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Biochemistry

Comparative analysis of Polyphenol Oxidase, Catalase and Lycopene production in *Lycopersicon esculentum* Mill.

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Abstract

Antioxidant activity of tomato has been extensively studied but only in context of Lycopene. This study relates the activity of antioxidant enzymes (Polyphenol Oxidase and Catalase) along with Lycopene in *Lycopersicon esculentum* Mill at different growth stages of (Seedling, Flowering and Fruiting). Polyphenol Oxidase, Catalase (both partially purified) and Lycopene were estimated spectrophotometrically and presence of Lycopene was further confirmed through Thin Layer and High-Performance Thin Layer Chromatography. Polyphenol Oxidase and Catalase could be partially purified with 2.61 (22.55% yield) and 2.11 (62.3% yield) fold purification respectively via ammonium sulphate precipitation respectively. Antioxidant enzymes showed maximum production at the seedling stage (Polyphenol Oxidases: 197.12U/ml and Catalase: 0.037U/ml) where Lycopene production was least; while Lycopene production was maximum in the fruiting stage (259.49mg/kg of fresh weigh) where enzyme activities were negligible. HPTLC analysis also supported the above findings. Linear Regression analysis of Lycopene, PPO and CAT were performed in which r (correlation coefficient) value for Lycopene and PPO was -0.9279108012 and for Lycopene and CAT was -0.7316992009; which indicated strong negative correlation between Lycopene and both the enzymes. It can be concluded that Antioxidant enzymes play their share at young stages while Lycopene at mature stage in antioxidant network of tomato.

Highlights

- Open Pollinated varieties of *Lycopersicon esculentum* Mill have been used for analysis.
- Enzymes have been partially purified via Ammonium Sulphate precipitation followed by dialysis and their physical parameters have been optimized.
- Quantitative methods, Thin Layer chromatography and High Performance Thin Layer Chromatography have been used to study the production of Polyphenol Oxidases, Catalase and Lycopene.

Keywords: Polyphenol Oxidase, Catalase, Lycopene, *Lycopersicon esculentum* Mill, Partial Purification and optimization.

Abbreviations: Polyphenol Oxidases (PPOs), Catalase (CAT)

Plants survive with various biotic and abiotic environmental stresses by altering their cellular metabolism and bringing into play different defense mechanisms. Reactive species are highly unstable structures; which are divided into four categories based on their central atom: Reactive Oxygen Species (ROS), Reactive Nitrogen Species, Reactive Chloride Species and Reactive Sulphur Species (Halliwell and Gutteridge, 2007). Foraging or detoxification of excess ROS is attained by a competent anti-oxidative system comprising of non-enzymatic as well as enzymatic antioxidants in plants (Noctor and Foyer, 1998). Antioxidants can act by rummaging reactive oxygen species (e.g. Sulphur Oxide Dismutase removing oxygen), by inhibiting their formation (e.g. by blocking phagocyte activation), by binding transition metal ions and preventing formation of hydroxyl ions and/ or decomposition of lipid hydro-peroxides, by repairing damage (e.g. α –tocopherol repairing peroxyl radicals and

so terminating the chain reaction of lipid peroxidation) or by any of the combination from the above (Niwa *et al.,* 2001). Antioxidants can be classified into three main types: first line defense antioxidants, second line defense antioxidants and third line defense antioxidants. Oxidases and Peroxidases come under first line of defense and carotenoids come under second line of defense.

Polyphenol Oxidases (catechol oxidase; E.C. 1.10.3.2) are widely distributed and well-studied oxidative enzymes and their effects on discoloration in damaged and diseased plant tissues have been known for many years. Polyphenol Oxidases (PPOs) are copper containing ubiquitous enzymes which utilize molecular oxygen to oxidize common ortho-diphenolic compounds such as catechol to their respective quinones. Quinones generated by PPOs are highly reactive and may cross-link or alkylate proteins which lead to brown pigment formation in damaged plant tissues. Based on these browning reactions, PPOs have been suggested to play a defensive role in plants (Constabel and Barbehenn, 2008).

CAT (EC 1.11.1.6) is an iron containing enzyme. CATs are most notably distinguished from the other enzymes in not requiring a reductant as they catalyze a dismutation reaction and readily degrade hydrogen peroxide. Oxidative stress is cumulative biological effect of oxygen radicals on plants. To minimize its effect plants have evolve various enzymatic defenses, CAT activity is one of them. It has also been reported that CAT mutant plants show a decrease in Pathogenesis Related (PR) proteins; which indirectly correlate role of CAT in plant defense mechanism. (Sanchez-Casas and Klessig, 1994; Chen *et al.,* 1995)

Tomato is one of the most important horticulture crops in India. According to Indian Agricultural Research Databook (2012); Area coverage of tomato has been increased from 5, 99,000 HA to 8, 65,000 HA in four years with 32.53% increase in production and 2.6% increase in productivity (Joshi *et al.,* 2012). Studies on tomato have been extensively done in regard to Lycopene. Lycopene is a pigment principally responsible for deep red colour of tomato. It has attracted attention because of its biochemical and physiological significance as natural antioxidant. Although it has no provitamin A activity, it has a physical quenching rate constant double to that of β- carotene and ten times as higher than α-tocopherol. As, Lycopene is associated with red colour of tomato, its studies have been focused in fruits only. (Karakaya and Yilmaz, 2007)

Considering the above facts; present study was conducted to explicate the presence of Lycopene at various vegetative stages along with two antioxidant enzymes to know which equivalent (enzymatic or non-enzymatic) can be the share of anti-oxidant network at different stages of growth studied. Present study also aimed in optimizing the physical conditions at seedling stage for partially purified PPOs and CAT.

Materials and Methods

Plant material

Seeds of *Lycopersicon esculentum* Mill were bought from Seedco Company, Jalna, India. Seeds were stored at 4^{o} C until used.

Seed germination

Seeds of *Lycopersicon esculentum* Mill were germinated in autoclaved sand containing 0.5X MS salts (Murashige and Skoog, 1962). Seeds were sown in medium sized plastic cups; 0.5 inch deep inside autoclaved sand (10 seeds per pot). These pots were kept in dark until they started germinating; afterwards pots were transferred to natural conditions (16hr/8hr light and dark).

Preparation of Homogenate for Enzyme Assay

Homogenate for PPO and CAT enzyme extraction was prepared according to Christopher *et al.,* (2010). For this, 1 gm of root sample was crushed with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 2.0 minutes at 10000 rpm. The supernatant obtained was used for enzyme assay. Enzyme homogenates were stored in deep freezer (-70°C) until utilized for biochemical analysis.

Ammonium sulphate fractionation and dialysis

PPO's and CAT's crude homogenate were subjected to 0-80% (w/v) saturation with ammonium sulphate at cold conditions. The saturated solutions were left overnight at 4^{o} C and the precipitated proteins were separated

by centrifugation at 7000 x g for 10 min at 4° C. The precipitate was dissolved in 13 ml of phosphate buffer. (0.9M sodium phosphate buffer pH 6.5 for PPO and 0.1M Potassium phosphate buffer pH 7.0 for CAT) The concentrated sample with maximum specific activity was dialyzed for 8 h against 1 L of above buffer for further use (Lokhandwala and Bora, 2013).

Quantification of Total Protein

Protein was quantitatively estimated by Lowry's *et al*., method (1951) using Bovine serum albumin as standard. (Coban *et al*., 2008)

PPO and CAT activity assay

The PPO activity in *Lycopersicon esculentum* Mill seedlings was measured using catechol as a substrate for the homogenate prepared, as mentioned earlier. PPO activity was determined following the procedure prescribed by Christopher *et al.,* (2010). The enzyme activity (in units) was expressed as change in absorbance min^{-1} mg^{-1} of protein at 410nm on UV-VIS 1800 spectrophotometer (Shimadzu).

CAT activity was determined by Beers and Sizer's method (1952). Disappearance of H_2O_2 was calculated spectrophotometrically at 240 nm on UV-VIS 1800 spectrophotometer (Shimadzu). One Unit decomposes one micromole of H_2O_2 per minute at 25°C and pH 7.0 under the specified conditions.

Optimum pH and pH stability profile

The optimum pH value for partially purified PPO and CAT activity was estimated by assaying enzyme activity at different pH levels. The assay was carried out in the presence of different buffers with different pH such as 0.2 M Glycine-HCl buffer (pH 1, 2, 3 and 4), 0.1 M Sodium Phosphate buffer (pH 5, 6, 7 and 8) and 0.2 M Glycine-NaOH buffer (pH 9 and 10) separately in an assay mixture. pH stability for PPO was assayed for pH ranging from 1 to 10 for 8 days and that for CAT for 5 days. 0.5 ml of enzyme extract and 0.5 ml of respective buffer was incubated for different time periods. Residual enzyme activity was measured by above mentioned method at every 24 hrs (modified method of Lokhandwala and Bora, 2013).

Optimum temperature and temperature stability profile

The enzyme activity for partially purified PPO and CAT was measured at different temperatures in the range of 0° C to 80° C. In order to determine thermal stability, 500µl of partially purified PPO and CAT was assayed at fixed time intervals (which varied according to the temperature). Residual activity was assayed by above mentioned method at fixed time intervals and compared to control (modified method of Ebiloma *et al.,* 2011).

Effect of ionic strength

The effect of ionic strength was assayed using different molarities (0.05 to 1 M) of Sodium phosphate buffer (pH 6.5) for PPO and Potassium Phosphate buffer (pH 7.0) for CAT (modified method of Şişecioğlu *et al.,* 2010)

Extraction of Lycopene

Lycopene was extracted from 1gm of seedling, mature flowering plant and fruit by modified method of Hyman *et al.,* (2004). In present study, instead of Tetrahydrofuran (THF), 8 ml 2:1:1 Hexane: ethanol: acetone was used as a solvent for extraction. Standard Lycopene was extracted by same protocol from Vista made Lycopene tablets (Lycopene-10000). 1 gm of powder was used for extraction (1 tablet = 10µg of Lycopene).

Quantitative analysis, TLC and HPTLC of Lycopene

Extracted Lycopene was spectrophotometrically quantified according to the method of Ravelo-Pérez *et al.,* (2008) and the amount of Lycopene was calculated using the formula:

Lycopene content (mg/kg of tissue) = A_{503}x 31.2/g tissue.

TLC and HPTLC were done using Acetone: Hexane (1:9) as mobile phase and Silica gel G (HiMedia) as stationary phase. 20µl of all the samples were injected on the 10x10 TLC plate. HPTLC analysis was done on Camag made HPTLC Instrument. CAT software was used for evaluation of TLC plate. TLC plate was observed and scanned under 285nm UV source.

Table 1: Partial Purification of PPO and CAT from *Lycopersicon esculentum* Mill seedling

	Fraction	Volume (ml)	Protein (mg/ml)	Total Protein (mg/ml)	Enzyme (Units)	Specific activity	Yield (%)	Purification fold
PPO	Crude Extract	100	4.5	450	511.8	1.13	100	1
	50% Ammonium sulphate fraction	13	7.6	98.8	328.67	3.32	21.95	2.93
	Dialyzed sample	14.5	7.0	101.5	300.18	2.95	22.55	2.61
CAT	Crude Extract	100	3.2	320	800.71	2,502	100	1
	60% Ammonium sulphate fraction	13	6.9	89.7	506.92	5.651	63.308	2.2
	Dialyzed sample	14.5	6.5	94.25	498.91	5.29	62.3	2.11

Table 2: Kinetic properties of partially purified PPO and CAT from *Lycopersicon esculentum* Mill seedling

Kinetic properties	PPO	CAT
Optimum pH range	7.0	7.0
Stable pH	8-10	7-9
Optimum temperature	40°C	70°C
Stable temperature range	10°C to 30°C	25°C to 40°C
Optimum Molarity	0.9 (sodium phosphate buffer pH 6.5)	0.1 (Potassium phosphate buffer pH 7.0)

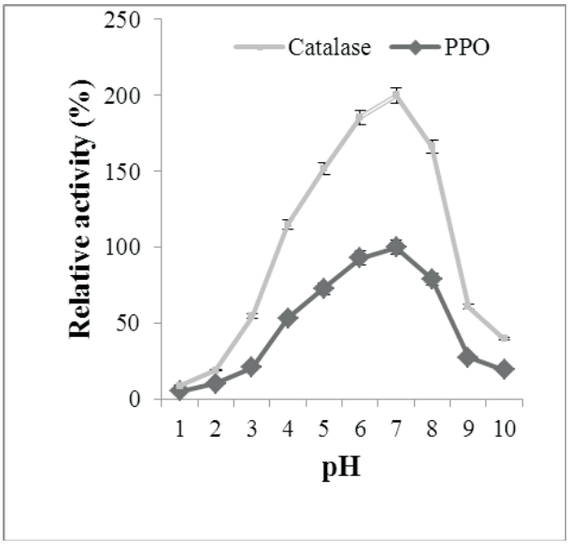


Figure 1. Optimum pH profile for PPO and CAT

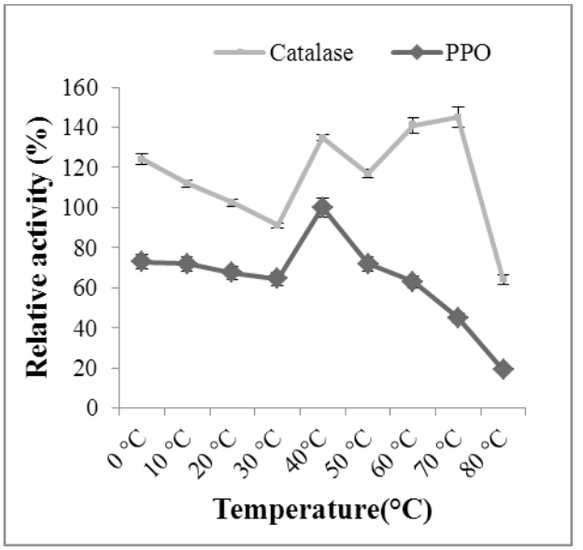


Figure 2. Optimum temperature for PPO and CAT

Statistical Analysis

Linear Regression analysis was performed to determine the relation between Lycopene and PPO and Lycopene and CAT produced at three different generative stages. All the experiments were performed in triplicates and standard error bars are mentioned in the graphs.

Results

Ammonium sulphate fractionation and dialysis

The fraction containing 50% ammonium sulphate concentration showed maximum specific activity (511.8µmoles/ml/min) for PPO and for CAT 60% ammonium sulphate concentration showed maximum specific activity (800.71µmoles/ml/min). This primary purification step resulted in 2.61 fold purification of PPO and 2.11 fold purification of CAT with 22.55% and 62.3% yield respectively (Table 1)

Kinetic studies for PPO

(i) pH: The optimum pH found by assaying enzyme activity at different pH levels was 7.0 (Figure 1, Table 2) in 0.1M Sodium Phosphate buffer. The enzyme activity measured at pH 7.0 was 53.54µmoles/ml/min. However, PPO was found more stable between the range of pH 8 to pH 10 in 0.1 M sodium phosphate buffer after incubation of 8 days (Figure 4a and 4b, Table 2). PPO activity reduced to zero at pH 1, 2 and 3 on 6th, 7th and 8th day respectively; which indicates that PPO was more stable at alkaline pH.

(ii) Temperature: The optimum temperature measured by assaying enzyme activity at various temperatures was 40^{o}C (Figure 2, Table 2). The activity obtained was 43.32 µmoles/min/ml. PPO activity reduced to zero at 70^{o}C and 80^{o}C after 50 min of incubation; which shows that it cannot tolerate temperature above 60^{o} C. The PPO enzyme was more stable between the range of 10^{o}C to 30^{o} C (Figure 5a, 5b, 5c and Table 2).

(iii) Ionic strength: The optimum molarity for PPO assayed was in 0.9M Sodium Phosphate buffer (pH 6.5), (Figure 3, Table 2).The activity measured was 35.53µmoles/min/ml.

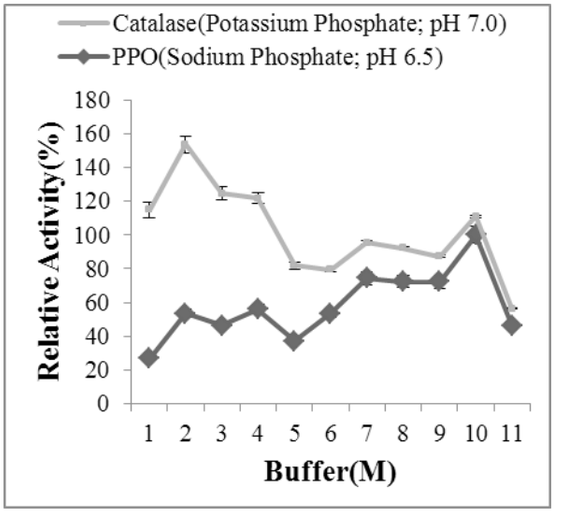
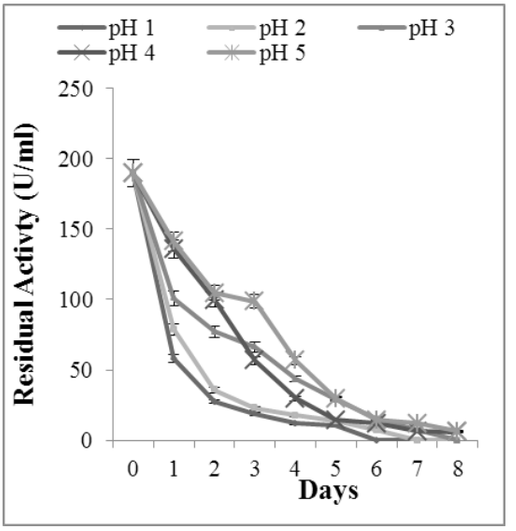


Figure 3. Optimum molarity profile for PPO and CAT



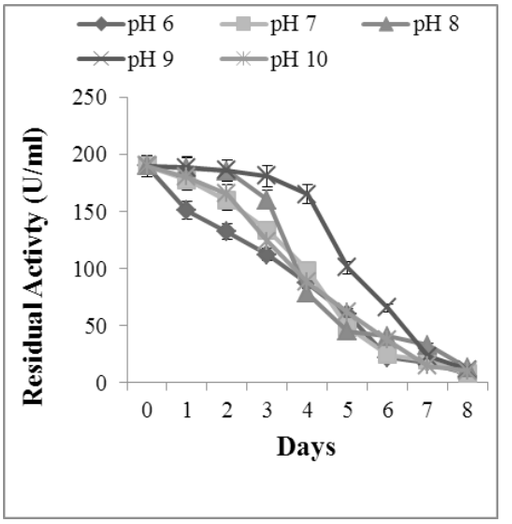


Figure 4a and 4b. pH stability profile for PPO.

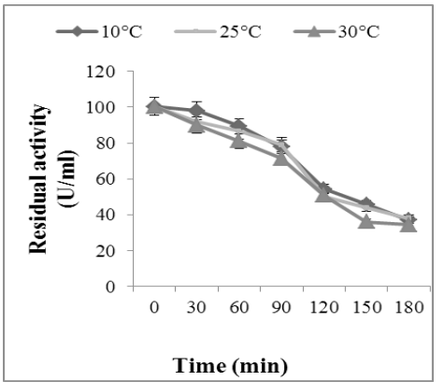


Figure 5a. Temperature stability profile for PPO (10°C, 25°C and 30°C)

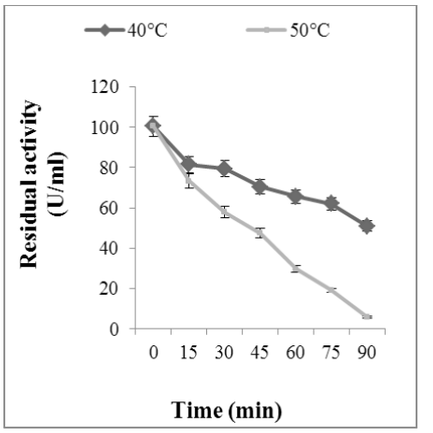


Figure 5b. Temperature stability profile for PPO (40°C and 50°C)

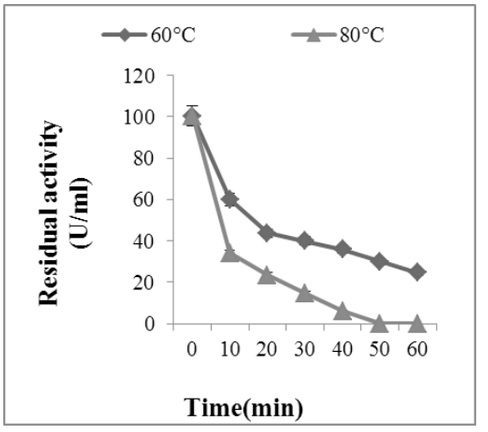
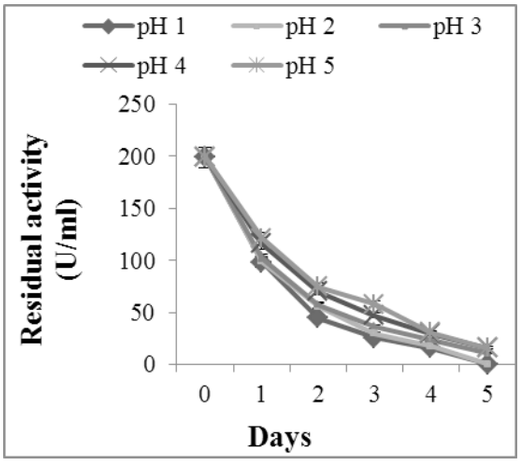


Figure 5c. Temperature stability profile for PPO (60°C, 70°C and 80°C)

Kinetic studies for CAT

(i) pH: The optimum pH found by assaying enzyme activity at different pH levels was 7.0 in 0.05 M Potassium Phosphate buffer (Figure1, Table 2).The activity measured was 129.76 µmoles/ml/min. CAT activity reduced to zero at pH 1 and 2 on 4th and 5th day of incubation respectively; which indicates that CAT was also stable at alkaline pH but PPO is more stable enzyme than CAT .CAT was found more stable between the range of pH 7 to pH 9 in 0.05 M Potassium phosphate buffer after incubating for 5 days (Figure 6a and 6b, Table 2).



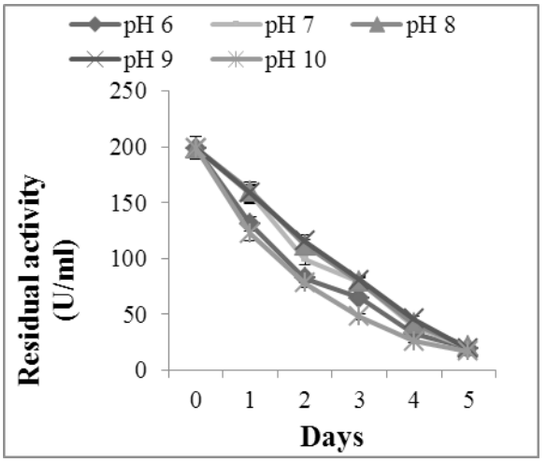


Figure 6a and 6b. pH stability profile for CAT.

(ii) Temperature: The optimum temperature measured by assaying enzyme activity at various temperatures was 70° C (Figure 2, Table 2). The activity obtained was 43.32 µmoles/ml/min. CAT activity reduced to zero at 50° C itself after 90 min of incubation; which indicates it is less stable at higher temperatures. The CAT enzyme was more stable between the range of 25° C to 40° C (Figure 7a, 7b, 7c and Table 2).

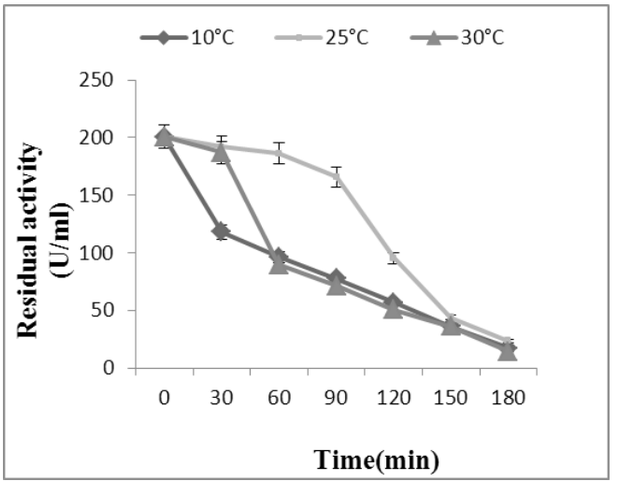


Figure 7a. Temperature stability profile for CAT (10°C, 25°C and 30°C)

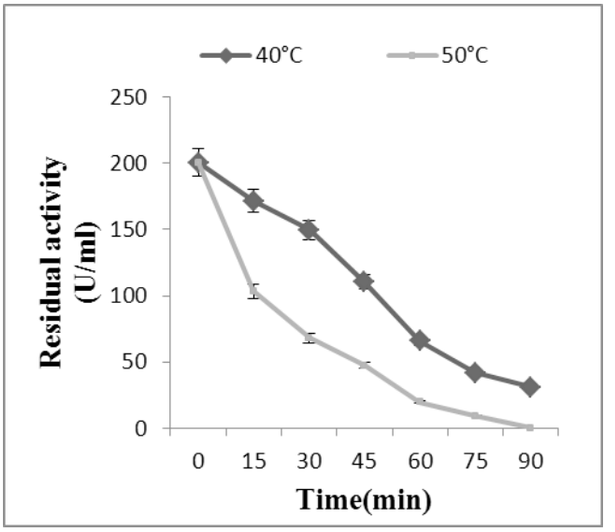


Figure 7b. Temperature stability profile for CAT (40°C and 50°C)

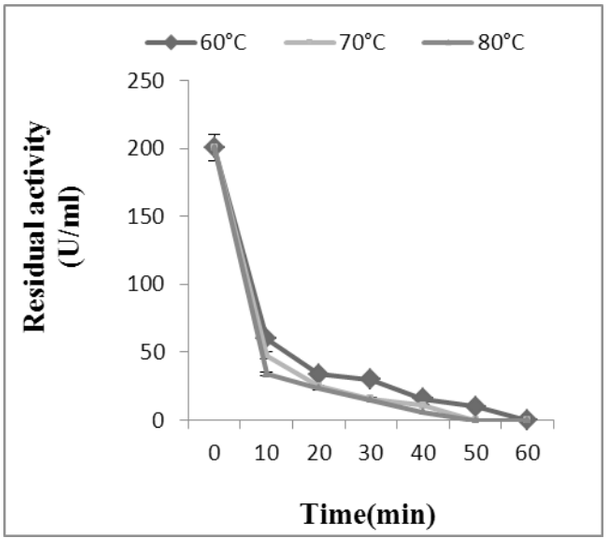


Figure 7c. Temperature stability profile for CAT (60°C, 70°C and 80°C)

(iii) Ionic Strength: The optimum molarity for CAT assayed was 0.1 M Potassium phosphate buffer (pH 7.0). The activity obtained was 98.72µmoles/min/ml (Figure 3, Table 2). CAT activity is higher than PPO under their respective optimum parameters though CAT is more unstable enzyme than PPO at a particular parameter.

Lycopene, CAT and PPO at three ontogenic stages

Lycopene production was highest at fruit stage (259.49mg/kg of fresh weight), while CAT and PPO activity was highest at seedling stage (197.12U/ml and 0.037U/ml). CAT and PPO activity was negligible at fruit stage, but Lycopene production was observed at both seedling and flowering stage; however, it was significantly less than that produced at fruit stage which was confirmed by HPTLC analysis also where Lycopene peak was highest at fruit stage. Analysis also showed that PPO production was extremely less as compared to CAT production at both seedling and flowering stage (Figure 8, 9, 10 and Table 3).

Statistical analysis

Correlation coefficient (r) obtained from Linear Regression analysis between Lycopene, PPO and CAT was -0.9279108012 for Lycopene and PPO while

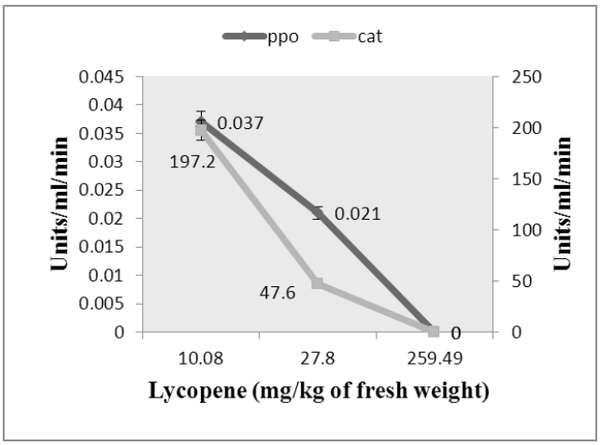


Figure 8. Lycopene, PPO and CAT production at different ontogenic stages in tomato plant.

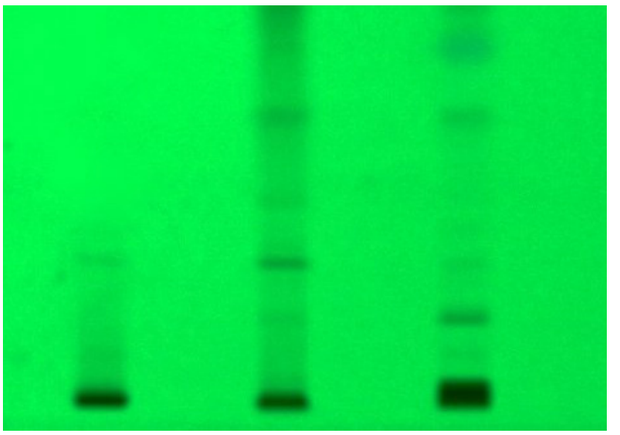


Figure 9. HPTLC plate for Lycopene at different stages under 285nm

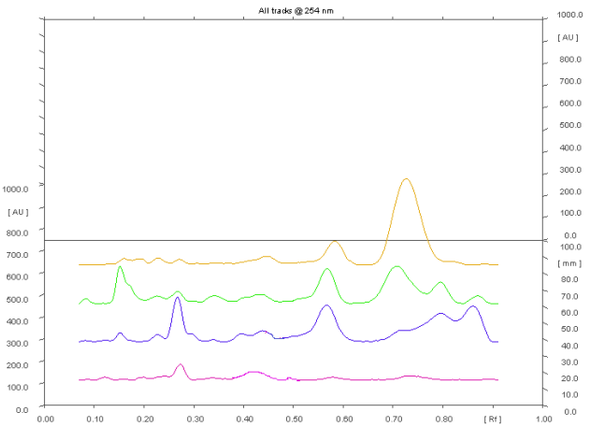


Figure 10. Peaks obtained from HPTLC plate (CAT software)

Table 3: Lycopene concentration, PPO activity and CAT activity at seedling, flowering and fruit stage in *Lycopersicon esculentum* Mill

Parameters Growth Stages	Lycopene Concentration (mg/kg of fresh weight)	PPO (Units/ml/min) Standard Deviation (σ)	CAT (Units/ml/min)
Seedling Stage	10.08	0.037	197.12
Flowering Stage	27.8	0.021	47.6
Fruit stage	259.49	0	0
Standard Deviation (σ)	139.1639	0.018556	102.9027

Table 4: Linear Regression Analysis data of Lycopene concentration, PPO activity and CAT activity at seedling, flowering and fruit stage in *Lycopersicon esculentum* Mill

	Lycopene and PPO	Lycopene and CAT
Equation	$y= -1.237283061\times 10^{-4} + 0.03159769546$	$y= -5.408076879 \times 10^{-1} + 135.1799934$
Correlation Coefficient (r)	-0.9279108012	-0.7316992009
Residual Sum of Squares	0.0000957	9831.614

-0.7316992009 for Lycopene and CAT. As the r value is negative and is above 0.7; it indicates that there is strong negative correlation between Lycopene and both the enzymes (Table 4).

Discussion

Generation of reactive oxygen species (ROS) in response to microbial pathogen attack; known as oxidative burst is one of the early resistance mechanisms by plant cells. Study of a number of plant–pathogen interactions and those modeled by elicitor treatment of cultured cells reveals that there may be more than one mechanism operating (Bolwell *et al.,* 2002). Plants activate such protective mechanisms by increasing the amount of ROS leading to evolvement of intricate antioxidant system for their self-protection (Nourimand *et al.,* 2012). Tomatoes are widely known for their antioxidant property. This Antioxidant property in tomatoes has been extensively researched in context of Lycopene. Present study focuses on characterization of two antioxidant enzymes in tomato at seedling stage; co-relating their antioxidant property with tomato antioxidant- Lycopene. PPO and CAT were partially purified with 2.61 and 2.11 purification fold and 22.55% and 62.3% yield respectively in present study. Saeidian (2013) also purified PPO from Tomato fruits with 3.7 purification fold and 69.52% yield via 80% ammonium sulphate precipitation. Mullen and Gifford (1993) partially purified CAT from Loblolly Pine seeds with 2.3 fold and 43% yield. Low yield of PPO in our study may be attributed with age of plant. Kinetic studies in present research are presented in Table 2. Spagna *et al.,* (2005) reported the optimum pH and temperature for PPO in tomato juice as 4.8 and 30°C respectively. Saeidian and Rashidzadeh (2013) showed that CAT from *Solanum lycopersicum* had two optimum pH (6.8 and 7) and was stable between temperatures of 25°C to 70°C. Trindade *et al.,* (1988) found that optimum pH and temperature of CAT from leaves of *Zantedeschiaa ethiopica* was 7.0 and 40°C. Sellés-Marchart *et al.,* (2006) reported that differences in optimum parameters for enzyme activity is dependent on the plant sources, extraction methods, and purity of the enzyme, buffers, and substrates. Similarly, variations in our results when compared to researched work also may be due to different species and variations in conditions provided like choice of buffer or ontogenic age. Spectrophotometric as well as statistical analysis of Lycopene, PPO and CAT production at three different stages also confirms that Lycopene plays its part as antioxidant after ripening while antioxidant enzymes play their role from seedling stage till maturity. Naghiloo *et al.,* (2012) analyzed that antioxidant activity was higher at fruiting stage than at vegetative stage in *Astragalus compactus* Lam. Polovnikova and Voskresenskaya (2008) showed that in lawn grasses PPO activity is highest in virginal (small) plants, while CAT was highest in

Generative plants (young plants). Likewise, in our study also both the enzymes showed maximum production at vegetative stages as seedling and flowering stage. Spagna *et al.,* (2005) showed that PPO activity is related with color changes associated with browning and Lycopene degradation, because Lycopene is an antioxidant agent that reconstitutes the polyphenols oxidized by the action of PPO. In our study, PPO was produced at both seedling and flowering stage where Lycopene production was very less; while at fruiting stage Lycopene production was maximum and PPO was not at all produced. As stated earlier; as PPO is associated with Lycopene degradation presence of one might negatively affect other's production.

In study done by us, CAT is present at both the vegetative stages. Haddad *et al.,* (2009) stated that there is no multicellular organism which does not contain at least some amount of CAT activity in it. They also reported that there are some plants like *Brassica napus*, Iris Flowers, Arabidopsis that show accumulation of CAT during senescence. So, presence of CAT at both the vegetative stages may be due to its ubiquitous nature as reported by Haddad *et al.,* (2009). However, it's increase or decrease in CAT production at different plant stages may not be related with Lycopene production but, its increase at young stages till maturity may be due to increased production of superoxide radicals during germination. (Job *et al.,* 2005)

Conclusion

Present study concludes that PPO and CAT can be partially purified and characterized at seedling stage from tomato. Anti-oxidant enzymes are produced at seedling stage and flowering stage only and not in mature fruit. So, it can be concluded that Lycopene acts as a sole strong antioxidant in mature fruit. We have studied only two antioxidant enzymes PPO and CAT; enzymes other than these may be involved in tomato's antioxidant network at these stages. As far as PPO, CAT and Lycopene in tomato are concerned; enzymatic part decreases with increase in non-enzymatic part and vice-versa.

Acknowledgement

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