Research Article

Biochemical characterization of esterases from the seeds of *Caesalpinia Mimosoides*

Boyidi Tirupateswara Rao[1*], Patnala Veeranjaneyulu[1], and Kempohalli S. Chandrashekharaiah[1]

[1] Department of Biochemistry, Jnana Kaveri PG Centre, Mangalore University, Chikka Aluvara - 571 232, Kodagu

(Received: 04-02-2025 Revised: 29-06-2025 Accepted: 01-07-2025)

Corresponding Author: ***Boyidi Tirupateswara Rao***. Email: venkat.boyidi@gmail.com

ABSTRACT

Caesalpinia mimosoides, a medicinally significant plant, harbors bioactive compounds, including hydrolytic enzymes such as esterases. Carboxyl esterases (CEs) play crucial roles in metabolic processes and have wide-ranging industrial relevance. However, limited information is available on the biochemical characterization of esterases from *C. mimosoides* seeds. This study aimed to isolate, purify, and characterize carboxyl esterases from the seeds of *C. mimosoides*, with an emphasis on understanding their properties for potential application in biotechnology and allied fields.

Protein extracts were obtained from seed acetone powder, followed by enzyme purification through ammonium sulfate precipitation (0–60%), ion-exchange chromatography using DEAE-cellulose, Sephadex G-100 gel filtration, and preparative polyacrylamide gel electrophoresis (PAGE). The enzyme was characterized using SDS-PAGE, native PAGE, and isoelectric focusing. Enzyme kinetics, substrate specificity, thermal and pH optima, stability, and inhibitor sensitivity were assessed using standard spectrophotometric assays with 1-naphthyl esters.

The purified CE showed a molecular mass was approximately 30.2–31.1 kDa, and the isoelectric point was 7.4. The enzyme demonstrated optimal activity at pH 7.5 and 45°C and retained activity between pH 4.0–9.0 and temperatures up to 60°C. Substrate specificity analysis revealed a preference for α-naphthyl butyrate. Kinetic parameters indicated a higher affinity for 1-naphthyl propionate compared to α-naphthol acetate. Inhibitory studies showed significant sensitivity to organophosphates (phosphomidon, dichlorvos), while resistance was observed to eserine sulfate and PCMB.

The carboxyl esterase from *C. mimosoides* seeds is a stable monomeric enzyme with distinct biochemical features, making it a promising candidate for applications in industrial catalysis and environmental bioremediation.

Keywords: *C. mimosoides*, Carboxyl esterases, Electrophoresis, Purification, SDS.

1. INTRODUCTION

Caesalpinia mimosoides, commonly known as the Mimosoides plant, belongs to the Caesalpinioideae subfamily and is known for its traditional medicinal uses. The seeds of this plant have been of particular interest due to their potential therapeutic properties. Among various sources of proteins, plants are extensively studied due to their abundance and comprehensive expression system. Microbes and animal sources, on the other hand, require complex handling procedures and ethical consent [1].

Plants serve as a valuable source of enzymes and proteins that have notable biological impacts. Proteins are essential macromolecules involved in various biological processes, including enzymatic activity, signaling, and structural support. The analysis of proteins present in *Caesalpinia mimosoides* seeds can provide valuable insights into their potential bioactive properties and pharmaceutical applications.

Carboxyl esterases (CE), on the other hand, are a diverse group of metabolic enzymes that accelerate the breakdown of complex molecules

into simpler compounds through hydrolysis reactions [2]. Carboxyl esterases are a part of numerous physiological functions, including seed germination, plant growth, and defense mechanisms against pathogens and pests [3]. Understanding the types and activities of carboxyl esterases present in *Caesalpinia mimosoides* seeds can shed light on their potential roles in various industries, including food processing, biotechnology, and pharmaceuticals [4].

The study aims to purify and characterize the carboxyl esterases in its seeds through comprehensive biochemical & molecular analyses, which are crucial for various biological processes & potential applications in pharmaceuticals, nutraceuticals & agriculture [5, 6].

By elucidating the carboxyl esterase profile of *Caesalpinia mimosoides* seeds, this study seeks to provide a foundation for further research on their therapeutic potential and industrial applications. Ultimately, the investigations of this research will be instrumental in understanding the existing knowledge on the biochemical properties of *Caesalpinia mimosoides* seeds and may uncover novel applications and potential benefits associated with these proteins and hydrolytic enzymes.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Genus Material

Caesalpinia mimosoides seeds were obtained from Madikeri Taluk, within Kodagu district, Karnataka. The seed collection period spanned from March to May.

2.1.2. Chemicals

The chemical substances used in the investigation, including acrylic amide, N,N-methylenebis(acrylic amide), 1-naphthyl acetate, BSA, Fast blue RR, and Diazo Blue-B purchased from Sigma-Aldrich, United States. Chemicals of AR grade were used.

2.2. Methods

2.2.1. Process of Germination

Caesalpinia mimosoides seeds were immersed for 24 hours in sterile distilled water, then placed over a moistened filter paper in a petri dish. Germination was conducted under sterile conditions in the dark at room temperature. Seedlings were collected at 24-hour intervals throughout the germination process.

2.2.2 Preparation of Crude Protein Extract

Acetone powder was prepared using the method established by Kumar and Sharma [7]. The seeds of C. mimosoides were soaked in distilled water for 20hrs and dehulled. 10% acetone powder of the dehulled seeds and germinated seedlings were prepared by blending in a homogenizer with chilled acetone for 5 minutes followed by filtration using a suction pump. The cake obtained was dried at 37°C, powdered and stored at 4°C until further use.

The 10% enzyme extracts were prepared from the acetone powder. These extracts were obtained by stirring the powder for 2 hours at 4°C in 25 mM sodium phosphate buffer with pH 7.0, followed by centrifugation at 10,000 rpm for 15 minutes at 4°C. The supernatants were separated and used for further analyses of protein content in total and activity of esterase.

2.2.3 Ammonium sulphate precipitation

The crude extract of enzyme underwent an ammonium sulfate precipitation ranging from 0 to 60%. The protein precipitated (0 – 60%) was isolated from the solution through centrifugation at 10,000 rpm for 30 minutes. The protein pellet was dissolved in 25 mM sodium phosphate buffer at pH 7.0, followed by dialysis against sodium phosphate buffer at the same pH. The ammonium sulfate fraction that underwent dialysis was further purified through ion exchange chromatography with diethylaminoethyl cellulose [8].

2.2.4 Assay of Esterase

The measurement of esterase activity was carried out using a protocol modified from Gomori's method [9], as further developed by Van Asperen [10]. The standard assay procedure entailed the combination of 5 parts of 0.3 mM solution of substrate (5ml) with one part of extracted enzyme (1 ml). The substrate solution was prepared by diluting a 30 mM 1-naphthol acetate stock

solution (acetone as solvent) 100-fold using 50 mM sodium phosphate buffer with pH 7.0. Following a 15-minute incubation period at 27°C, the enzymatic reaction was halted by introducing DB-LS reagent (1 ml), composed of 1% diazo blue B with 5% SDS in a 2:5 ratio. After a 30-minute development phase, the resultant color intensity was quantified spectrophotometrically at 600 nm. Esterase activity was ultimately expressed as micromoles of product formed per minute at 27°C, determined using a calibration curve constructed with 1-naphthol as the standard.

2.2.5 Assay of Protein

Proteins were quantified using the method established by Lowry *et al.,* [11] with bovine serum albumin serving as the standard [12, 13].

2.2.6 Electrophoresis

The anionic discontinuous gel electrophoresis procedure was implemented following the Ornstein gel electrophoresis method and Davis's method [14, 15]. The experimental setup utilized a disc electrophoresis, containing a 7.5% resolving gel and a 4% stacking gel. Tris-glycine buffer functioned as the electrode buffer. The electrophoretic separation was performed under cold conditions (0-4°C) by supplying2 mA current per each well of a sample for 4 hours.

2.2.7 Staining of esterolytic activity

The activity of esterases on PAGE was detected following a procedure based on the methodology published by Hunter and Markert [16]. Fast Blue RR coupled with alpha-naphthol group, which is produced during the hydrolysis of alpha-naphthol acetate. After electrophoresis, the polyacrylamide gels were detached from the glass plates. For subsequent analysis, detached gels were stained to visualize the estero-lytic activity.

2.2.8 Staining of proteins

The separated polyacrylamide gels were immersed in a staining solution containing 0.5% Coomassie Brilliant Blue R-250 for one hour. It is prepared in 25% methyl alcohol and 7.5% ethanoic acid in distilled water. The gels were placed in a destaining solution consisting of 25% methyl alcohol and 7.5% ethanoic acid in water for overnight.

3. RESULTS

3.1 Ammonium sulphate precipitation

All centrifugations were carried out in centrifuge (Beckman Coulter 64 R) at 4°C. The crude extract obtained from acetone powder was subjected to fractional precipitation. Ammonium sulfate, refrigerated acetone, and a pH change were employed to conduct fractional precipitation. The addition of acetone and a change in pH resulted in a substantial reduction in esterase activity. Conversely, ammonium sulphate fractionation resulted in a favorable yield along with an enhancement in fold purification. Therefore, ammonium sulfate was chosen for the fractional precipitation of esterase from the primary enzyme extract.

3.2 DEAE-Cellulose Ion Exchange Chromatography

The 0 – 60% ammonium sulfate fraction that had been dialyzed (25 ml) was applied to the DEAE-Cellulose column and subsequently rinsed with 150 ml of the initial buffer. The column was subsequently eluted through a gradual increase in ionic strength, utilizing a starting buffer with 0.1 M and 0.3 M NaCl, and 10 ml portions were gathered. Fraction I, fraction II, and fraction III were the three esterase activity peaks that were eluted. Fraction I was not adsorbed onto the column and, as a result, was eluted out with the beginning buffer. Fraction II was eluted with 0.1 M sodium chloride, while fraction III was eluted with 0.3M sodium chloride in the starting buffer. The fractions that contained a significant quantity of esterase activity were pooled separately, dialyzed against double-distilled water, and subsequently concentrated using ammonium sulfate.

Size exclusion chromatography was performed on the DEAE-cellulose fraction II using a Sephadex G-100 column. The protein and the esterolytic activity were eluted in a single peak. The flow chart illustrates the purification procedure that has been modified for *C. mimosoides*. Table 1 provides a purification table

that displays the specific activity, fold purification, and recovery at each stage.

3.3 Sephadex G-100 Gel-filtration chromatography

A column measuring 1.0 cm diameter and 140.0 cm long was filled with Sephadex G-100 gel using gravity. The column was balanced using two column volumes of 0.025 M sodium phosphate buffer, pH 7.0, at a flow rate of 12 ml/hr. The pooled DEAE-cellulose first fraction (1.5 ml) was applied onto the gel and the proteins were eluted with 0.025 M sodium phosphate buffer, pH 7.0 and 2ml fractions were collected. The peak fractions containing esterase activity obtained from Sephadex G-100 were pooled and subjected to preparative gel electrophoresis.

Table 1: Extraction of esterases from the seeds of *Caesalpinia mimosoides*

Purification Step	Total volume (mL)	Total Protein (mg)	Total Activity (µmol/ min)	Specific Activity	Fold Purification	% Yield
Crude	168	1026.00	41.27	0.0402	1.00	100
0-60% Ammonium sulphate fractionation	60	718.20	36.83	0.0513	1.27	89.23
DEAE Cellulose Fraction II	60	95.02	26.10	0.2747	5.36	70.87
Sephadex G-100 Fraction	10	10.04	16.30	1.6235	5.91	62.45

3.4 Isoelectric focusing

The method of Wringley [17] was employed to conduct isoelectric focusing on glass tubes containing 8% polyacrylamide gels.

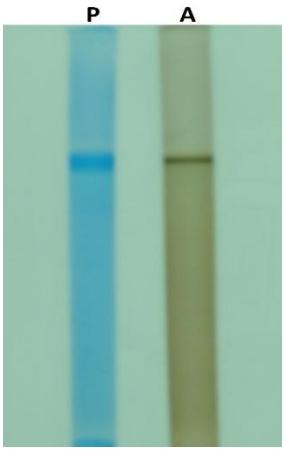

Figure 1: Gel electrofocussing pattern of purified *C. mimosoides*. (P) Protein (A) Activity.

The voltage progressively increased to 250 volts during the electrophoresis process, while the current remained at 2 mA/tube. Following the run, the gels were extracted from the containers and stained for esterolytic activity in accordance with the previously described procedure. Staining solution (0.02% Coomassie brilliant blue G-250 (w/v) and 10% trichloroethanoic acid) was employed to stain the gels for proteins. Distilled water was then used to remove the stain (Fig.1).

3.5 Gel Electrophoresis with SDS-PAGE

Electrophoresis was performed with sodium dodecyl sulphate polyacrylamide gel using the method described by Smith [18] on 10% gels, both with and without 2-mercaptoethanol. The fraction obtained from preparative PAGE underwent SDS-PAGE analysis. The uniformity of CE was determined through native PAGE, GEF, and SDS-PAGE. Both PAGE and GEF displayed a single protein band along with a related esterase band. Sodium dodecyl sulphate polyacrylamide gel electrophoresis conducted with and without 2-hydroxyethylmercaptan revealed single protein band, indicating that the CE is monomeric in nature (Fig.2).

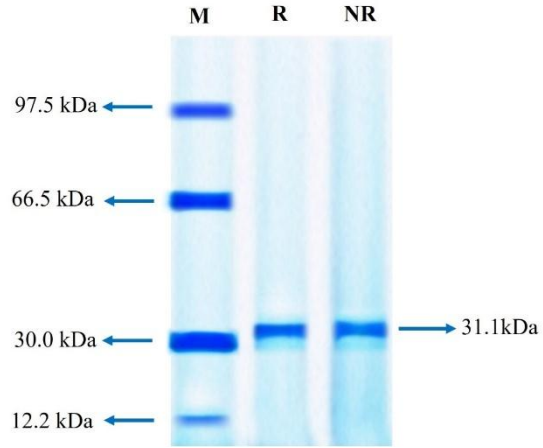

Figure 2: SDS–PAGE pattern of (M) Standard proteins (R) Purified esterase of *C. mimosoides* CE in the presence and (NR) absence of b-mercaptoethanol.

3.6 SDS-PAGE analysis to measure the molecular mass

Electrophoresis of the isolated carboxylesterases was performed as described previously. The different molecular weight marker proteins were used: (Cytochrome c, Carbonic anhydrase, BSA, and Phosphorylase b).

The graph of relative mobility versus logarithmic molecular mass was used to ascertain the

molecular mass of the purified CE. The graph determined the molecular mass of CE to be 31.1 kDa (Fig. 3).

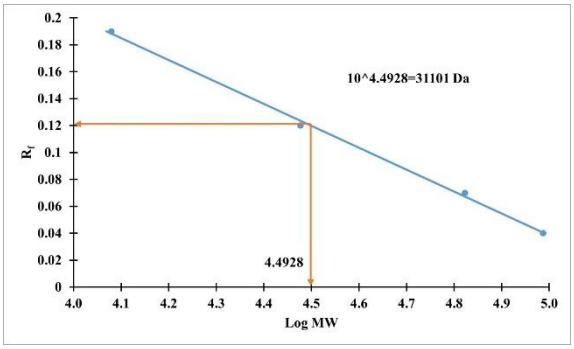

Figure 3: Molecular mass determination of purified CE of C. mimosoides by SDS–PAGE

3.7 Gel Permeation chromatography (Sephadex G-100) analysis to measure the molecular mass

Gel-permeation chromatography of standard proteins and purified esterases was carried out on a column of Sephadex G-100, as described earlier. The standard marker proteins such as cytochrome c (12.2kDa), carbonic anhydrase (30kDa), BSA (66.5kDa), and phosphorylase b (97.5kDa) were used. The marker proteins and purified CE (1.0 ml) were loaded individually onto the column in 0.01M sodium phosphate buffer pH 7.0 and the same buffer was used to elute them. Samples of 2.0 ml were gathered at a flow rate of 10 ml per hour. The absorbance at 280 nm was used to examine the elution patterns of marker proteins. The assay of purified CE was carried out using 1-naphthyl acetate.

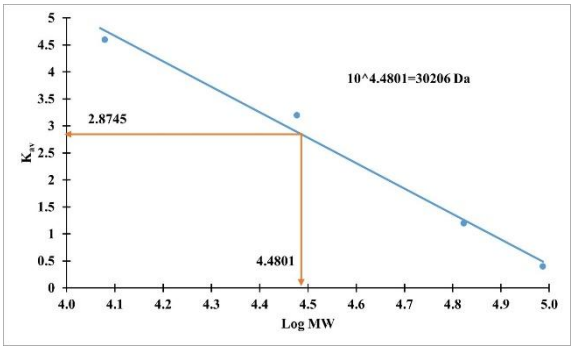

Figure 4: Molecular mass determination of purified CE of *C. mimosoides* by GPC

The molecular mass of CE of *Caesalpinia mimosoides* was assessed by GPC on Sephadex G-100 using the calibration curve obtained by constructing a graph of Kav versus logarithmic molecular mass of different standard proteins. The CE found it to have a molecular weight of 30.2kDa (Fig. 4).

3.8 Proteolytic activity

The proteolytic activity of the isolated esterase was assessed as per the method of Kakade *et al*., [19]. The purified CE did not exhibit any proteolytic activity.

3.9 Isoelectric point

The IEF was carried out as described earlier for the isolated CE. IEF measured the isoelectric point of CE using ampholytes of pH range 3 – 10. The isoelectric pH (pI) value of CE was 7.4.

3.10 Effect of time

The purified esterase was incubated separately with 1-naphthyl acetate for 10, 20, 30, 40 and 50minutes at 27°C. The catalytic activity was assayed and the amount of 1-naphthol released for each time of incubation was determined from the standard graph of 1-naphthol.

The velocity of the esterase catalyzed reaction with time was determined for purified esterase of *C .mimosoides*. The graph of activity versus time indicated that the activity was linear up to 45min for CE.

3.11 Temperature stability and optimal temperature determination

The purified esterase's activity was investigated at temperatures varying from 4°C to 65°C in relation to temperature.

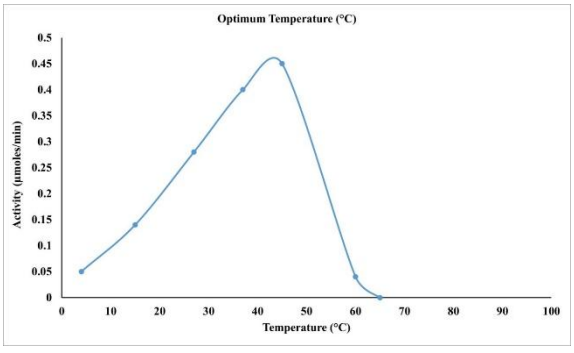

Figure 5: Effect of temperature on esterolytic activity

The temperature stability was investigated for a duration of an hour at varying temperatures (4°C–65°C). The enzyme remained stable at temperatures ranging from 4°C to 60°C, with 45°C being the optimal temperature (Fig. 5).

3.12 Effect of enzyme

An esterase assay was conducted as previously outlined for purified esterase with protein concentrations of 20, 40, 60, 80, 100, and 120 µg. The standard graph of 1-naphthol was used to determine the quantity of 1-naphthol released in each case. The linearity was achieved up to a protein concentration of 120 µg.

3.13 Identification of ideal pH and pH resilience

The influence of pH on the CE activity was examined with sodium acetate (50 mM, pH 4–5), sodium citrate (50 mM, pH 5.5–6.0), sodium phosphate (50 mM, pH 6.5–7.5), Tris-HCl (50 mM, pH 8–9), and sodium carbonate (50 mM, pH 9–11). The esterolytic activity of the enzyme in the above buffers was determined with α-naphthyl acetate as substrate.

The pH stability of CE was assessed by incubating it with buffers (0.2 M) of varying pH levels (pH 4– 10) at 4°C for 24 hours. Following the incubation, the pH was adjusted to the optimal level, and the assay was conducted at the optimal temperature for 15 minutes. The calorimetric determination of 1-naphthol formed was performed. The optimum pH obtained from the graph is 7.5. and the CE was stable between pH 4.0 to 9.0 (Fig.6).

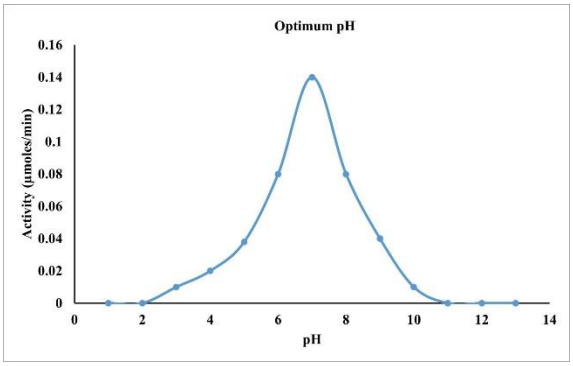

Figure 6: Effect of pH on esterolytic activity

3.14 Substrate specificity determination

The purified esterase's substrate specificity was evaluated using 1-naphthyl acetate, 1-naphthyl propionate, 1-naphthyl butyrate, and acetylthiocholine chloride.

The activities of purified carboxylesterase towards different naphthyl esters are tabulated in table 2. The enzyme hydrolyzed α-naphthyl esters but showed more activity towards α-naphthyl butyrate. Nevertheless, no activity was observed when acetylthiocholine chloride was used as the substrate.

Table 2: Substrate specificity of the purified CE of *C. mimosoides* seeds

Enzyme	1-naphthyl acetate	1-naphthyl propionate	1-naphthyl butyrate	Acetylthiocholine chloride
CE (nmoles/min)	1120	2380	4130	0

3.15 Determination of K_m and Vmax

The purified esterases were incubated with varying concentrations of 1-naphthyl acetate, 1-naphthyl propionate, and 1-napthyl butyrate for 15 minutes at their respective optimum temperatures, and the assay was conducted in accordance with the previous protocol. The colorimetric determination of the 1-naphthol produced in each concentration was conducted. The Lineweaver-Burk plots were used to ascertain the Km and Vmax values. Table 3 presents the results.

Table 3: K_m and Vmax of purified CE of *C. mimosoides* seeds

Substrate	Km (µM)	Vmax (µmole/min)
1-naphthyl acetate	26.4	7.01×10^{-3}
1-naphthyl propionate	20.4	7.31×10^{-3}
1-naphthyl butyrate	24.5	8.10×10^{-3}

3.16 Study on esterase inhibition

The inhibitor experiments were conducted colorimetrically by incubating the enzyme with varying concentrations of inhibitors for 30 minutes at 27°C before the addition of substrate. In place of the inhibitor, the control vial contains 1% (w/v) Triton X-100. Stock solutions of various inhibitors were prepared in 1% Triton X-100 and subsequently serially diluted to achieve the desired concentrations. The effect of various inhibitors on the purified esterase was tabulated. CE was inhibited by phosphomidon and dichlorvos (organophosphates) but is tolerant to eserine sulphate and PCMB. Dichlorvos and phosphamidon inhibited the enzyme in the pI range 4 and 7.

4. DISCUSSION

Purification and characterization of carboxylesterase (CE) from *Caesalpinia mimosoides* have provided valuable insights into its enzymatic properties and potential

applications [20-22]. The ammonium sulfate fractionation method proved to be the most effective for isolating the enzyme, as it preserved esterase activity while enhancing enzyme purity. Through a combination of DEAE-cellulose ion-exchange chromatography, Sephadex G-100 gel filtration, and isoelectric focusing, the CE was successfully purified and characterized, revealing its monomeric nature and molecular weight of approximately 31 kDa.

The enzyme demonstrated optimal catalytic activity at pH 7.5 and 45°C, with stability across a wide range of temperatures (4°C to 60°C) and pH levels (4.0 to 9.0). These properties suggest that CE is suitable for use in various biotechnological and industrial applications [23], particularly in environments where moderate pH and temperature fluctuations are common. Furthermore, the enzyme exhibited a clear preference for α-naphthyl esters, with the highest activity observed for α-naphthyl butyrate, confirming its substrate specificity.

Kinetic analysis revealed that CE operates efficiently with a low Km for α-naphthyl butyrate, indicating strong affinity for this substrate. The enzyme's activity could be further optimized by considering factors such as pH, temperature, and substrate concentration, which are critical for maximizing its efficiency in industrial processes. Additionally, the enzyme's inhibition profile demonstrated its susceptibility to organophosphate inhibitors like phosphomidon and dichlorvos, this inhibitory interaction itself forms the basis for its potential role in bioremediation. The enzyme can act as a stoichiometric scavenger, where each enzyme molecule sacrificially binds to and sequesters a molecule of the toxin, thus neutralizing it. This detoxification-by-sequestration is a recognized mechanism, distinct from efficient catalytic breakdown [24].

5. CONCLUSION

Overall, the findings from this study lay a strong foundation for future research into the biochemical properties and applications of CE from *Caesalpinia mimosoides*. Further investigations into the enzyme's activity under varied conditions, as well as its role in industrial and pharmaceutical processes, will be essential for unlocking its full potential in practical applications.

Acknowledgement

The author wishes to thank Dr. K.S. Chandrashekharaiah, the Chairman, D.O.S in Biochemistry, Jnana Kaveri PG Centre, Mangalore University, Chikka Aluvara, Kodagu for providing research facility to carry out my work.

Conflict of Interest

The authors declare no conflict of interest.

Funding Resources

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Ethical Report

This study was conducted using plant material and did not involve animal or human subjects; therefore, ethical approval was not required.

REFERENCES

1. Grist PA, Lovell JT. Plant-based proteins: From traditional uses to modern applications in industrial processes. *J Agric Food Chem.* 2014;62(2):317-25.
2. Feng Y, Li Z. Carboxyl esterases: Structure, catalytic mechanism, and applications. *Biotechnol Adv.* 2017;35(6):858-72. doi: 10.1016/j.biotechadv.2017.10.007.
3. Cao Y, Yang H. Carboxyl esterases and their roles in plant growth, defense, and seed germination. *Plant Physiol Biochem.* 2016;99:105-13. doi: 10.1016/j.plaphy.2015.12.007.
4. Lad SS, Rao MA. Carboxyl esterases: Biological functions and industrial applications. In: *Handbook of Enzymes in Biotechnology.* Springer; 2015. p. 512-34.
5. Hughes SP, D'Abrosca BM. Purification and characterization of carboxyl esterases from plant sources: Applications in biotechnology and agriculture. *Biochem Biophys Res Commun.* 2013;440(4):520-5. doi: 10.1016/j.bbrc.2013.09.089.

6. Naylor CE, Knapp JA. Carboxyl esterases in biotechnology: Properties and applications. *In: Enzymes in Food Biotechnology*. Springer; 2018. p. 230-55.
7. Kumar N, Sharma M. Variations in the esterase activity during the germination period of Jatropha curcas seeds. *J Phytol*. 2018;10(1):22-7.
8. Wingfield PT. Protein precipitation using ammonium sulfate. *Curr Protoc Protein Sci*. 2001 May;Appendix 3F:1–9. doi:10.1002/0471140864.psa03fs13.
9. Gomori G. Methods of enzymatic analysis. In: Bergmeyer HU, editor. Vol 1. New York: Academic Press; 1953. p. 258-65.
10. Van Asperen K. A study of some properties of esterases and their substrates. *J Insect Physiol*. 1962;8(3):341-5. doi: 10.1016/0022-1910(62)90032-9.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-75.
12. Ali SA, Bendre G, Ojha S, Krishnamurthy V, Swamy NR, Chandrashekharaiah KS. Esterases from the seeds of an edible legume, Phaseolus vulgaris L.; variability and stability during germination. *Phytochemistry*. 2013;92:101-7. doi: 10.1016/j.phytochem.2013.04.010.
13. P BK, Swamy MN, Swamy NR, S CK. Purification and characterization of esterase from the seeds of *Caesalpinia mimosoides*. *Biochem J*. 2014;463(2):233-40. doi: 10.1042/BJ20140387.
14. Ornstein L. Disc electrophoresis. I. Background and theory. *Ann N Y Acad Sci.* 1964 Dec 28;121: 321–349. doi:10.1111/j.1749-6632.1964.tb14207.x.
15. Davis BJ. Disc electrophoresis. II. Method and application to human serum proteins. *Ann N Y Acad Sci.* 1964 Dec 28;121 :404–427. doi:10.1111/j.1749-6632.1964.tb14213.x
16. Hunter SH, Markert CL. The effect of buffer composition on enzyme activity in the extraction of liver. *J Biol Chem*. 1957;226(2):753-8.
17. Wrigley C. Gel electrofocusing-a technique for analyzing multiple protein samples by isoelectric focusing. *Sci Tools*. 1968;15:17.
18. Smith BJ. SDS Polyacrylamide Gel Electrophoresis of Proteins. *Methods Mol Biol*. 1984;1:41–55.
19. Kakade ML, Simons NR, Liener IE. An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem*. 1969;46:518-26.
20. Swamy BK, Swamy NR, Chandrashekharaiah KS. Purification and characterization of esterase from the seeds of *Caesalpinia mimosoides*. 2014.
21. Zanin JLB, Carvalho BA de, Martineli PS, Santos MH dos, Lago JHG, Sartorelli P, Viegas C, Soares MG. The genus *Caesalpinia* L. (Caesalpiniaceae): *Phytochemical and pharmacological characteristics*. *Molecules*. 2012;17(7):7887-906.
22. Chandrashekharaiah KS, Murthy KRS, Swamy NR. Carboxylesterases (Purification and Characterization). Saarbrücken: LAP Lambert Academic Publishing GmbH & Co. KG; 2011. p. 13-150.
23. Zhang Y, Wang X, Liu J, Chen H, Zhao L. Thermostable plant esterases and their potential in industrial applications. *J Biotechnol*. 2022;345:112–20.
24. Kim D, Lee S. Protein engineering of carboxylesterases for enhanced bioremediation: advances and challenges. *Appl Microbiol Biotechnol*. 2021;105(3):987–95.