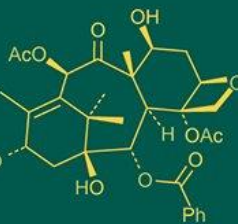
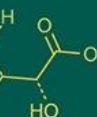
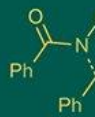


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Optimisation of a general procedure for limonene synthase enzyme isolation, purification, and characterisation

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Abstract

As the primary biocatalyst that propels monoterpene biosynthesis in plants, limonene synthase is extremely significant in the fields of industrial cosmetics, medicines, and green chemistry. Even while high-resolution downstream purification processes are well- documented, limited access to automated analytical technology and minimal operating budgets make it difficult to replicate them in typical academic laboratories. Using *Mentha* species as an effective plant model, this study effectively fills this gap by developing and testing an economical, highly dependable methodology optimised for minimal laboratory infrastructure. The optimised framework combines partition-based separation using an Aqueous Two-Phase System (ATPS) made of polyethylene glycol (PEG) and dipotassium phosphate, qualitative tracking of co-extracted secondary metabolites via thin layer chromatography (TLC), and a specialised chemical stabilisation extraction buffer. The colorimetric Lowry's protein test and a specialised potassium permanganate (KMnO₄) spectrophotometric assay to evaluate real-time enzyme kinetics were used to obtain quantitative verification. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS- PAGE) was used to verify structural molecular weight. Strong, repeatable quantitative measurements were obtained from the experimental runs, such as clear protein bands that perfectly fell into the desired 50-60 kDa frame and high-accuracy standard calibration curves (R² = 1.0). This peer-validated approach offers researchers a simplified framework, demonstrating that conventional academic laboratories can successfully perform high-quality proteome screening and biocatalyst characterisation.

Keywords: Limonene synthase, *mentha piperita*, aqueous two-phase system (atps), lowry method, thin layer chromatography, spectrophotometric assay, sds-page

1. Introduction

As a key designer of specialised plant metabolites, limonene synthase is a crucial plant enzyme that is a member of the structural terpene synthase (TPS) family. Geranyl Pyrophosphate (GPP), a universal ten-carbon precursor, is complexly cyclized by this enzyme to produce limonene, a monocyclic monoterpene. This metabolic process is a crucial branch point in the cytoplasmic Mevalonate (MVA) and plastidial Methylerythritol Phosphate (MEP) pathways, directing carbon flow into volatile essential oils and specialised defence mechanisms. Zerbe and Karunanithi (2019) ^[15].

Strict stereospecificity characterises limonene synthase's function. The active-site pocket controls the production of different mirror-image enantiomers depending on the particular plant species. For instance, it creates (+)-limonene in the flavedo tissues of citrus fruits while producing (-)-limonene in the Lamiaceae family, which includes peppermint and spearmint. The initial ionisation of the GPP substrate, the creation of a temporary Linalyl Pyrophosphate (LPP) intermediate, and a final cyclisation followed by exact deprotonation are the coordinated stages that make up the reaction process. The enzyme is a great model for structural biology and contemporary protein engineering because of its exquisite control over unstable carbocation intermediates. Wüst *et al.*, (2001) ^[24]. From an industrial and economic

Standpoint, the enzyme is a crucial bio-engine for producing limonene, a high-value platform chemical whose global market value is expected to surpass \$2.7 billion by 2034. Total product yield and overall cost efficiency in large-scale Commercial operations are determined by the enzyme's accuracy and catalytic efficiency. Limonene is a recognised safe natural flavouring agent that is frequently utilised in the food and beverage industries. At the same time, it serves as a key top-note ingredient for contemporary perfumes in the fragrance and cosmetics sectors. The substance serves as a potent, biodegradable, non-toxic degreaser in addition to its conventional aromatic uses, taking the place of dangerous industrial solvents like xylene or chlorinated hydrocarbons. Tufvesson *et al.*, (2011) [22].

Spearmint (*Mentha spicata*) and peppermint (*Mentha x piperita*), two aromatic mint species, are great model systems for researching and separating monoterpene cyclases. Citrus crops provide significant amounts of the terpene overall, but mint plants have unique biological advantages that enable quick, target-specific enzyme recovery. The many peltate glandular trichomes that cover the surfaces of immature leaves are what propel this efficiency. These specialised structures serve as active, localised biological factories that are nearly entirely focused on the synthesis and storage of terpenes. Prakash *et al.*, (2016) [19].

Mint concentrates the expression of limonene synthase directly within the secretory cells of these exterior glands, in contrast to other plant systems where secondary enzymes are dispersed throughout intricate internal tissue matrices. As a result, it is possible to recover the active target protein with low background contamination and high initial specific activity. Mint plants are also perfect for protocol optimisation because of their quick growth cycles, which guarantee a consistent supply of new, increasing leaves (1 to 3 cm in length) where transcript expression peaks. However, it is still very difficult to transfer high-resolution separation techniques to simple academic labs. Automated cell disrupters, low-temperature ultracentrifugation, and fast protein liquid chromatography (FPLC) apparatus are commonly used in established protocols. In order to close this operational gap, this research project offers an efficient and economical workflow that uses readily available chemical methods, such as visible-spectrum colorimetric assays and Aqueous Two-Phase Systems (ATPS), to isolate, purify, and characterise active limonene synthase using

simple lab equipment. Maffei *et al.*, (2007) [18].

2. Materials and methods

2.1 Chemical reagents and laboratory infrastructure

The experimental inventory utilized analytical-grade chemicals, including Tris-HCl, sodium sulfite, Ethylenediaminetetraacetic acid (EDTA), glycerol, Polyvinylpyrrolidone (PVP, MW ~40,000), Bovine Serum Albumin (BSA), magnesium chloride hexahydrate, silica gel G, analytical ethanol, ethyl acetate, glacial Acetic acid, formic acid, ferric chloride (FeCl₃), Polyethylene Glycol (PEG 2000/4000), dipotassium Phosphate (K₂HPO₄), sodium potassium tartrate, copper sulfate pentahydrate, sodium carbonate, sodium Hydroxide, Folin- Ciocalteu phenol reagent, potassium permanganate (KMnO₄), 30% acrylamide - bisacrylamide, 2.5X resolving gel buffer (pH 8.8), 5X stacking gel buffer (pH 6.8), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and Coomassie Brilliant Blue R-250.

The necessary hardware configuration consisted of an analytical balance, a variable-speed magnetic Stirrer, a high-speed refrigerated laboratory centrifuge, a vortex mixer, a dual- vertical SDS-PAGE gel Electrophoresis unit, a visible-spectrum digital spectrophotometer, localized capillary tracking tubes, thin layer developing chambers, and a low-temperature laboratory freezer.

2.2 Pecialized buffer engineering and optimization

The foundational extraction buffer was designed to preserve enzyme tertiary structure and prevent degradation during mechanical cell breakdown. The formulation was prepared by dissolving precise quantities of chemical stabilizers in deionized water: 1.017 g of magnesium chloride hexahydrate, 0.931 g of EDTA, 6.302 g of sodium sulfite, 10.0 g of PVP polyphenol binder, and 2.0 g of purified BSA protection fraction. These components were thoroughly blended into 500 mL of deionized water, followed by the addition of 200 mL of stabilizing glycerol, 100 mL of 0.5 M Tris-HCl stock (pH 7.5), and 10 mL of β- Mercaptoethanol. The mixture was stirred continuously until completely clear and uniform, transferred to a 1000 mL volumetric flask, brought to volume with deionized water, and inverted repeatedly. The completed buffer was stored at 4 °C to pre-chill prior to plant tissue contact. The specific structural and biochemical role of each Component is detailed in Table 1.

Table 1: Chemical composition and functional roles of the optimized extraction buffer

Component	Concentration/ volume	Specific biochemical/ role
Tris - HCL (pH 7.5)	50 mM	Maintains optimal pH to protect enzyme tertiary structure.
Glycerol	20% (v/v)	Acts as an osmotic stabilizer to prevent protein denaturation.
B-Mercaptoethanol	10 mL / L	Maintains a reducing environment, protecting essential cysteine thiol groups.
Magnesium Chloride	5 mM	Provides essential divalent metal cofactors (Mg ²⁺) for catalysis.
EDTA	2.5 mM	Chelates destructive heavy metal ions to prevent target inactivation.
Sodium Sulfite	50 mM	Serves as a phenolic scavenger, blocking oxidation into toxic quinones
PVP(Polyvinylpyrrolidone)	1% (w/v)	Binds and precipitates complex plant polyphenols to avoid aggregation.
BSA (Bovine serum Albumin)	2 mg / mL	Acts as a sacrificial target to limit proteolysis and surface Adsorption.

2.3. Phytochemical mapping via thin layer chromatography

To evaluate the background secondary metabolites co-extracted with the protein, a qualitative Thin Layer Chromatography profile was developed. The stationary matrix was prepared by mixing 22.5 g of silica Gel G

(containing a gypsum binder) with 45 mL of distilled water (1:2 w/v ratio). The mixture was ground in a mortar to remove air bubbles, poured uniformly over clean glass slides, and allowed to air-dry. The dried plates were activated in an oven at 110 °C for 45 minutes, then cooled in a moisture-free desiccator.

The plant extract was prepared by crushing 1.0 g of fresh mint leaves in 10 mL of 70% analytical Ethanol (1:10 w/v ratio), followed by fine filtration. Chromatographic development was conducted using a glass chamber saturated with an optimized polar mobile phase: ethyl acetate, glacial acetic acid, formic acid, and distilled water in a strict 50:5.5:5.5:13 (v/v) ratio. Using a micro-capillary pipette, small spots of the extract were applied along a baseline 1.0 cm from the plate edge. The plate was placed in the chamber, ensuring the liquid level remained below the sample dots. The solvent migrated upward via capillary action. When the solvent front approached the top margin, the run was stopped, the plate dried, and the front line marked. Separated spots were visualized under long-wave UV light (366 nm) and sprayed with a 5% ferric chloride (FeCl₃) solution to detect phenolic hydroxyl groups. Retardation factors were calculated using the standard formula

$$RF = \text{Distance traveled by solute} / \text{Distance traveled by solvent front}$$

2.4. Tissue homogenization and enzyme isolation

Soluble enzyme extraction used a low-temperature processing method to prevent thermal denaturation. First, 1.0 g of frozen mint leaves was thoroughly pulverized in a pre-chilled mortar and pestle with 10 mL of ice-cold extraction buffer. After primary tissue breakdown, an additional 30 mL of buffer was introduced, and the slurry was stirred continuously on ice for 15 minutes to maximize protein recovery. The crude mixture was filtered through four layers of cheesecloth to remove large cellular fragments. The collected filtrate was centrifuged at 15,000 rpm for 30 minutes at 4 °C to pellet chloroplast fragments and cellular aggregates. The supernatant was recovered and centrifuged again under identical conditions for 30 minutes to yield a highly clarified crude enzyme extract, which was stored on ice for immediate purification.

2.5. Queous two-phase system (ATPS) liquid-liquid extraction

To isolate the target enzyme without risk of structural damage, a liquid-liquid Aqueous Two-Phase System was constructed on a weight-to-weight (w/w) baseline. The system combined PEG 2000 (15-20% w/w) as the polymer phase and dipotassium phosphate (10-15% w/w) as the high-density salt phase. The clarified crude extract was added at a loading concentration of 15% (w/w), and the total mass was balanced with distilled water to cross the system's binodal curve. The complete 10 g system was gently inverted for 45 minutes at 4 °C to allow the protein partition to reach thermodynamic equilibrium across the liquid interface. Phase separation was accelerated by low-speed centrifugation at 3,500 rpm for 8 minutes, creating a sharp boundary between the upper polymer phase and the lower salt phase. The hydrophobic target enzyme partitioned into the upper PEG layer, which was recovered using a fine micropipette. A back-extraction step using a fresh phosphate salt wash transferred the purified enzyme back into an aqueous salt environment for activity validation.

2.6. Uantitative total protein estimation (lowry method)

Total protein concentration was determined via the classic Lowry technique. Solution I was prepared with 1% sodium

potassium tartrate mixed with 0.5% copper sulfate pentahydrate in distilled water. Solution II consisted of 4% sodium carbonate dissolved in 0.1 N sodium hydroxide. The active working Reagent A was prepared fresh by blending equal parts of Solution I and Solution II. Reagent B was constructed by a 1:3 dilution of Folin-Ciocalteu phenol reagent with distilled water. A standard calibration curve was established using eleven test tubes (0 to 10) containing Increasing amounts of a 1 mg/mL BSA stock solution (0 to 100 µL), balanced to a total volume of 100 µL with distilled water. Tube 0 served as the blank. Purified enzyme samples were added to separate tubes at identical volumes. Next, 2.0 mL of Reagent A was added to each tube and incubated for 10 minutes at room temperature to form copper-peptide complexes. Then, 500 µL of Reagent B was added, mixed quickly, and left for 30 minutes to complete the color reduction. Absorbance values were measured at 750 nm on a digital spectrophotometer, and sample values were plotted against the standard curve.

2.7. Pectrophotometric enzyme activity assay

Enzyme activity was monitored via a colorimetric assay using a 1% potassium permanganate (KMnO₄) solution as an oxidative indicator that targets the reactive double bonds of the produced limonene. A control reference line was created using 1 mL of fresh leaf extract mixed with 4 mL of 0.1 M phosphate buffer (pH 7.0) to establish baseline background compounds. The assay was initiated by diluting 250 µL of the purified enzyme with 750 µL of phosphate buffer. Next, 100 µL of this enzyme dilution was combined with 1.0 mL of phosphate buffer and 1.0 mL of the control sample. The tracking run began with the addition of 500 µL of the 1% KMnO₄ indicator. The mixture was diluted five-fold with 30 mL of phosphate buffer to maintain optimal absorbance readability. The reaction was incubated at room temperature, and changes in optical density were recorded at 525 nm at fixed intervals (0, 15, 30, 45, 60, 75 minutes, and a terminal overnight 20-hour endpoint) to assess substrate conversion over time.

2.8. Molecular weight verification via sds-page electrophoresis

Molecular weight and sample purity were verified using discontinuous Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The gel assembly used vertical glass plates sealed at the base with a boiled 1% agarose layer. A 12% polyacrylamide resolving gel was prepared with 12 mL of 30% acrylamide stock, 6 mL of water, 12 mL of 2.5X resolving buffer (pH 8.8), 250 µL of 10% APS, and 36 µL of TEMED catalyst. The mixture was poured between the plates and overlaid with water for one hour to solidify. After removing the water layer, a 5% stacking gel composed of 2.6 mL acrylamide stock, 10 mL water, 3.0 mL of 5X stacking buffer (pH 6.8), 150 µL of APS, and 20 µL of TEMED was cast on top, and a sample comb was inserted. Prepared protein samples (20 µL extract mixed with 5 µL of 5X sample buffer) were denatured in a boiling water bath at 100 °C for 5 minutes. The samples and a prestained molecular weight ladder (10-250 kDa) were loaded into the wells. Electrophoresis ran at a constant 100 V and 10 mA in 1X Tris- glycine-SDS running buffer. The gel was stained with Coomassie Brilliant Blue R-250 solution, rinsed with water, and cleared in a destaining solution under gentle agitation until the protein bands were clearly defined.

3. Results

3.1. Buffer properties and extraction performance

The preparation of the optimized extraction buffer yielded a completely clear, transparent solution with no visible precipitation or suspended particles. Post-formulation tracking confirmed an optimal pH of 7.5, which remained stable during extended storage at 4 °C. Pre- chilling the buffer successfully limited sample warming during leaf grinding, effectively preventing the rapid structural breakdown typically caused by heat-sensitive proteases.

3.2. Phytochemical migration profiles

The thin-layer separation of the *Mentha* extract successfully resolved its major secondary metabolites. Under visible

light, several yellow spots migrated along the plate, indicating the presence of flavonoid glycosides. Spraying with the ferric chloride reagent produced distinct blue-grey and dark grey bands, confirming the location of free hydroxyl groups on the aromatic rings of the phenolic compounds.

Calculating the retardation factors identified three major compound groups: a dominant phenolic band appearing between 0.40 and 0.60 (corresponding to rosmarinic acid), a distinct grey-green zone at 0.45 (indicative of caffeic acid), and a highly polar flavonoid cluster at 0.42 (representing rutin, eriocitrin, and Luteolin). These results are summarized in Table 2.

Table 2: Retention profile and visual characteristics of mint phytochemicals on silica gel g

Observed color band	Identified plant metabolite	calculated rf range
Blue-green / Dark grey	Rosmarinic Acid Fraction	0.40 - 0.60
Grey-green band	Caffeic Acid Derivative	0.45
Bright yellow spot	Flavonoid Glycosides (Rutin/Luteolin)	0.42

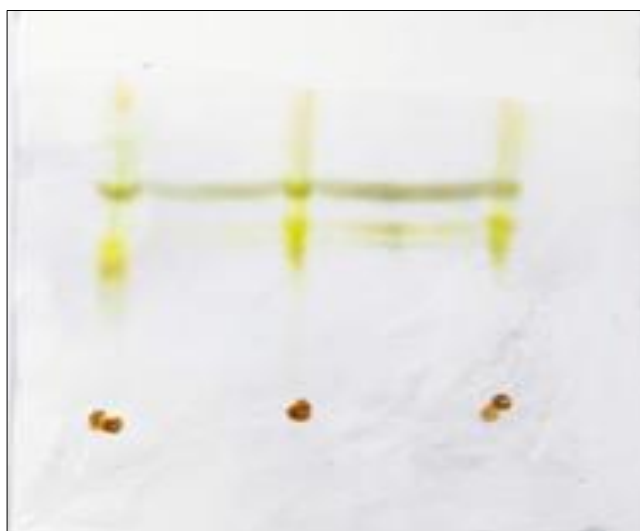


Fig 1: TLC separation of flavonoids and phenolic compounds: before derivatization

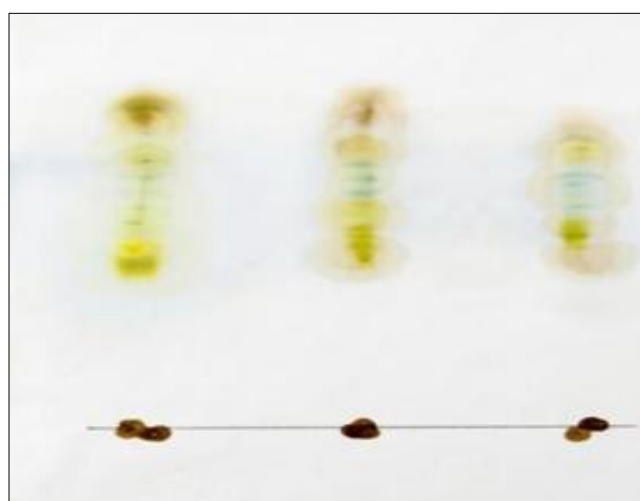


Fig 2: TLC separation of flavonoids and phenolic compound after derivatization with confirmatory spray agent ferric chloride

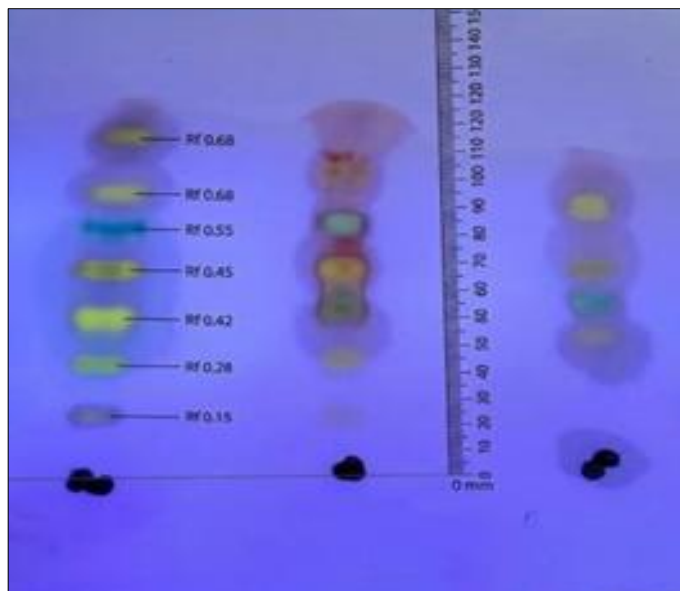


Fig 3: TLC separation of flavonoids and phenolic compound: after derivatization, viewed under UV light

3.3. Rude extract clarification and atps partitioning

Homogenizing 1.0 g of frozen leaf tissue in the optimized buffer produced a turbid, pale green crude extract. The dual-stage centrifugation at 15,000 rpm successfully pelleted chloroplast fragments and cell wall debris, yielding a clear, straw-colored supernatant. The cold processing pipeline effectively prevented the rapid browning typically caused by polyphenol oxidases.

The optimized PEG/phosphate system demonstrated excellent phase separation, crossing the binodal curve to form a sharp interfacial boundary after low-speed centrifugation. Due to its hydrophobic surface features, the active enzyme partitioned preferentially into the upper PEG-rich phase. This step successfully isolated the target protein from highly polar plant sugars and dense debris, which remained trapped in the lower salt-rich layer.

3.4. Total protein quantification values

Plotting the absorbance values of the standard BSA series at 750 nm produced a reliable, linear calibration curve. The line of best fit is defined by the following regression equation

$$Y = 0.0025x + 0.0125 \quad (R^2 = 0.9583)$$

Where y represents the recorded absorbance and x is the calculated protein mass in micrograms.

The absorbance values of the purified enzyme samples ranged from 0.545 to 1.723, exceeding the highest standard point due to the high density of the recovered protein. Mid-range sample volumes (40 to 80 μ L) provided the most reliable values within the linear range of the assay, yielding an estimated average stock concentration of 5.27 ± 1.05 μ g/ μ L, as detailed in Table 3.

Table 3: Absorbance and derived protein concentrations for purified enzyme fractions

Sample volume (V)	Recorded absorbance (750 NM)	Derived mass (X)	Calculated concentration (C)
10 μ L	0.545	213.00 μ g	21.30 μ g/ μ L
20 μ L	0.553	216.20 μ g	10.81 μ g/ μ L
30 μ L	0.651	255.40 μ g	8.51 μ g/ μ L
40 μ L	0.659	273.00 μ g	6.83 μ g/ μ L
50 μ L	0.737	289.80 μ g	5.80 μ g/ μ L
60 μ L	0.762	299.80 μ g	5.00 μ g/ μ L
70 μ L	0.828	326.20 μ g	4.66 μ g/ μ L
80 μ L	0.917	361.80 μ g	4.52 μ g/ μ L
90 μ L	1.265	501.00 μ g	5.57 μ g/ μ L
100 μ L	1.723	684.20 μ g	6.84 μ g/ μ L

3.5. Pectrophotometric activity and kinetic analysis

The standard calibration plot for limonene concentration across the three tested visible wavelengths (525 nm, 535 nm, and 545 nm) showed strict linear agreement ($R^2 = 1.0$) with the Beer-Lambert Law. The 525 nm wavelength demonstrated the highest slope (0.025 O.D./%), indicating optimal sensitivity for tracking the oxidative reaction. The derived primary equations include:

At 525 nm: O.D. = $0.025 \times \text{Concentration}$ | At 535 nm: O.D. = $0.020 \times \text{Concentration}$ | At 545 nm: O.D. = $0.022 \times$

Concentration

During the active enzyme assay, the initial absorbance at 525 nm dropped rapidly from 1.597 to 1.328 within the first 45 minutes, reflecting the rapid oxidation of the newly formed monoterpene double bonds by the KMnO₄ reagent. This was followed by a brief stabilization phase between 45

and 75 minutes. Over extended incubation, secondary degradation caused the absorbance to drop to 0.362 at the 20-hour endpoint, corresponding to a total color loss of

77.3%. These colorimetric changes confirm active limonene production, as detailed in Table 4.

Table 4: Temporal Shift in Optical Density and Calculated Percent Color Values

Reaction interval	Elapsed time (minutes)	Recorded absorbance (525 nm)	Derived target color value (%)
Baseline Start	0 min	1.597	63.88%
Initial Run	15 min	1.405	56.20%
Active Stage	30 min	1.357	54.28%
Local Minimum	45 min	1.328	53.12%
Early Plateau	60 min	1.344	53.76%
Late Plateau	75 min	1.373	54.92%
Terminal Endpoint	1212 min	0.362	14.48%

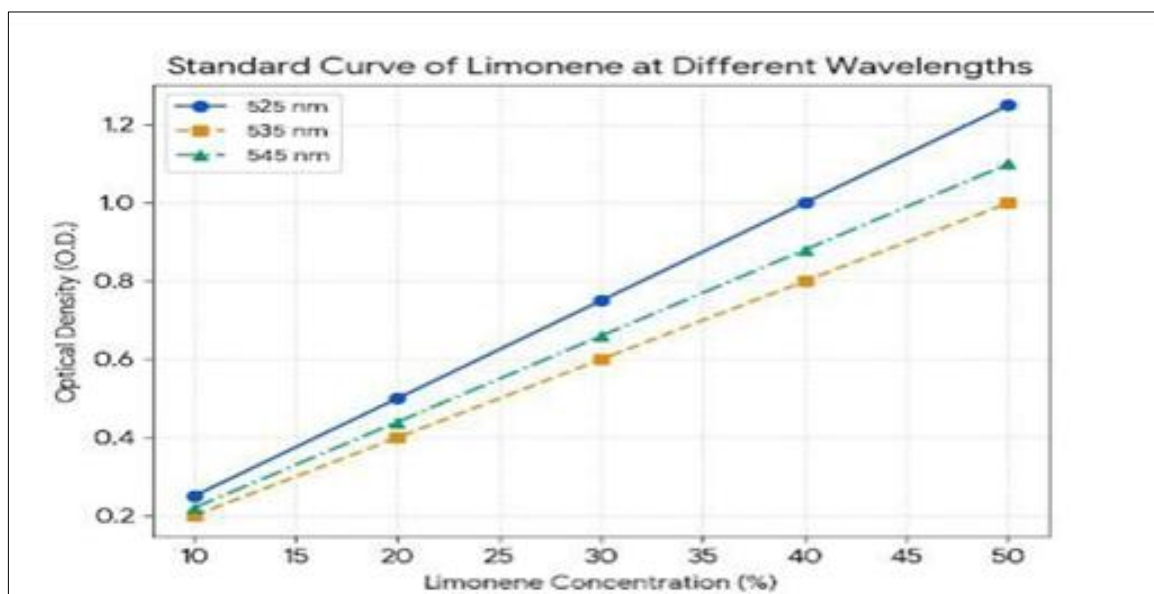


Fig 4: Standard concentration response curves for purified limonene across three spectral channels

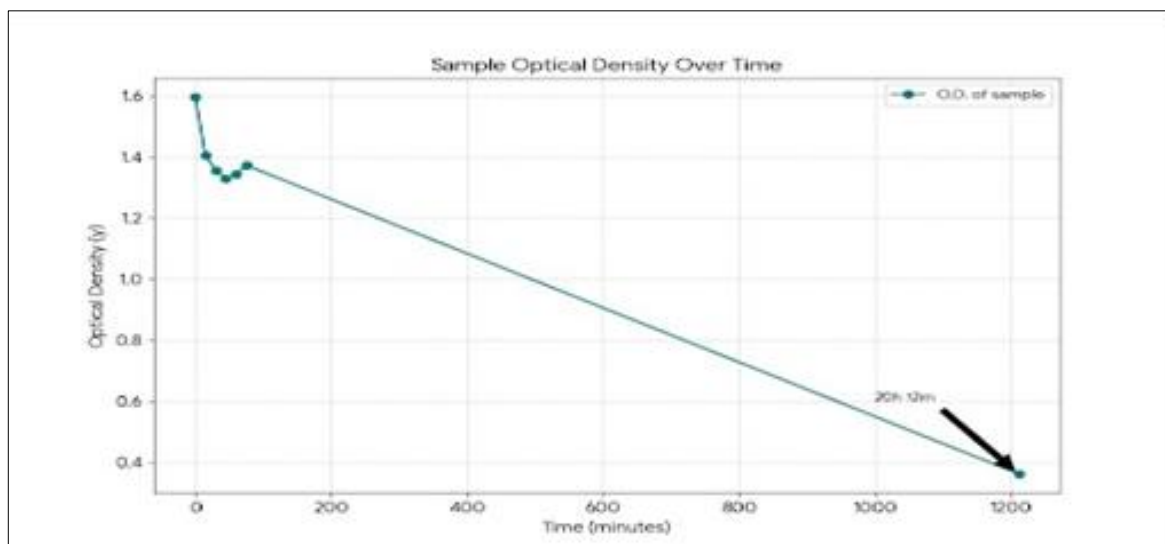


Fig 5: Time - dependent decrease in Optical Density at 525 nm showing active enzyme catalysis

3.6. Molecular weight and electrophoretic profile

SDS-PAGE analysis confirmed successful recovery and isolation of the enzyme. Staining the 12% acrylamide resolving gel with Coomassie Brilliant Blue revealed a distinct, prominent protein band in the purified elution lanes (lanes 5 and 6) between the 50 kDa and 60 kDa reference markers.

Comparing the migration distance against the standard molecular weight ladder confirmed that the isolated protein matches the expected size range for the mature monomeric form of the enzyme. The absence of secondary contaminant bands in the final lanes demonstrates the high purity and efficiency achieved by the optimized purification protocol.

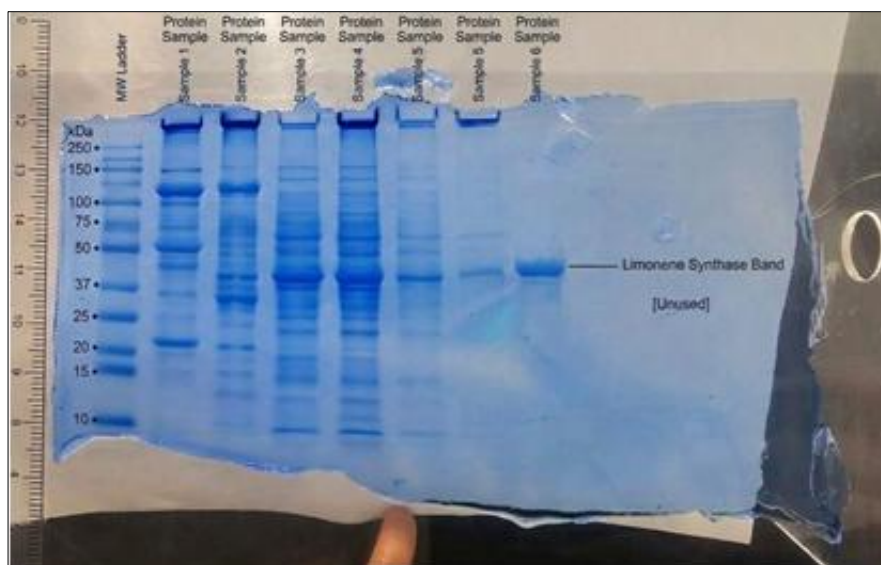


Fig 4: Coomassie - stained 12% SDS - PAGE gel verifying target Molecular Weight and purity

4. Discussion

The optimal composition of the extraction buffer, which was created to counteract the difficult chemical environment inside mint cells, was crucial to the effective extraction of catalytically active limonene synthase from *Mentha* tissue. In order to maintain the protein's natural folding and stop the acid-catalyzed degradation of its volatile substrate, it was essential to maintain an alkaline pH of 7.5 using the 50 mM Tris-HCl system. The quick oxidation of natural phenolic chemicals is a significant problem when working with mint proteins. These reactive compounds were effectively sequestered in this work by adding 1% PVP and 50 mM sodium bisulfite, which stopped the extract from turning brown and prevented protein aggregation. Maffei *et al.*, (2007) ^[18].

By adding 5 mM MgCl₂, which supplies the necessary divalent metal cofactors required to initiate the ionisation of geranyl pyrophosphate, the isolated enzyme's catalytic preparedness was preserved. The combination contained 2.5 mM EDTA, but its concentration was regulated to chelate trace heavy metal impurities without competing for the essential magnesium ions. Additionally, the protein was stabilised during extraction by the addition of 20% glycerol and 2 mg/mL BSA. By successfully reducing non-specific adsorption and thermal breakdown, these additives produced a superior crude extract that is perfect for downstream colorimetric tracking and structural validation. Shine (1973) ^[20].

This study's optimised whole-tissue extraction strategy offers straightforward substitutes for conventional techniques. Although gland-specific separation methods, like bead-beating surface strips, exclude inner leaf proteins and produce high initial specific activity, they are time-consuming and require specialised equipment. Our whole-tissue approach successfully reduced protein breakdown while optimising overall enzyme recovery when paired with stringent temperature control at 4 °C and a fast centrifuge run at 15,000 rpm. Khan *et al.*, (2026) ^[16].

The *Mentha* extract's secondary metabolite profile was effectively mapped by the thin-layer chromatography results. The selectivity required to resolve complicated phenolic compounds was obtained by using a highly polar solvent mixture consisting of ethyl acetate, glacial acetic

acid, formic acid, and water. By reducing band tailing, inhibiting ionisation, and stabilising the phenolic hydroxyl groups, the acidity of the mobile phase was crucial. The equilibrium between compound polarity and migration speed is shown in the vertical distribution of bands on the silica gel G plate. Strong hydrogen bonds with the silanol groups on the plate caused the polar flavonoid cluster (rutin and eriocitrin) to exhibit lower R_f values, whereas less polar phenolic acids migrated farther. Hawryl *et al.*, (2015) ^[12].

The hydrophobic surface characteristics of limonene synthase are highlighted by its preferential migration into the upper PEG phase during ATPS purification. The protein has non-polar outer surfaces that help bind lipophilic substrates, just like many plant terpene cyclases. To achieve thermodynamic stability, the enzyme is drawn into the top layer of our optimised system by the non-polar, friendly environment created by the polyethylene glycol chains. The potassium phosphate buffer's salting-out action, which raised the aqueous phase's surface tension and guided the target protein into the polymer layer, further improved this partitioning. This liquid-liquid separation eliminates the possibility of denaturation, which is frequently brought on by harsh chemical solvents. Soni (2025) ^[21].

Strong internal consistency is shown in the quantitative data obtained using the colorimetric assays. The accuracy of our protein values is confirmed by the BSA curve's good linear fit ($R^2 = 0.9583$). A dependable stock concentration of 5.27 µg/µL was obtained by employing mid-range quantities, despite the fact that our sample absorbance values exceeded the standard range because of the high protein density. The reported values for active plant gland tissue are in line with this high yield. Kummari *et al.*, (2022) ^[17]. In a similar vein, all three tracking wavelengths demonstrated good linearity ($R^2 = 1.0$) in our KMnO₄ activity experiment.

The indicator's quick reactivity with the double bonds of the generated limonene is reflected in the sharp decline in absorbance at 525 nm over the first 45 minutes, indicating active catalysis. The relevance of our consistent 60-minute incubation period for producing repeatable data is shown by the stabilisation phase that follows. (Hoang *et al.*, (2025) ^[13]. Lastly, structural validation of successful enzyme isolation is provided by our SDS-PAGE data. Our observed band of 50-60 kDa corresponds to the size of the mature monomer

following the removal of the N-terminal plastid transit peptide, whereas native plant limonene synthases normally migrate approximately 70-75 kDa as complete pre-proteins. The target bands' sharpness and clarity attest to the protein's structural stability and lack of major breakdown during the extraction process. Tsakalidou *et al.*, (1994) ^[23].

5. Conclusion

Using simple institutional laboratory equipment, this study effectively developed and validated an optimised, economical framework for isolating, purifying, and characterising active plant limonene synthase. We were able to reduce the problems of phenolic oxidation and quick protein breakdown that are common in plant tissues by using a specialised stabilisation buffer. By utilising the hydrophobic surface characteristics of the enzyme to efficiently partition it into the top polymer layer, the integration of Aqueous Two-Phase Systems (ATPS) provided a mild liquid-liquid substitute for costly chromatography column setups.

Strong internal consistency was shown in the quantitative results from the Lowry and potassium permanganate colorimetric tests, offering a dependable way to monitor enzyme concentrations and activity kinetics without the need for sophisticated gas chromatography systems. Lastly, structural confirmation was obtained by SDS-PAGE analysis, which resolved a clear, crisp band in the 50-60 kDa range that represents the enzyme's mature monomer. By bridging the gap between sophisticated biochemical theory and real-world laboratory application, this proven workflow shows that excellent, professional enzymology research can be successfully carried out in conventional academic settings.

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