to identify homologous proteins in public databases. Sequence comparison and molecular characterization are widely employed to establish enzyme novelty and evolutionary relationships among plant peroxidases (Freitas et al., 2024).

**Statistical Analysis**

All experiments were conducted in triplicate and the results were expressed as mean ± standard deviation. Statistical significance among treatments was determined using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$.

## RESULTS

**Callus Induction and Proliferation**

Leaf explants of *Vitex negundo* responded successfully to in vitro culture conditions and produced callus on Murashige and Skoog (MS) medium supplemented with different combinations of plant growth regulators. Among the treatments tested, MS medium containing 0.5 mg $L^{-1}$ 2,4-D and 0.3 mg $L^{-1}$ BA produced the highest callus induction frequency. The induced callus was compact, yellowish, and actively proliferating.

Subsequent subculturing of the callus on medium containing 1.0 mg $L^{-1}$ BA resulted in the formation of compact callus aggregates (CCAs). These aggregates exhibited rapid growth and maintained their structural integrity during repeated subcultures. Both compact and friable callus forms were obtained depending on the hormonal composition of the culture medium.

**Effect of Growth Regulators on Peroxidase Production**

Peroxidase production varied considerably with the type and concentration of growth regulators used. Calli cultured in hormone-free medium exhibited relatively high peroxidase activity but showed poor biomass accumulation. Auxins such as IAA, NAA, and 2,4-D generally reduced enzyme production and callus growth when supplied individually.

In contrast, cytokinin supplementation with BA significantly enhanced both biomass production and enzyme activity. The combination of 1.5 mg $L^{-1}$ BA and 1.0 mg $L^{-1}$ 2,4-D was found to be optimal for simultaneous biomass accumulation and peroxidase production.

**Regeneration and Peroxidase Activity**

The addition of coconut water (CW) induced organogenesis in the non-organogenic callus cultures. Among the concentrations tested, 1% (v/v) CW produced the highest regeneration response. Progressive development of shoots was observed during the regeneration process.

Analysis of peroxidase activity during different stages of regeneration revealed a gradual decline in enzyme production with increasing differentiation of the callus tissue. The highest enzyme activity was recorded in undifferentiated callus cultures, whereas regenerated tissues exhibited substantially lower activity levels.

**Growth Kinetics and Enzyme Production**

The suspension cultures of *V. negundo* demonstrated active biomass accumulation throughout the cultivation period. Peroxidase production increased concomitantly with cell growth and reached maximum levels during the late exponential phase of culture growth. Thereafter, enzyme production stabilized with only minor fluctuations.

The relationship between biomass accumulation and enzyme production indicated that peroxidase synthesis was closely associated with active cellular proliferation (Fig 1).

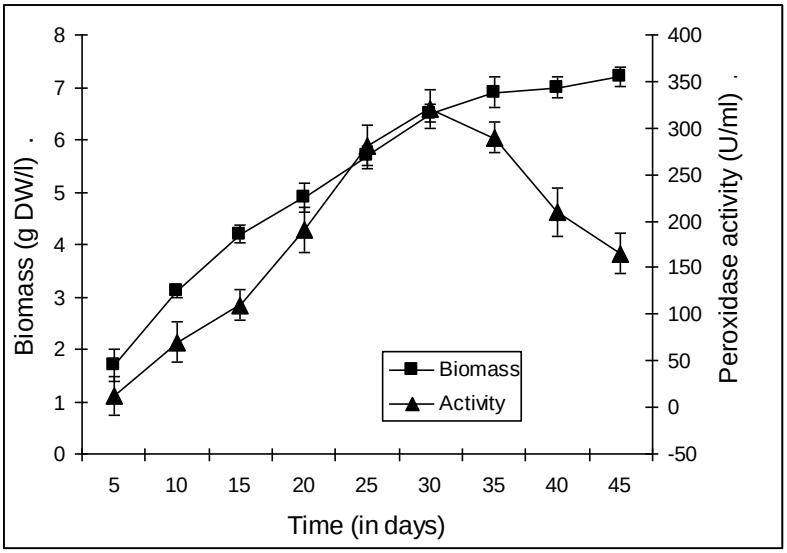


**Fig 1:** Impact of time course on peroxidase activity and biomass growth of *Vitex*

**Purification of Extracellular Peroxidase**

The extracellular peroxidase produced by suspension cultures was successfully purified through ultrafiltration, lyophilization, and preparative isoelectric focusing. Multiple isozyme fractions were detected; however, the major active fraction was focused at an isoelectric point (pI) of approximately 9.0.

Purification resulted in a substantial increase in enzyme specific activity. The purified enzyme exhibited nearly nine-fold enrichment compared with the crude extract, indicating the effectiveness of the purification procedure.

**Molecular Weight Determination**

SDS–PAGE analysis of the purified enzyme revealed a single prominent protein band, indicating a high degree of purity. The molecular mass of the purified *Vitex* peroxidase was estimated to be approximately 34 kDa (Figure 2).

This molecular weight was lower than that reported for commercial horseradish peroxidase, which typically exhibits a molecular mass of approximately 40 kDa.

**Biochemical Characterization of Purified Peroxidase**

**Optimum Temperature**

The purified peroxidase exhibited maximum catalytic activity at 45°C. Enzyme activity gradually declined at temperatures above the optimum, indicating thermal sensitivity of the protein structure (Fig 2)

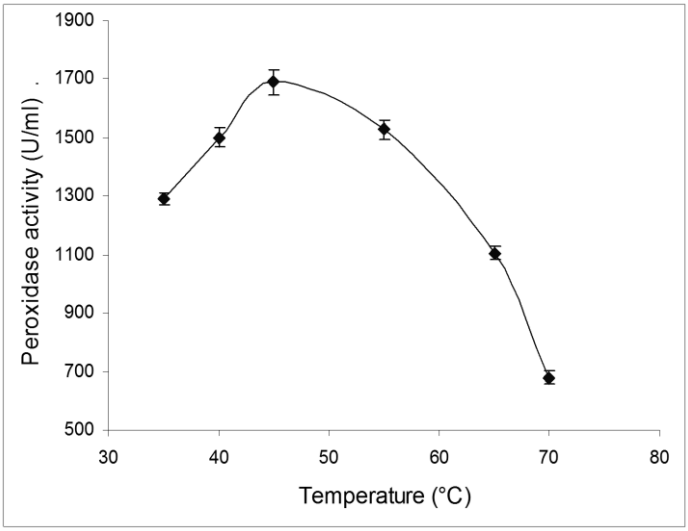


**Fig 2:** Optimum temperature of purified *Vitex* peroxidase

**Optimum pH**

Maximum enzyme activity was observed at pH 5.5. Activity decreased significantly at both acidic and alkaline extremes, suggesting that the catalytic efficiency of the enzyme is strongly influenced by hydrogen ion concentration (Fig 3)

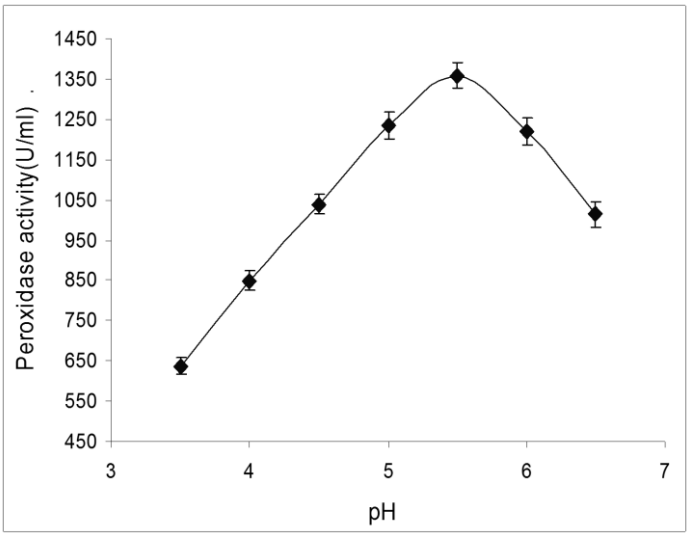


**Fig 3:** Optimum pH of purified *Vitex* peroxidase

**Effect of Metal Ions and Chemical Reagents**

The influence of selected metal ions and inhibitors on purified peroxidase activity is summarized in Table 1. $ZnSO_4$ produced the highest activation effect, increasing enzyme activity to 138% of the control value. $MnSO_4$ and $CaCl_2$ also enhanced activity, resulting in 131% and 130% relative activity, respectively.

Conversely, $NaN_3$ and $HgCl_2$ strongly inhibited enzyme activity, reducing activity to 73% and 62% of the control, respectively. KI slightly stimulated enzyme activity, whereas $MgSO_4$ and NaCl produced only marginal effects.

**Table 1.** Effect of metal ions on purified *Vitex negundo* peroxidase activity

| **Reagent (1 mM)** | **Relative Activity (%)** |
|---|---|
| Control | 100 |
| KI | 116 |
| $NaN_3$ | 73 |
| $MgSO_4$ | 97 |
| $ZnSO_4$ | 138 |
| $MnSO_4$ | 131 |
| $CaCl_2$ | 130 |
| $HgCl_2$ | 62 |
| NaCl | 103 |

**Comparison with Commercial Horseradish Peroxidase**

The crude extracellular enzyme preparation from *V. negundo* exhibited specific activities of 1572 U $mg^{-1}$ protein using ABTS and 544 U $mg^{-1}$ protein using guaiacol as substrates. Following purification, the specific activity increased to 14,730 U $mg^{-1}$ protein using ABTS (Table 2).

Remarkably, even the crude enzyme preparation demonstrated activity levels comparable to those of commercial horseradish peroxidase (HRP). The purified enzyme showed substantially higher specific activity than the commercial reference enzyme, highlighting its potential industrial significance.

**Table 2.** Comparative chart of nirgundi peroxidase with known Horseradish peroxidase

| **Properties** | **Horseradish peroxidase (Sigma)** | ***V. negundo*** | |
|---|---|---|---|
| | | crude | pure |
| *Molecular weight (kDa)* | 40 | - | 34 |
| R.Z (Reinheitszahl) value | 2.0 | | 2.10 |
| pH optimum | 6–7 | 4.5 | 5.5 |
| Optimum temperature (˚C ) | 50 | 50 | 45 |
| Specific activity (Units/mg of protein): | | | |
| Using ABTS: | | | |
| Using Gaiacol: | 1820 | 1572 | 14730 ND* |
| | 520 | 544 | |

**N-Terminal Amino Acid Sequence Analysis**

The N-terminal amino acid sequence of the purified peroxidase was determined as:

**ADIPYNGNGAV**

BLAST analysis revealed very low sequence similarity with previously reported plant peroxidases. The highest similarity observed was approximately

18%, indicating that the enzyme possesses a unique amino acid sequence (Fig 4)

Sequence alignment with known peroxidases from horseradish, soybean, barley, wheat, tobacco, Arabidopsis, and other plant sources confirmed the distinct nature of the *Vitex* enzyme. The low homology strongly suggests that the enzyme represents a novel peroxidase isoform.

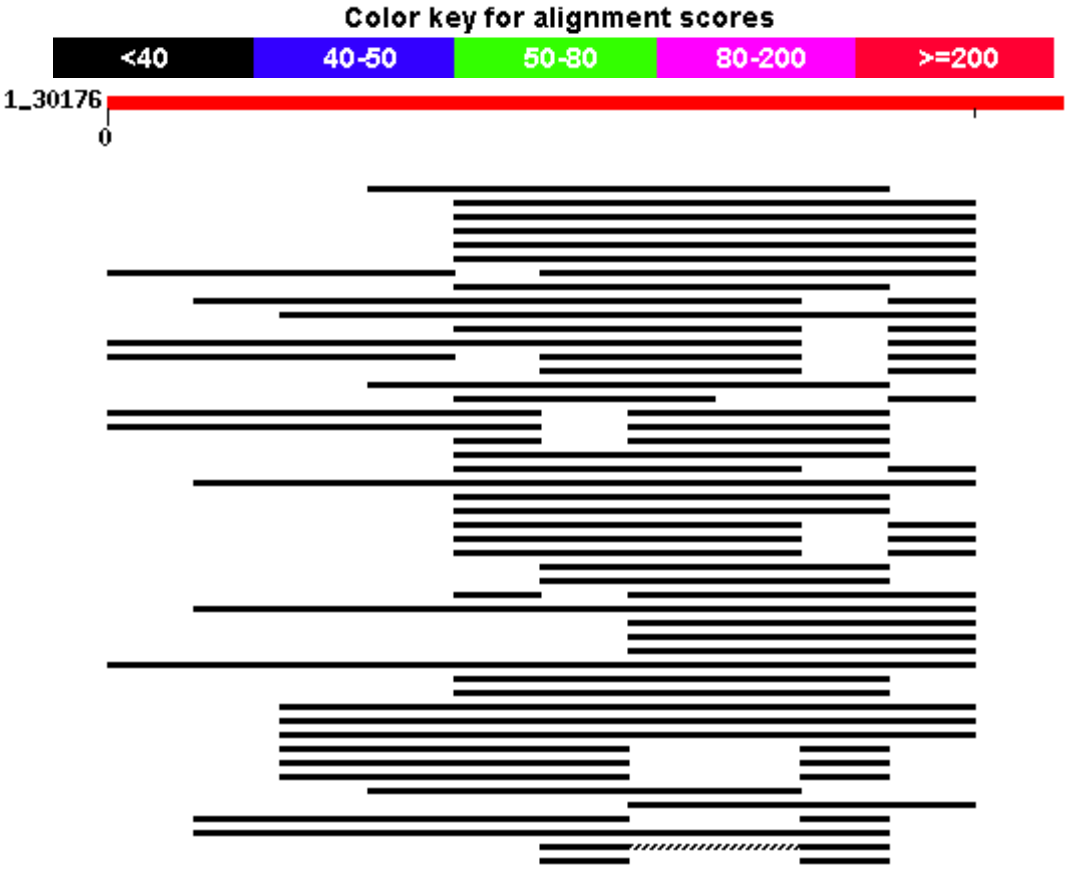


**Fig 4:** BLAST results of query amino acid sequence, ADIPYNGNGAV

Most Related Structures

| Sequences producing significant alignments: | **Score** | (**bits**) Value |
|---|---|---|
| gi\|55701055\|tpe\|CAH69336.1\| TPA: class III peroxidase 94 pr... | 18 | 12 |
| gi\|166807\|gb\|AAA32842.1\| peroxidase | 18 | 12 |
| gi\|16323436\|gb\|AAL15212.1\| putative peroxidase [Arabidopsis... | 18 | 12 |
| gi\|62319823\|dbj\|BAD93845.1\| peroxidase like protein [Arabid... | 18 | 12 |
| gi\|17530564\|gb\|AAL40849.1\| class III peroxidase ATP34 [Arab... | 18 | 12 |
| gi\|129812\|sp\|P17180\|PER3_ARMRU Peroxidase C3 precursor | 18 | 12 |
| gi\|26398007\|sp\|P59121\|PERE5_ARMRU Peroxidase E5 | 18 | 12 |
| gi\|55700917\|tpe\|CAH69267.1\| TPA: class III peroxidase 25 pr... | 17 | 21 |
| gi\|4504183\|ref\|NP_000843.1\| glutathione transferase [Homo s... | 16 | 28 |
| gi\|13475896\|ref\|NP_107466.1\| hypothetical protein mll7076 [... | 16 | 37 |
| gi\|25285624\|pir\|\|B98301 non-heme chloroperoxidase (chloride... | 16 | 37 |
| gi\|55700915\|tpe\|CAH69266.1\| TPA: class III peroxidase 24 pr... | 15 | 50 |
| gi\|1781334\|emb\|CAA71494.1\| peroxidase [Spinacia oleracea] | 15 | 67 |
| gi\|60687493\|gb\|AAX35769.1\| fatty acid oxygenase [Emericella... | 15 | 67 |
| gi\|17135659\|dbj\|BAB78205.1\| all7121 [Nostoc sp. PCC 7120] >... | 15 | 90 |
| gi\|55700883\|tpe\|CAH69251.1\| TPA: class III peroxidase 8 pre... | 14 | 121 |
| gi\|24196840\|gb\|AAN50172.1\| Methylamine utilization protein ... | 14 | 121 |
| gi\|37962659\|gb\|AAR05646.1\| alpha-DOX1 [Lycopersicon esculen... | 14 | 121 |
| gi\|55700957\|tpe\|CAH69287.1\| TPA: class III peroxidase 45 pr... | 14 | 121 |

| | | | |
|---|---|---|---|
| gi\|444058\|prf\|\|1906181A | lignin peroxidase | 14 | 121 |
| gi\|732970\|emb\|CAA59484.1\| | pox1 [Triticum aestivum] >gi\|2117... | 14 | 163 |
| gi\|30793970\|gb\|AAP40436.1\| | putative peroxidase [Arabidopsis... | 14 | 163 |

The following Fig 5 shows a comparison of amino acid sequences of some of the reported peroxidases from selected sources.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AKPC | N | P | T | D | V | V | A | L | S | G | G | H | T |
| TOB | T | P | T | D | V | V | A | L | S | G | G | H | T |
| ATP | N | I | T | D | V | V | A | L | S | G | A | H | T |
| HRPC | R | S | S | D | L | V | A | L | S | G | G | H | T |
| SBP | N | T | L | D | L | V | T | L | S | G | G | H | T |
| BP1 | D | A | T | D | L | V | T | I | S | G | G | H | T |
| WP | L | T | I | D | M | V | A | L | S | G | A | H | T |
| APX | S | D | Q | D | I | V | A | L | S | G | G | H | T |
| LiP 2 | P | A | P | D | G | L | V | P | E | P | F | H | T |
| ARP | S | P | D | E | V | V | D | L | L | A | A | H | S |

**Fig 5:** Amino acid sequence alignment of 13 amino acids sequence of a fragment for different peroxidases.

## DISCUSSION

The present study demonstrated that callus cultures of *Vitex negundo* constitute an efficient source of extracellular peroxidase. A particularly noteworthy observation was the differential expression of peroxidase between the intact plant and its callus cultures. While the general plant tissues exhibited negligible enzyme activity, the callus cultures produced substantial amounts of peroxidase. Such differential expression is commonly associated with cellular dedifferentiation and stress-induced activation of metabolic pathways during in vitro culture. Recent studies have shown that plant tissue cultures frequently exhibit altered gene expression patterns and enhanced synthesis of stress-responsive enzymes compared with differentiated tissues (Freitas *et al.,* 2024).

The successful induction of callus using BA and 2,4-D confirms the importance of auxin–cytokinin interactions in regulating cellular proliferation and metabolic activity. Similar findings have been reported in several medicinal plants where optimized hormone combinations significantly enhanced biomass production and accumulation of valuable metabolites (Sharma *et al.,* 2023). The superior performance of compact callus aggregates observed in the present study may be attributed to improved cellular organization and physiological stability, which facilitate enhanced enzyme biosynthesis.

The gradual decline in peroxidase activity during regeneration suggests that enzyme production is associated primarily with the undifferentiated state of the tissue. Peroxidases are known to participate in stress signaling, cell wall remodeling, lignification, and reactive oxygen species (ROS) detoxification, processes that are generally more active in proliferating callus cells than in differentiated tissues (Li *et al.,* 2024). Similar reductions in enzyme activity during organogenesis have been reported in other plant tissue culture systems and are often linked to metabolic reprogramming accompanying cellular differentiation.

The growth-associated pattern of enzyme production observed in suspension cultures indicates that peroxidase synthesis is closely related to active cellular metabolism. Such findings are advantageous for industrial enzyme production because biomass accumulation and enzyme synthesis can be optimized simultaneously. Cell suspension cultures have been increasingly recognized as sustainable platforms for large-scale production of enzymes and secondary metabolites due to their scalability, controlled growth conditions, and year-round productivity.

Purification studies revealed a major extracellular peroxidase isoform with a molecular mass of approximately 34 kDa and an isoelectric point near pI 9.0. Plant Class III peroxidases typically exhibit molecular masses ranging from 30 to 45 kDa, depending on their amino acid composition and glycosylation patterns (Freitas *et al.,* 2024). The molecular weight observed for *V. negundo* peroxidase therefore falls within the expected range but differs from commercial horseradish peroxidase, which generally possesses a molecular mass of approximately 40 kDa. Such differences may reflect species-specific structural variations and post-translational modifications.

The purified enzyme displayed optimum catalytic activity at pH 5.5 and 45°C. These characteristics are consistent with the acidic pH preference commonly reported for plant peroxidases. Similar optimum conditions have been documented for peroxidases isolated from horseradish, soybean, tobacco, and

several other plant sources (Freitas *et al*., 2024). The ability of the enzyme to retain substantial activity at elevated temperatures further supports its potential utility in industrial applications requiring moderate thermal stability.

The effects of metal ions on enzyme activity provided additional insights into the biochemical characteristics of the purified enzyme. Zinc, manganese, and calcium ions enhanced catalytic activity, whereas sodium azide and mercury ions strongly inhibited the enzyme. Sodium azide is a well-known inhibitor of heme-containing peroxidases because it binds directly to the heme prosthetic group and interferes with electron transfer reactions. Similar inhibition patterns have been reported for other Class III plant peroxidases (Li *et al.*, 2024). The stimulatory effects of divalent cations may be associated with stabilization of enzyme conformation and enhancement of substrate binding efficiency.

One of the most significant findings of this investigation was the exceptionally high specific activity of the purified enzyme. Even the crude extracellular preparation exhibited activity comparable to commercial horseradish peroxidase, while purification resulted in an approximately nine-fold increase in specific activity. The growing demand for highly efficient peroxidases in biosensors, clinical diagnostics, wastewater treatment, and biocatalytic processes has stimulated the search for alternative enzyme sources (Freitas *et al.*, 2024). Therefore, the high catalytic efficiency exhibited by *V. negundo* peroxidase highlights its potential industrial significance.

The N-terminal amino acid sequence analysis revealed the sequence ADIPYNGNGAV, which exhibited only limited similarity with previously reported plant peroxidases. BLAST analysis showed a maximum sequence identity of approximately 18%, suggesting that the enzyme may represent a novel peroxidase isoform. Class III plant peroxidases constitute a highly diverse multigene family characterized by substantial sequence variation despite conservation of catalytic function (Li *et al.*, 2024). The low sequence homology observed in the present study, together with the unusually high specific activity of the enzyme, supports the hypothesis that *V. negundo* callus cultures express a structurally distinct peroxidase.

Although *Vitex negundo* has been extensively investigated for its medicinal properties, including antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, and anticancer activities (Dube *et al.*, 2024), information regarding its industrial enzyme potential remains scarce. The present study therefore expands the biotechnological significance of this medicinal species by demonstrating that its callus cultures can serve as a sustainable source of a highly active and potentially novel peroxidase.

Overall, the results establish *Vitex negundo* callus cultures as a promising platform for peroxidase production. The unique expression pattern, high catalytic activity, favorable biochemical properties, and distinctive N-terminal sequence collectively suggest considerable potential for industrial and biotechnological applications. Future studies involving complete gene sequencing, recombinant expression, kinetic characterization, and structural analysis will be valuable for further understanding the molecular basis of the enzyme's exceptional activity and novelty.

## CONCLUSION

Very effective protocol could be established for the callus induction and proliferation of some selected plants that are potent sources of peroxidases. Phenolic substances were found to increase peroxidase production, though it inhibited callus formation. The calluses showed differences in peroxidase production depending on the type of growth regulator used and also the nature of callus. *Vitex* peroxidase was very unique as the callus cultures expressed tremendous enzyme activity even though the general plant body lacked it. The N-terminal sequencing of the *Vitex* peroxidase was found to be different from the other reported sequences and that may be accounting for the high specific activity of the enzyme.

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