RESEARCH ARTICLE

# EXTRACTION AND PURIFICATION OF CARBOXYLESTERASES FROM THE SEEDS OF *SAMANEA SAMAN*

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**Abstract:** The enzyme carboxyl esterases was extracted from the *Samanea saman*seeds using different buffers at different pH. Highest activity was obtained with 33 mM phosphate buffer, pH 7.0. The enzyme assay was carried out using α-naphthyl acetate as substrate. The activity of carboxyl esterases was shown to have an optimal operating condition at pH 7.0 and a temperature of 50$^{o}$C. The thermo stability of the enzyme was in the range of 7$^{o}$C - 37$^{o}$Cwith the pH stability in the range of 4.0 – 8.0. The $K_m$ and $V_{max}$ values was determined as 0.157 mM and 1.785µM / minute. IC 50 of dicholorovas for *Samanea saman* esterase was found to be 2.23x10$^{-10}$M. Dicholorovas was found to be an irreversible inhibitor as the time of incubation increase, the percentage of activity decreases.

**Keywords:** Carboxylesterases, Samanea saman, Dicholorovas, α - naphthyl acetate

## INTRODUCTION

Carboxylesterases (EC.3.1.1.1) are carboxylic-ester hydrolases mostly of group serine hydrolases,structurally belonging to alpha/beta hydrolase fold proteinsuperfamily that catalyze the hydrolysis of endogenous and exogenous carboxyl esters via the addition of water to form an alcohol and an acid metabolite, as shown in Fig.1. Carboxylesterases contains the conserved catalytic triad of a serine (Ser), an aspartate (Asp) or a glutamate (Glu) and a histidine (His) residues.(Putterill *et al.* 2003; Satoh and Hosokawa 2006; Yang *et al.* 2008)

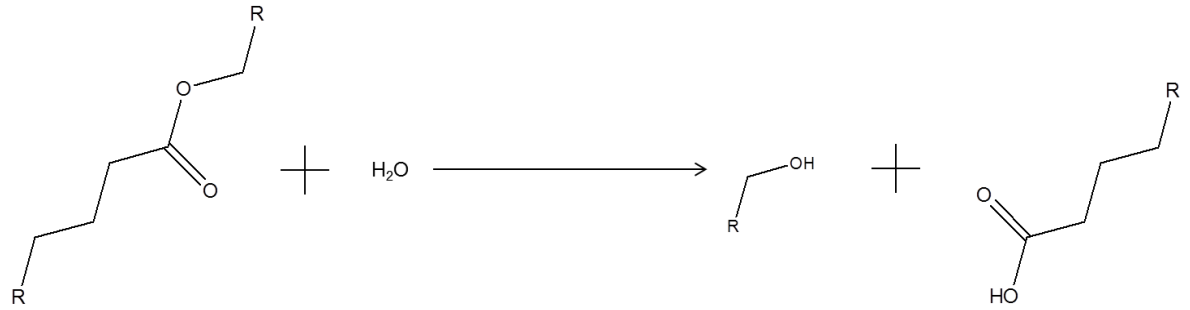


**Figure 1:** Basic carboxylesterase hydrolysis reaction.

According to Enzyme Commission [EC] numbering systemby the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), the numbers define the following classes: EC 3 - hydrolases (enzymes that catalyzes hydrolysis reactions), EC 3.1 - hydrolases that act on ester bonds, e.g., esterases, lipases, nucleases, phosphodiesterases andphosphatase, EC3.1.1 - Carboxylic Ester Hydrolases,catalyzes hydrolysis of a wide range of carboxylic ester substrates, EC3.1.1.1 –Carboxylesterases.

Esterases have been classified into three categories (A, B and C) as proposed in(Aldridge 1993; ALDRIDGE 1953a, 1953b) on the basis of interaction of esterases with organophosphates (OPs). A-esterases, or arylesterases are those which are capable of hydrolysing OPs, but are not inhibited by them and other acylating inhibitors (mechanism shown in figure 2). A-esterasesare mainly phosphotriesterases (PTEs). B-esterases or carboxylesterases, are those which are inhibited by OPs. Presence of serine as nucleophiles residue in the catalytic site of B-esterases compared to a cysteine residue on the active site in A-esterases indicates that cysteine instead of serine in the active site are capable of hydroxylsing OPs rather than phosphorylation, usually seen in the presence of cysteine residue.(Kong et al. 2017; Krejci et al. 1991; Satoh et al. 2002; Yu et al. 2009)C-esterases do not interact with OPs.

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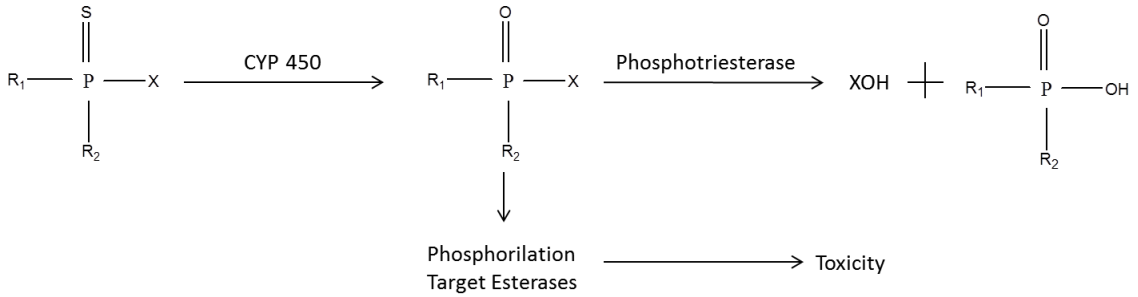



**Figure 2:** Scheme for metabolism of OPs.

Carboxylesterases family of evolutionarily related proteins includes a number of proteins with different substrate specificities, such asali-esterase, B-esterase, butyrate esterase, butyryl esterase, carboxylesterase 1 carboxylesterase 2,carboxylesterase 3, esterase A, esterase B, esterase D, methylbutyrase, methylbutyrate esterase, monobutyrase, procaine esterase, propionyl esterase, triacetin esterase, vitamin A esterase and cocaine esterase, etc.

Carboxylesterases interacts differently with different class of insecticides, it hydrolyzespyrethroids (Abernathy and Casida 1973; Stok *et al*. 2004) and bindsstoichiometrically to carbamates(Gupta and Dettbarn 1993; Sogorb and Vilanova 2002)and organophosphates(Casida and Quistad 2004; Kao, Motoyama, and Dauterman 1985). Thus can act as scavengers to protect against insecticides, facilitated by spontaneous reactivation after inhibition. Subsequently playing a significant role in metabolism and detoxification of many agrochemicals(Potter and Wadkins 2006; Redinbo and Potter 2005).

Carboxylesterases are widely distributed in nature and are common in animals, plants and microbes. Carboxylesterases in mammalian liver participate in phase I metabolism of xenobiotics such as toxins or drugs, resulting carboxylates are then conjugated by other enzymes to increase solubility and eventually excreted.

Carboxylesterases are important in the metabolism of a number of therapeutical molecules(Williams n.d.), including the cholesterol-lowering drugs lovastatin, simvastatin, clofibrate, fenofibrate(Godfrey, Digiacinto, and Davis 2011; Halpin et al. n.d.; Tang and Kalow 1995; Vickers et al. n.d.), the antiviral drugsoseltamivir (Tamiflu), tenofovirdisoproxil, adefovirdipivoxil, valacyclovir(Van Gelder et al. 2000; MacDougall and Guglielmo 2004; Shi et al. 2006; Tong et al. 2007), the narcotic analgesic meperidine (Demerol) (Zhang et al. 1999), cocaine and heroin (Pindel et al. 1997). Carboxylesterases are also used in resolutionof racemic mixtures by transesterification, orenantioselectivehydrolysis of esters for obtaining optically pure compounds(Bornscheuer 2002).

Carboxylesterase activity is extensively used in designing soft and prodrugs(Bodor and Buchwald 2000), as demonstrated by activation of the cancer therapeutic pro-drug Camptothecin-11 (CPT-11 or irinotecan)through its conversion toactive metabolite,7-ethyl-10-hydroxy camptothecin (SN-38)(Hatfield et al. 2011; Potter et al. 1998).Capecitabine hydrolyzed to intermediate metabolite (5'deoxy-5-fluorocytidine), is further metabolized by cytidinedeaminase and thymidine phosphorylase to form the active antitumor moiety, 5-fluorouracil.(Quinney et al. 2005)

*Samaneasaman* (Jacq.) Merr, commonly known as rain tree or monkey pod belongs to the family Leguminosae and they have synonyms such as *Albiziasaman*, *Enterolobiumsaman*, *Inga saman*, *Pithecellobiumsaman* and *Mimosa saman.* They are large sized, fast growing tree which is native to Central America and South America(Staples 2006). Rain tree attains a height between 15-25m. They are ornamental tree which offers excellent shade, wood and produces great quantity of fruits of high nutrition and nitrogen fixing capability. *S. saman* is used as an alternative feed for ruminants and monogastrics.*S. saman* shows several bioactive compounds which possess remarkable pharmacological properties such as antioxidant, antibacterial, anti-diabetic, analgesic, anti-ulcer, insecticidal, antifungal, and cytotoxic activities.

*Hydrolysis mechanism and insecticide interaction* –

Hydrolysis reaction of the carboxyl esterase is a two-step process. Catalytic mechanism begins with nucleophilic attack by catalytic serine hydroxyl on the carbonyl carbon of the ester bond, forming a tetrahedral intermediate. Nucleophilicity of this hydroxyl group is increased and the reaction is stabilised through hydrogen bonding to the catalytic histidine. Two glycine residues are important in stabilizing the reaction and in maintaining the substrate in place. During the reaction, the histidine is stabilised by the carboxyl group of the acidic member of the catalytic triad.

The first step of the catalysis reaction liberates the alcohol metabolite, while the enzyme becomes acylated.

The second step involves returning of enzyme to its active state by releasing the acid molecule, histidine residue's affinity to water molecules enables this step.(Russell *et al*. 2011; Testa and Krämer 2007).

## MATERIALS AND METHODS

***Plant material***

The seed pods of *Samanea saman* were collected using random sampling technique (RST) from JnanaBharathi Campus, Bangalore University. The seeds were removed apart from the pod, after removing immature and damaged seeds matured seeds were soaked overnight in water for the easy dehulling of seed coat. Endosperm is the source of protein for germination and thus exploited as a source of enzyme in our study and the following methods are employed for the isolation and purification of carboxylic esterase.

***Chemicals***

Analytical grade chemicals were employed in the study. acetone, acetic acid, α-naphthol, α-naphthyl acetate, Bovaine Serum Albumin, coomassie brilliant blue, DEAE cellulose, dichlorvos (inhibitor), diazo blue, dialysis membrane, disodium hydrogen phosphate, dihydrogen sodium phosphate, fast blue RR salt ,hydrochloric acid, sodium lauryl sulphate, sephadex G-50, sodium hydroxide, sodium chloride were from Sigma Aldrich chemicals.

***Enzyme extraction***

The dehulleddicotyledons were taken from the overnight soaked seeds and a 10% extract of *Samanea saman* was prepared using33mM Sodium phosphate buffer pH7.0. The homogenized solution was prepared by blending in a mixer for 5 mins,centrifuged at 10,000 rpm for 20 minutes at 4°C and then filtered through Whatman filter paper. The supernatant obtained was measured and used for further purification or stored at -20 °C for further use.

***Protein assay***

Protein content was determined according to the method ofLOWRY et al., 1951using bovine serum albumin (BSA) as standard. The protein content in the fractions obtained from chromatographic columns was monitored by measuring absorbance at 280 nm.

***Enzyme assay***

Esterase activity was assayed according to the method of GOMORI, 1953 as modified by van Asperen, 1962.

Esterase activity was carried out using α-naphthyl acetate as substrate. 1.0 ml of suitably diluted enzyme extract was added to 5.0 ml of 0.3 mM α - naphthyl acetate (a stock solution of 30 mM α - naphthyl acetate prepared in acetone and diluted in 33mM sodium phosphate buffer pH 7.0) and incubated for 15 minutes at room temperature. The reaction was stopped by the addition of 1.0 ml of DBLS reagent (2 parts of 1% diazo blue B and 5 parts of 5% sodium lauryl sulphate) and incubated for 20 minutes for color development.

Esterases assay is based on the measurement of the amount of α-naphthyl liberated.The amount ofα-naphthyl liberated is measured at 600 nm.One unit of enzyme activity was defined as the amount of enzyme that releases one µmol of $\alpha$-naphthol per min at a given pH and temperature.

**Polyacrylamide Gel Electrophoresis (PAGE)**

Native PAGE was performed, according to the method as described in REISFELD et al., 1962. SDS-PAGE was performed after denaturing the proteins with SDS and β-mercaptoethanol.

Gel-electrofocussing was performed according to the method reported inWrigley, 1968 in 10% polyacrylamide gels. The electrophoresis was performed at $4^0$C for 2 h.

After the completion of run, gels were stained for proteins according to the method of Davis using 0.5% solution of coomassieBrilliant Blue R-250 in 25% methanol and 7.5% acetic acid in water for 1 hour and were destained in 25% methanol and 7.5% acetic acid in water for overnight.

Gel-localization of esterase activity on polyacrylamide slab gels was detected by the method of HUNTER & MARKERT, 1957. The electrophoresed gels were stained for esterase activityby placing in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 40 mg Fast Blue RR salt and 20 mg $\alpha$-naphthyl acetate (dissolved in 1 ml of acetone) for 15 minutes at 37℃. The gels are fixed using 7% acetic acid and stored.

**Purification**

**pH precipitation / Isoelectric point precipitation**

The crude enzyme extract obtained was taken in an ice bath and the pH was adjusted to 5.0. The extract was incubated for 30 minutes at 4 °C and centrifuged at 6000 rpm for10 minutes at 4 °C. The pellet was separated from the supernatant and mobilized using extraction buffer. The volumes of mobilized pellet and supernatant were noted. Activity assay and protein assay for both pellet and supernatant were carried out.

**Acetone precipitation / fractionation**

The crude enzyme extract obtained was subjected to 0-40% acetone precipitation and centrifuged at 6000 rpm for 10 minutes at 4 °C. Separate the pellet and supernatant. Supernatant was subjected to 40 – 80%acetone precipitation and centrifuged at 6000 rpm for 10 minutes at 4 °C Mobilize the pellets obtained with suitable volume of extraction buffer. The volumes of mobilized pellets and supernatant weremeasured; activity assay and protein assay were carried out for mobilized pellets.

**Ammonium sulphate precipitation**

Ammonium sulphate was carried out by adding small amounts of ammonium sulphate to the crude extract by constant stirring over magnetic stirrer at 4 °C to obtain 0-40% saturation. The solution was allowed to stand for 30 minutes at 4 °C followed by centrifugation at 6000 rpm for 10 minutes at 4°C. Pellet obtained was mobilized in chilled extraction buffer. Then supernatant was collected and subjected to 40-80% ammonium sulphate fractionation as described above, followed by centrifugation at 6000

rpm for 10 minutes at 4°C .The pellet obtained was dissolved in chilled extraction buffer. The mobilized pellets (0-40% and 40-80%) were dialysed against 6.0 mM phosphate buffer pH 7.0 for 12 hours with two changes of buffers. The dialysates were centrifuged at 6000 rpm for 10 minutes at 4°C to remove the insoluble residue and the supernatants were assayed for esterase activity and protein content.

**Ion exchange chromatography**

For this purification strategy, an anion exchange resin, DEAE cellulose was used with a buffer of pH 7.0.

DEAE cellulose matrix activation was done bysoaking it overnight in 33mM phosphate buffer pH 7.0. The swollen matrix was washed 3-4 times with distilled water followed by 33mM phosphate buffer. The column was set up and packed using the thick suspension of matrix. Equilibrated the column by washing with 1 bed volume of 33mM phosphate buffer and the flow rate 30-40 ml/hour.

Ammonium sulphate saturated (40-80%) sample was loaded onto the column. The bound proteins were eluted with stepwise increase in the ionic strength by adding NaCl, phosphate buffer in increasing concentration (0.1M NaCl - 0.4M NaCl). Fractions of 1 ml were collected and assayed for esterase activity and protein content.

**Gel filtration**

Sephadex G-50 was soaked overnight in 33mM phosphate buffer. The swollen matrix was washed 3-4 times with distilled water followed by 33mM phosphate buffer. A thick suspension was made and packedinto 50ml column without any air bubbles. The matrix was washed with 1 bed volume of buffer before sample was loaded. The flow rate was set to 8-12 ml/hour.

Ion-exchange pooled sample was loaded to the Sephadex column, the flow-rate was adjusted to 8-12ml/hr and 1ml fractions were collected by continuous addition of 0.1M NaCl in 33mM phosphate buffer pH 7.0.

Finally the fractions obtained were read at 280nm using UV–spectrophotometer for protein concentration and absorbance read at 600nm for activity.

**Void volume determination**

About 1ml of blue dextran (1 mg/ml) was loaded to the column. 1ml fractions were collected until all the blue dextran is eluted out. The absorbance of the eluted fractions was read at 600nm. The maximum absorbance of respective fraction will give the appropriate void volume.

**Kinetic studies**

**Effect of time on activity**

The purified esterase was incubated with α-naphthyl acetate for 0, 5, 10, 15, 20, 25 and 30 min at room temperature. The reaction mixture in each case contained 5 mL of 0.3 mM substrate solution; reaction was started by the addition of 1.0 ml of enzyme and assayed as described above. The graph was plotted using the values obtained.

**Effect of Substrate Concentration**

In order to estimate $K_m$ and $V_{max}$, assay is carried out by incubating the enzyme with different concentration of the substrate for 15 mins as described above. Km and Vmax for esterase is determined by construction of LB-plot.

**Influence of pH and Temperature**

The effect of pH on the activity of the purified esterase was studied using the following buffers:

sodium acetate (0.1 M, pH 4.0, 4.5, 5.0 and 5.5), sodium phosphate (0.1 M, pH 6.0 , 6.5 , 7.0, and 7.5) andTris–HCl (33 mM, pH 8.0, 8.5 and 9.0). The catalytic activity of the enzyme in the above buffers was determinedwith α -naphthyl acetate as substrate. The pH stability of the purified esterase was determined by incubating with buffers of different pHsfor 30 minutes.

Similarly, the effect of temperature on the activity of the purified esterase was studied at different temperatures ranging between 7 $^{o}$C and 60 $^{o}$C. The temperature stability of the purified esterase was studied by incubating the purified enzyme at different temperature (7 $^{o}$C - 60 $^{o}$C) for 30 minutes. The reaction was carried out as described above.

**Inhibition Studies**

The inhibitor studies were carried out by incubating the enzyme with different concentrations of inhibitors for 10 min at room temperature prior to the addition of substrate. Inhibition studies were performed using organophosphate (dichlorovos) as inhibitors. Effect of time on inhibition was carried out by stopping the reaction at different time interval.

## RESULTS AND DISCUSSION

**Enzyme assay**

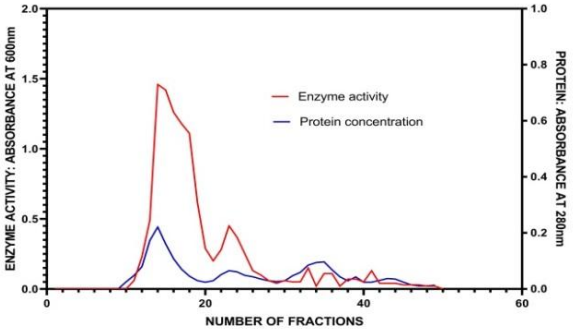
The supernatant obtained was used for further purification.

| Purification methods | Total activity (µmoles/min/g tissue) | Total protein (mg) | Specific Activity (IU/mg protein) |
|---|---|---|---|
| **Crude** | 4.69 | 152.69 | 0.031 |
| **pH precipitation,** Pellet1 | 0.1073 | 19.49 | 0.0050 |

| | | | |
|---|---|---|---|
| Supernatant | 3.69 | 66.60 | 0.0050 |
| **Acetone precipitation,** Pellet 1 | 0.036 | 10.22 | 0.0035 |
| Pellet 2 | 0.105 | 46.29 | 0.0022 |
| **Ammonium sulphate precipitation,** Pellet 1 | 0.0501 | 9.34 | 0.0054 |
| Pellet 2 | 1.56 | 26.80 | 0.058 |

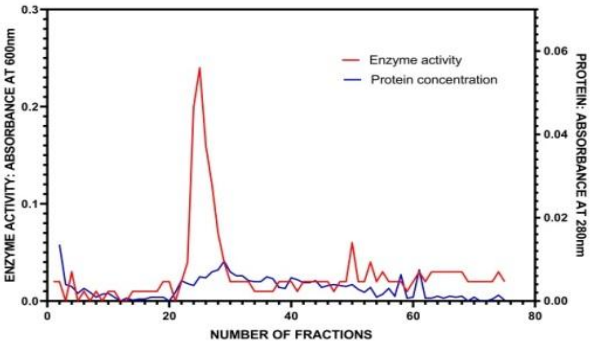
**Ion exchange Chromatography**

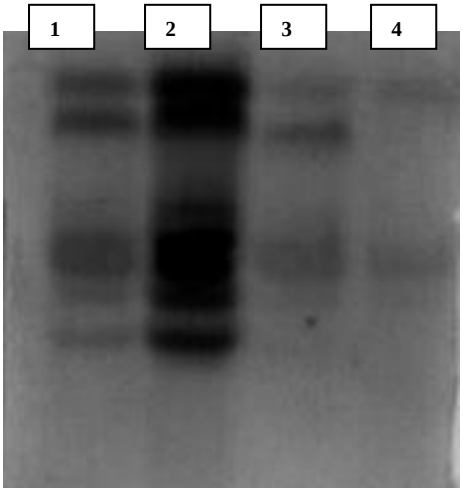
| Sample | Total activity (µmoles/min/g tissue) | Total protein (mg) | Specific activity (IU/mg) |
|---|---|---|---|
| DEAE ion exchange fraction | 0.3432 | 2.48 | 0.258 |




**Gel filtration Chromatography**

| Sample | Total activity (µmoles/min/g tissue) | Total protein (mg) | Specific activity (IU/mg) |
|---|---|---|---|
| Gel filtration fraction | 0.055 | 0.133 | 0.411 |

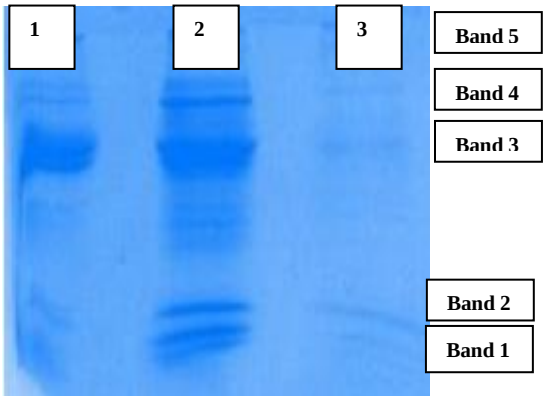



**Band 2**

**Band1**

1. Crude Extract
2. Ammonium Sulphate fraction
3. Ion Exchange fraction




1. Crude Extract
2. Ammonium Sulphate fraction
3. Ion Exchange

**Purification table**

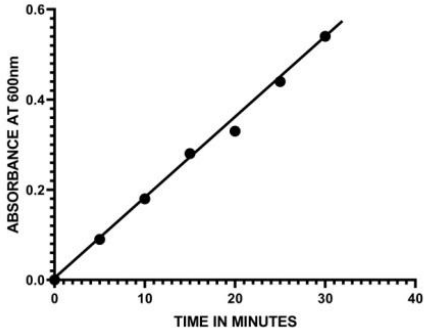
| | **Total activity** | **Total protein** | **Specific activity** | **Fold Purification** | **% Yield** |
|---|---|---|---|---|---|
| Crude | 2.52 | 44.34 | 0.057 | 1 | 100 |
| Ammonium Sulphate | 0.625 | 3.34 | 0.187 | 3.29 | 24.82 |
| Ion exchange | 0.343 | 1.33 | 0.26 | 4.55 | 13.69 |
| Gel filtration | 0.055 | 0.133 | 0.412 | 7.26 | 2.17 |

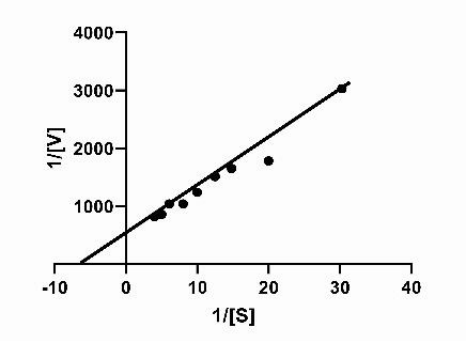
**Kinetic studies**

**Effect of time and substrate concentration on activity**

The effect of time on activity was determined by incubation of reaction mixture for different time intervals. Optimum incubation time was 15 minutes. For all further experiments, the optimum time of incubation was maintained.

*Samanea saman* esterase exhibited $K_m$ value of 0.157 mM and $V_{max}$ value of 1.785µM / minute.

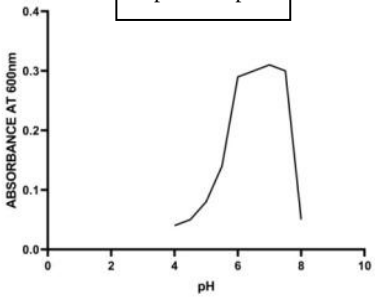


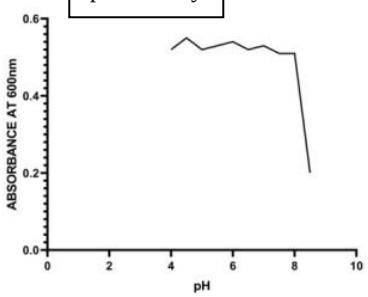

**pH optimum and stability**

*Samanea saman* esterase showed maximum activity at pH 7.0 and pH stability ranged from pH 4.0 to pH 8.0.

Optimum pH

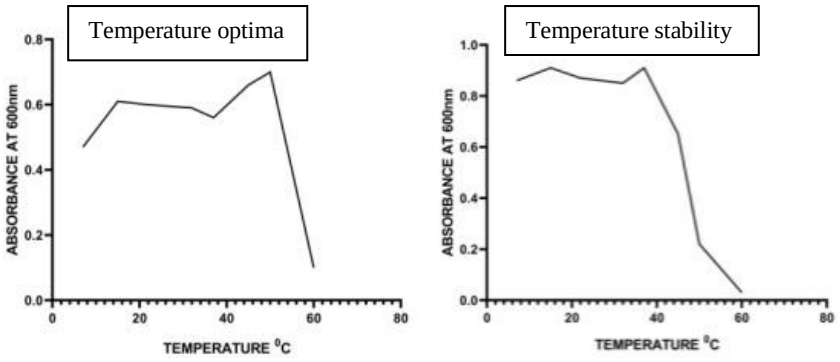



pH stability



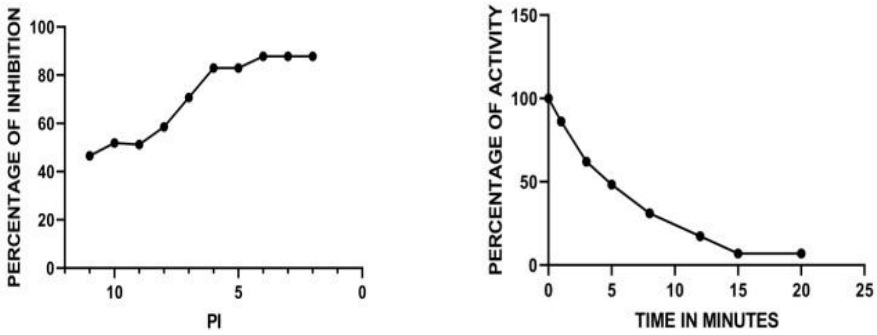
**Temperature optima and stability**

*Samaneasaman* esterase showed highest activity at 50 °C and the stability ranged between 7°C - 37 ° C.




**Effect of inhibitor on the activity and time on inhibition**

IC 50 of dicholorovas for *Samanea saman* esterase was found to be $2.23x10^{-10}$M. Dicholorovas was found to be an irreversible inhibitor as the time of incubation increase, the percentage of activity decreases.




## CONCLUSION

This study is the first to determine the activity of carboxylesterasefrom the seeds of*Samanea saman*. The enzyme was partially purified using ion-exchange (CM-cellulose) and gel filtration (sephadex G- 50) chromatography. The enzyme was stable at room temperature. Stability at wide range of pH and temperature makes it more suitable to perform in adverse industrial environment. The characterization of enzyme helps to know about their biochemical and catalytic properties.

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