Enzymatic antioxidants in potato (*Solanum tuberosum* L.): Determination of Polyphenol oxidase and Peroxidase activities in different organs

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IN
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BY

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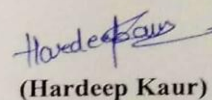
Dedication

This report is dedicated foremost to God almighty for best owing His mercy and grace upon me especially during this dissertation work at Thapar Institute of Engg. & Technology, Patiala. I would also like to dedicate it to my parents, brother and teachers for their love and support.

Candidate's Declaration

I, hereby declare that the work which is being presented in dissertation entitled **"Enzymatic antioxidants in potato (*Solanum tuberosum* L.): Determination of Polyphenol oxidase and Peroxidase activities in different organs"** for the partial fulfillment of Master of Science in Biochemistry submitted to Thapar Institute of Engineering & Technology, Patiala is an original record of my own research work carried out under the guidance of **Dr. N. Das**, professor, Department of Biotechnology, Thapar Institute of Engineering &Technology, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.



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CERTIFICATE

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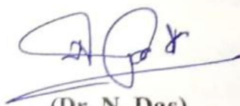
I certify that the dissertation entitled "**Enzymatic antioxidants in potato (*Solanum tuberosum* L.): Determination of Polyphenol oxidase and Peroxidase activities in different organs**" comprises research work carried out by **Ms. Hardeep Kaur** (Regd. No. 301707008**)** under my supervision and guidance during the period between 7th January 2019 to 10th July 2019 for the partial fulfillment of the Master of Science in Biochemistry submitted to Thapar Institute of Engineering & Technology, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.



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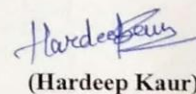
Therefore, I consider myself as a very lucky individual as I was provided with an opportunity to be a part of it. I am also grateful for having a chance to meet so many wonderful people and professionals who led me through this dissertation period.



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List of Abbreviations

Abbreviations	Name
ROS	Reactive oxygen species
$O_2^{\cdot -}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
$^1O_2$	Singlet Oxygen
H_2O_2	Hydrogen Peroxide
LPO	Lipid peroxidation
SOD	Superoxide Dismutase
APX	Ascorbate peroxidase
GPX	Guaiacol peroxidase
CAT	Catalase
DHAR	Dehydroascorbate reductase
AA	Ascorbic Acid
GSH	Reduced glutathione
GR	Glutathione Reductase
CS-1	Kufri Chipsona-1
CS-2	Kufri Chipsona-2
PR	Kufri Pukhraj
AS	Kufri Ashoka
KJ	Kufri Jyoti
DE	Desiree
PPO	Polyphenol oxidases
POD	Peroxidases
TPP	Three phase partitioning
SDS	Sodium dodecyl sulphate
BSA	Bovin Serum Albumin
IAA	Indole acetic acid
Na_2CO_3	Sodium bicarbonate
NaOH	Sodium hydroxide
PVP	Polyvinylpyrrolidone
APS	Ammonium persulphate
kDa	kilo-dalton
Nm	Nanometer
mL	Millilitre
mg	Milligram
g	Gram
mM	Millimolar
M	Molar
µg	Microgram

Abstract

Potato is very nutritious and a major non-grain food crop consumed worldwide. It contains biologically active substances that affect the human health positively and reduce degenerative chronic disorders. Although, cutting and peeling of potato induces black or brown pigments of damaged tissues, results from enzymatic browning. This reaction is a result of catalytic conversion of phenolic compounds into quinones by the enzymes namely, polyphenol oxidase (PPO) and peroxidase (POD). PPO uses molecular oxygen (O_2) whereas POD uses hydrogen peroxide (H_2O_2) for oxidation. Both PPO and POD are involved in the ROS defence mechanism and are the crucial part of enzymatic antioxidants. The objectives of the study were a) extraction and estimation of PPO and POD in different organs of potato cultivars at different stages of growth under field conditions along with different storage conditions of the harvested tubers; b) to purify both the enzymes conveniently by simple and rapid three phase partitioning (TPP). The specific activity of PPO and POD was notably higher in mature leaves, stem and tuber in comparison to young leaves, stem and tuber. The specific activities of PPO and POD showed a considerable increasing trend during the storage for 60 days at low temperatures. Prolonged storage at −20ºC was associated with more accumulation of the enzymes as compared to 4ºC and room temperature. Facile TPP method was adopted for purification of the enzymes. The focus was on mainly two parameters such as ammonium sulphate concentrations and crude to t-butanol ratios. 40% ammonium sulphate concentration and 1:1 crude to t-butanol ratio worked effectively for PPO where, the enzyme was purified up to 1.64 fold (with 96% recovery) and 2.73 fold (with 86% recovery) for the cultivars CS-1 and DE, respectively. Whereas 50% ammonium sulphate concentration and 1:0.67 crude to t-butanol ratio worked well for POD. In this case, the enzyme was purified up to 3.21 fold (with 95% recovery) and 1.98 fold (with 134% recovery) for the cultivars CS-1 and DE, respectively. Molecular weight of the TPP-purified PPO and POD subunits were found to be in the range of 20-25 kDa as revealed by SDS-PAGE.

Keywords: Potato (*Solanum tuberosum* L.) cultivars; Enzymatic antioxidants; Polyphenol oxidase; Peroxidase; Three phase partitioning

1.1 About potato

Potato (*Solanum tuberosum* L.), is fourth most cultivated non-grain food crop in the world after wheat, rice and maize (Haan S et al., 2016). It was introduced in India from Europe in the 17th century by British missionaries or Portugese traders (Averyanov and Lapikova, 1989). Now, India ranks third in term of area and second in term of its production, after China. Potato is regarded as valuable food crop due to its nutritive value and ease of cultivation (Singh et al., 2016). Potato belongs to family solanaceae and genus *Solanum* which consist of 3,000-4000 species and placed within 90 genera (Huaman et al., 1986). It is an annual herb, surviving only one growing season and is cultivated under temperate, subtropical and tropical climate conditions. Temperature is the main factor affecting the growth and production of potato. The optimum temperature range found to be 18ºC- 20°C whereas; optimum soil temperature is 16ºC-19ºC. This crop requires continues water supply and adequate soil aeration and grown in different varieties of soil (alluvial, hill, black and red) with pH range of 5- 7 (Reddy et al., 2018)

The potato plant grows up to 0.4 to 1.4 m long and having pinnately compound and alternate leaves. It is a nightshade plant which consists of fibrous root system. The stems may or may not contain hairs and are medium to dark green in color as leaves. Potato plant possesses white, pink, blue, or purple flowers with yellow stamens inserted on short corolla tube. Tubers are the storage organs that originate from swollen underground stem called stolon (Dennison et al., 1997). Potato is autogamous plant and having small inedible berries which contains poisonous alkaloids (Solanine) (Berg et al., 1998). It plays major role in human consumption as it is a rich source of amino acids, minerals and carbohydrates. Freshly harvested potatoes consist of 75-80% water, 16-22% carbohydrates, 2.6-3.1% proteins, 0.8-1.3% minerals, 0.1-0.2% crude fats, 0.6% crude fiber and also some vitamins (Schoenemann et al., 1977). However, potato contains only relatively little protein but its nutritional quality is better than cereals. It also contains some essential amino acids i.e. leucine, isoleucine, tryptophan and other dietary antioxidants, dietary fiber, thiamine, iron, folic acid and various polyphenols and phenolic acids (Kim et al., 2019).

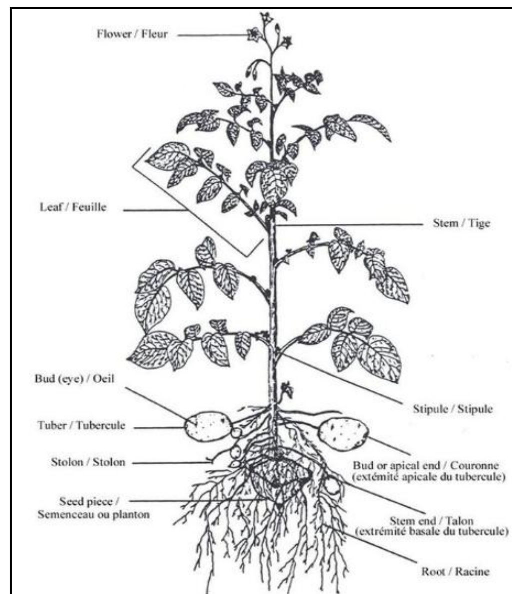


Fig. 1: Morphology of potato plant (Ref: https://www.quora.com)

1.2 Reactive oxygen species

Reactive oxygen species (ROS) are considered as toxic by-products of metabolic process that includes oxygen such as photosynthesis and respiration. From the past few years of investigation, it has been reported that ROS plays an important signaling role in plants, its growth, development and also response to biotic and abiotic stress conditions. ROS family include two major members, free radicals ($O_2^{\cdot-}$, $OH^{\bullet}$) and non-radicals (H_2O_2 and $^1O_2$) (Roychoudhury and Basu, 2012). The location of ROS production in plants occurs mainly in the chloroplast, mitochondria and peroxisomes. Major site of ROS found in the reaction centre of PSI and PSII in chloroplast (Das et al., 2014). Other secondary sites include cell wall, cell membrane, endoplasmic reticulum and apoplast (Roychoudhury and Basu, 2012). They are very lethal and damages the (genetic material) DNA, proteins and lipids which leads to affect the cellular functioning and even cell death.

Cell has acquired well defined antioxidant machinery to overcome the stress of ROS which is done by enzymatically that consist of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPX) and the non-enzymatically via reduced glutathione (GSH), ascorbic acid (AA), α-tocopherol, proline, carotenoids and phenolic compunds (Miller et al., 2010).

1.3 Sites of ROS production

ROS are generally termed as consequence of electron transport activities of cell organelles under stress conditions. ROS is produced at different location in cell including mitochondria, chloroplast, plasma membrane, endoplasmic reticulum, peroxisomes and cell wall under both stress and normal conditions. Site of ROS production depends on light as well as dark conditions. Chloroplast, peroxisomes produces ROS in the presence of light whereas in dark conditions mitochondrion is mainly responsible for ROS production (Das, K., & Roychoudhury et al., 2014). But, above the threshold level they become toxic for the cell, leading to damage of cell organelles and even cell death.

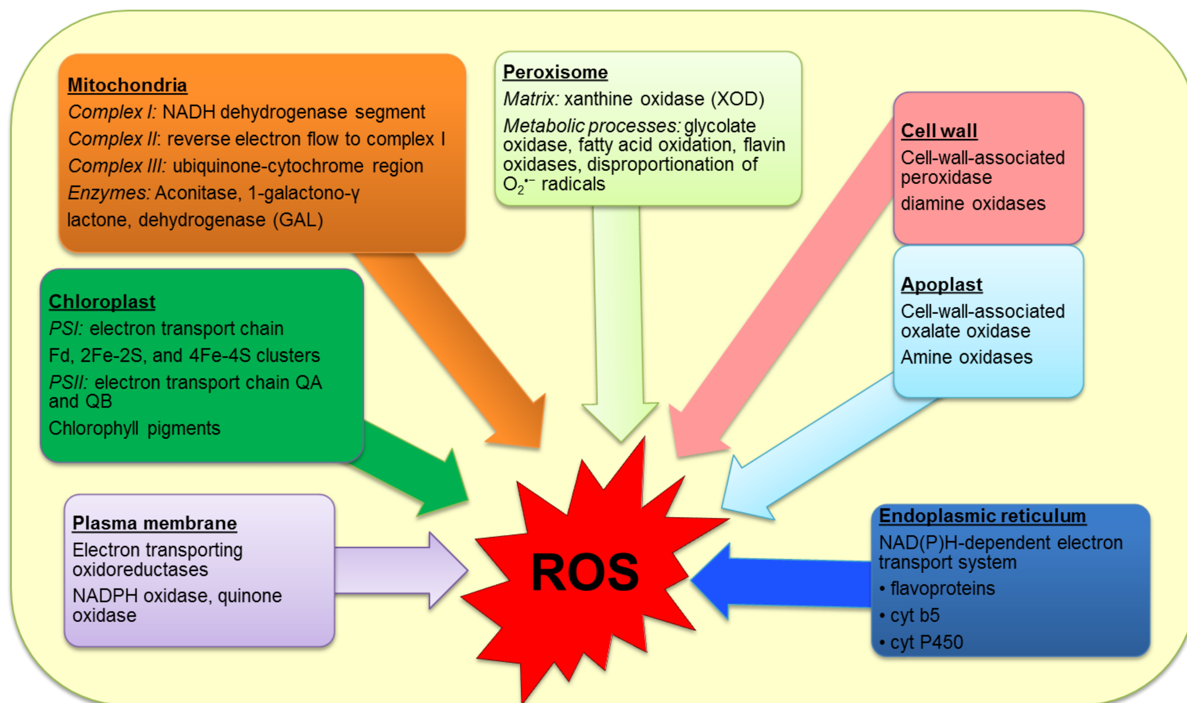


Fig. 2: Site of ROS production in plants (Hasanuzzaman et al., 2013).

1.4 Types of ROS

Different types ROS are responsible for oxidative damage which involves $^1O_2$ (singlet oxygen), H_2O_2 (hydrogen peroxide), $OH^•$ (hydroxyl radical) and $O_2^{•-}$ (superoxide radical). ROS are generated from 1-2% of total O_2 taken by plants (Bhattacharjee et al., 2005).

Superoxide radical: Partial reduction of oxygen in the cell generates the ROS family. The superoxide radical mainly generated inside the chloroplast and mitochondria and other cell organelles. It is the firstly formed ROS during non-cyclic reaction of electron transport chain (ETS). Superoxide dismutase (SOD) acts as a scavenger of this radical (Mittler et al., 2002).

Singlet oxygen ($^1O_2$): Singlet oxygen is produced by reactions occurs in chlorophyll with oxygen. It is generated in membranes, mitochondria and chloroplasts. Half life of singlet oxygen is very short i.e. 3μs yet it causes harm to both the photo-systems, PSI and PSII. It is scavenged by non enzymatic antioxidants i.e. carotenoids and α- tocopherol (Hatz et al., 2007).

Hydroxide radical ($OH^•$): The hydroxyl radical is highly reactive and toxic ROS produced in mitochondria, chloroplast and other organelles. It damages the cellular components by membrane destruction, protein damage and lipid peroxidation (LPO) (Pinto et al., 2003).

Hydrogen peroxide (H_2O_2): H_2O_2 is generated in plant cells in both normal conditions and oxidative stress condition such as drought, intense light, chilling, pathogen infection and UV radiations (Sharma et al., 2012). Major source of production of H_2O_2 include membranes, peroxisomes, ER, mitochondria and chloroplasts. Catalases and various peroxidase are the enzymatic scavengers of hydrogen peroxide.

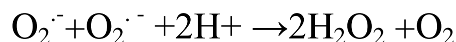
1.5 ROS defence machinery

ROS consists of antioxidants machinery to deal with oxidative damage, which is done by enzymatically and non- enzymatically.

Enzymatic antioxidants: The enzymes play a major role in antioxidant machinery localized in the various cellular organelles include Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) and Guaiacol Peroxidase (GPX).

Superoxide dismutase (SOD): SOD [E.C.1.15.1.1] is a metallo-enzyme present in all aerobic organisms. SOD act as first defense machinery against ROS under stress condition. The SOD dismutase the $O_2^{•-}$ into hydrogen peroxide (H_2O_2) and oxygen (O_2). There are three isozymes

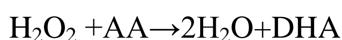
classes of SOD on the basis of metal ion binding, this includes Mn-SOD (mitochondria), and Cu/Zn-SOD (cytosol, chloroplasts and peroxisomes) and Fe-SOD (chloroplasts) (Mittler et al; 2002).



$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow 2H_2O_2 + O_2$$

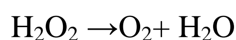
Polyphenol oxidase (PPO): Polyphenol oxidase is a copper containing enzyme which consists of two forms namely; tyrosinase [E.C 1.14.18.1] and laccase [E.C 1.10.3.1] that are widely distributed among plants, animals and micro-organisms (Mayer et al., 2006). In plants, this enzyme is located in chloroplast and plastids and is mainly responsible for undesired enzymatic brown which shows the negative impact on color, flavor, nutritional value and shelf life of food products (Taranto et al., 2017). PPO helps in oxidation of phenolic compound at different pH and temperature ranges and also plays a crucial role in the defense mechanism in arthropods and pathogenesis in plants and fungi (Flurkey et al., 2008). It catalyses the oxidation of monophenols and diphenols to a reactive compound o-quinone, which leads to formation of black or brown pigments on reacting with amino acid group of cellular proteins (Boeckx et al., 2015).

Ascorbate peroxidase (APX): APX [E.C.1.11.1.1] works wonder against ROS, found in almost every cell organelles. APX undergoes the reduction of hydrogen peroxide into water and dehydroascorbate with the help of ascorbic acid that act as a reducing agent. APX scavenges hydrogen peroxide mainly in cytosol and chloroplast. It is considered as powerful scavenger of hydrogen peroxide with the comparison of others.



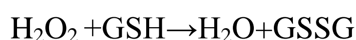
$$H_2O_2 + AA \rightarrow 2H_2O + DHA$$

Catalase (CAT): Catalase [E.C.1.11.1.6] is a heme containing enzyme of tertameric structure located in the cytosol, mitochondria and chloroplast. It catalyzes the dismutation of H_2O_2 to form water and oxygen. It is highly specific for H_2O_2 and shows weak activity against peroxides.



$$H_2O_2 \rightarrow O_2 + H_2O$$

Guaiacolperoxidase (GPX): GPX [E.C.1.11.1.7] is a heme-containing antioxidant which consist 40-52 kDa monomers. It is a key enzyme in extracellular removal of hydrogen peroxide and its degradation under both normal and stress conditions.



$$H_2O_2 + GSH \rightarrow H_2O + GSSG$$

Peroxidase (POD): Peroxidase (POD) [EC 1.11.1.7] is a heme-containing enzyme majorly distributed in higher plants, bacteria, fungi and algae and localized in cell walls, some transport organelles, vacuoles and also on rough endoplasmic reticulum (Azevedo et al., 2003). Peroxidase is involved in various physiological processes in plants, including responses stress conditions. It helps in combating effects of ROS by scavenging hydrogen peroxide. POD is mainly responsible for enzymatic browning of fruits and vegetables and lignin biosynthesis (Motamed et al., 2009). They have been used in industrial and biochemical process like estimation of uric acid, cholesterol, glucose, detoxification of industrial waste and polluted water with phenols, aromatic amines, and dyes (Srinivas et al., 1999).

Non-enzymatic antioxidants: Non-enzymatic component act as another half of antioxidant machinery. It consist of plant metabolites (polyphenols, flavonoids), vitamins (ascorbic acid (AA), carotenoids, α- tocopherols) and amino acid (proline) regulates the growth and development of plant (Pinto et al., 2004).

Polyphenols: Polyphenols are secondary metabolites of plants and are beneficial for human health. They affect taste, color and texture of plant products. Polyphenol act as antioxidant, anti-cancerous, anti- microbial, anti-inflammatory agents and also protects different types of abiotic and biotic stress (Andreu et al., 2018).

Ascorbic Acid (AA): Ascorbic acid also called as vitamin C. It is considered as the most abundant and powerful antioxidant as it can donate electrons and act as first line of defense against ROS. Ascorbate is found both in cytosol and apoplast. It helps in the detoxification of H_2O_2 via the catalytic action of ascorbate peroxidase. It is a water soluble antioxidant and found as a major component in human nutrition. Ascorbate is act as a coenzyme and used as a reducing agent in various metabolic pathways (Upadhayaya et al., 2009).

Carotenoids: Carotenoids are lipophilic antioxidants like α-tocopherol and found in plastid of plant tissue (photosynthetic and non-photosynthetic). Carotenoids exhibit their antioxidants capacity by scavenging free radicals and inhibit lipid peroxidation. These are also reported in mico-organisms. Carotenoids regulate the photosynthetic activity and transfer the energy to cholorophyll.

Flavonoids: Flavonoids are phenolic compound which inhibit lipid peroxidation and lipoxygnase activity. It is found in leaves, pollen grains and floral organs. On the basis of the structure it is

classified into four classes such as flavonols, isoflavones, flavonols and anthocyanin. Major role of flavonoid is to scavenge damaged caused by ROS to photosynthetic apparatus. It performs various roles like defense machinery against plants pathogen, pollen germination and flowers pigmentation (Agati et al., 2012).

Table 1: List of enzymatic antioxidants

Enzymatic antioxidants	**Code**	**Subcellular Location**
Superoxide dismutase (SOD)	1.15.1.1	Mitochondria, Chloroplast, Cytosol, Peroxisomes
Polyphenol oxidase (PPO)	1.10.3.2	Mitochondria, Chloroplast, Plastid
Ascorbate peroxidase (APX)	1.11.1.1	Mitochondria, Cytosol, Chloroplast
Catalase (CAT)	1.11.1.6	Mitochondria, Peroxisome
Guaiacol peroxidase(GPX)	1.11.1.7	Mitochondria,Cytoplasm,Chloroplast
Peroxidases (POD)	1.11.1.7	Cell walls, Vacuoles, RER, Cytoplasm.

Table 2: List of non-enzymatic antioxidants

Non-enzymaticAntioxidants	**Subcelluar location**
Polyphenols	Chloroplast and Mitochondria
Ascorbic Acid	Cytosol, Mitochondria ,Apoplast
Carotenoids	Chloroplasts
Flavonoids	Vacuole

ROS-"a dual edged sword" are now considered to play a dual role in cell metabolism. At low concentration, below threshold level they play an important role in cell signaling and various other processes (Mittler et al., 2002). Therefore it becomes essential and crucial to understand the components of the antioxidant machinery of the cell that works to overcome the effect of ROS. So in the next chapter some attributes of the two essential candidates of the antioxidant defense machinery i.e, **Polyphenol oxidase and Peroxidase** are discussed.

2.1 Brief introduction to Polyphenol oxidase (PPO)

Potatoes are an important source of polyphenols (phenolic compounds) in the human diet. Phenolic compounds are partially responsible for color, bitterness, texture and nutritional values in fruits and vegetables (Niphadkar et al., 2015). Polyphenol oxidase (PPO) [EC 1.10.3.2], is a copper-containing enzyme present in plants, animals and microorganisms. PPO is the major cause of enzymatic browning in higher plants and involved in melanin biosynthesis in animals which protects skin and hair against harmful solar radiations (Arnnok et al., 2010). Its localization in plant cell depends on the age, species of fruits and vegetables. In green leaves, PPO is mainly found in the chloroplast, in potato tuber it is found in all sub-cellular fractions and in freshly harvested potato, it is localized mainly in chloroplast and mitochondria (Vamos-Vigyazo et al., 1981). It is also located in the plastids of plant cells and the phenolic compounds are stored in the vacuoles (Holderbaum et al., 2010). PPO activity is more prominent in the skin of fruits and vegetables but during ripening its activity gets lower. In plants, PPO works as a defense system but its exact function is still unknown.

2.2 Biochemical properties of PPO

PPO catalyzes the two types of oxidative reaction: the first is the hydroxylation of monophenols to o-diphenols in the presence of monophenol oxidase and second is the oxidation of o-diphenols to o-quinones in the presence of catecholase/ diphenolase in the presence of molecular oxygen (O_2). This reaction leads to the formation of black or brown pigments (Mishra et al., 2016). PPO is substrate specific where some of substrates for potato are p-Coumaric acid, L-DOPA, p-coumaryl glucose, caffeic acid Chlorogenic acid, catechol, and p-cresol. PPO enzyme activity depends on the pH that affects the binding of substrates and the catalysis. Generally, this enzyme works at optimal pH range of 4.0 to 8.0. Temperature is most important factor that affects the catalytic activity of PPO, as it affects the oxygen solubility and may cause denaturation of enzyme. The optimal temperature range of PPO varies for different plant sources found to be 25–35°C (Taranto et al., 2017).

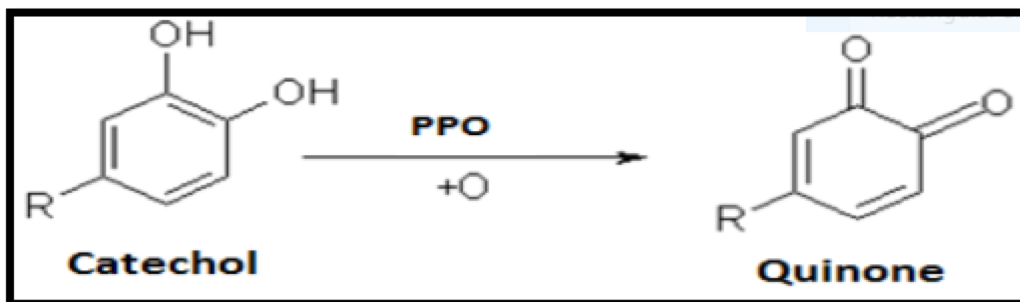


Fig. 3: Conversion of catechol into quinone by PPO (Mishra et al., 2016)

2.3 Mechanism of PPO

Mechanism of PPO involved two substrates and their binding in specific order. The active site of PPO has two copper ions and both the ions are bonded by histidine residue (His). Active site shows cyclic transitions in oxy and deoxy and met-forms. Hydroxylation of monophenol to o-diphenol and to further oxidation of reactive o-quinone molecular oxygen is required (Mukherjee et al., 2013). In each cycle, two catechol molecules gets oxidized with the reduction of molecular oxygen into water and this leads to the formation of two quinone products. Molecular oxygen binds to the CuA (deoxy form) by replacing the solvent molecule (H_2O) and the catechol molecule binds to CuB (Oxy form) by deprotonating the one of two hydroxyl groups. The electron transfer from the substrate to the peroxide is done by protonating the peroxide group along with O-O bond cleavage (Mishra et al., 2016). The other hydroxyl group of the substrate donates a proton with the formation of the o-quinone product along with the loss of water molecule. Another catechol molecule act as “co-substrate” that reduces dicupric (CuII) form to the dicuprous form (CuI) (Eicken et al., 1999).

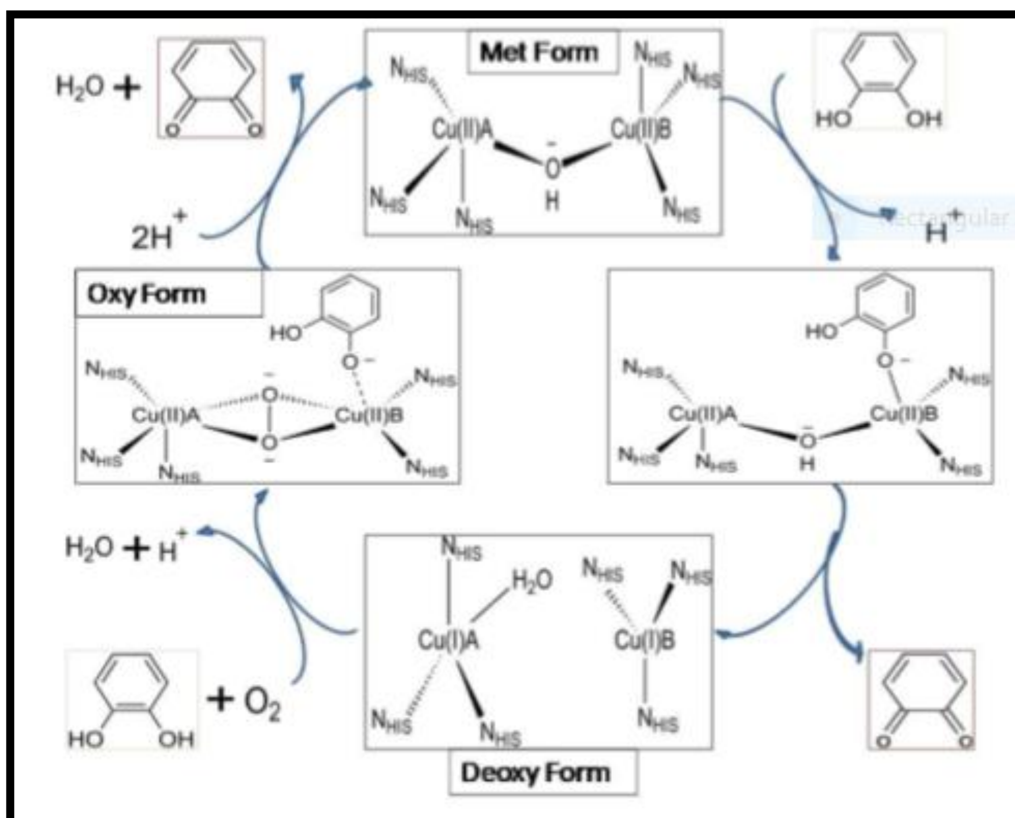


Fig 4: Mechanism of action of Polyphenol oxidase (PPO) (Eicken et al., 1999)

2.4 Role of PPO

Polyphenol oxidase has been largely studied by researchers because of its commercial importance of browning. It performs several functions of plants such as hormone regulation, lignin biosynthesis, degradation of indole acetic acid (IAA) and defense mechanism (Niphadkar et al., 2015). It has been reported that PPO show positive expression and resistant to biotic stress. Similarly, PPO could also be used as a biomarker for different interactions among genotype and pathogens (Taranto et al., 2017). Also, this enzyme plays a major role in biodegradation of phenols and purification of polluted water (Mukherjee et al., 2013).

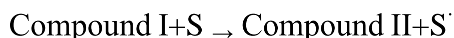
2.5 Brief introduction to Peroxidase (POD)

Peroxidase (POD) [E.C.1.11.1.7] is a heme-containing enzyme localized in cell walls, some transport organelles, vacuoles and also on rough endoplasmic reticulum. POD is widely distributed enzyme in nature which catalyzes the oxidation of large varieties of substrate with the help of hydrogen peroxide (H_2O_2) as an electron acceptor (Azevedo et al., 2003). Enzymatic browning is generally catalyzed by POD that leads to the formation of quinone products results in brown pigmentation (Dehorn et al., 2002).

2.6 Biochemical properties of POD

POD is substrate specific which uses some of phenolic substrate such as guaiacol, m-cresol, p-cresol, anisole and hydroquinone (Yadav et al., 2017). POD reduces H_2O_2 into water with the oxidation phenolic and other substrates. The POD works in multistep reaction as shown in the following general scheme (Nokthai et al., 2010).

$$POD + H_2O_2 \rightarrow Compound\ I + H_2O$$

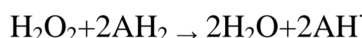


$$Compound\ I + S \rightarrow Compound\ II + S^{\cdot}$$

$$Compound\ II + S \rightarrow POD + S^{\cdot}$$

Where, S and $S^{\bullet}$ represents substrate and radical form
AH_2 and $AH^{\bullet}$ represent a reducing substrate and its radical product, respectively.

A general equation for this bio-chemical reaction is as follows:



$$H_2O_2 + 2AH_2 \rightarrow 2H_2O + 2AH^{\cdot}$$

2.7 Mechanism of POD

Native form of enzyme (Fe^{III}) is oxidized by H_2O_2 to give an active intermediate form of enzyme (Fe^{IV}) as complex compound I. This complex compound oxidizes the substrate to form free radical in the reaction mixture and attain complex compound II state. This compound II state oxidise the second substrate to give a free radical product followed by returning back to native state.

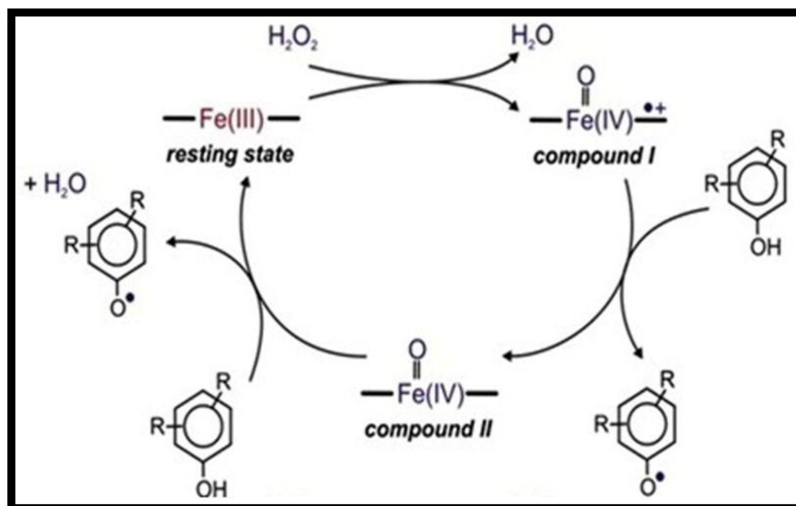


Fig 5: Catalytic cycle of peroxidase (Yadav et al., 2017)

2.8 Role of Peroxidase

POD is a key enzyme that causes browning of fruits and vegetables. It has been found that peroxidase play varieties of role in plant physiological responses which includes wound healing, pathogen defense, catabolism of auxin, lignifications and cell wall modifications. Peroxidase are widely used in research field like clinical biochemistry, enzyme immunoassay, organic chemistry, decolorization of dyes, oxidation of IAA and proline hydroxylation (Zhang et al., 2015).

2.9 Studies on PPO and POD till date

Flurkey et al., 1826 studied the PPO and POD activity in developing peaches. Poulos et al., 1980 investigated the stereochemistry of POD catalyst. Kahn et al., 1981 studied the soluble and bound peroxidase in potato and their biochemical properties. Vaughn et al., 1984 studied the role of PPO in higher plants. Bachem et al., 1994 showed the inhibition of enzymatic browning in potato by antisense expression of PPO gene. Multigene family of PPO in potato was studied by Thygensen et al., 1995. Internal damaged occurs due to enzymatic browning was examined by McGarry et al., 1996. Dietary role of potato polyphenols was studied by Friedman et al., 1997. Coetzer et al., 2001 studied the effect of enzymatic browning in potato through sense and anti-sense RNA from tomato PPO. Dehon et al., 2002 studied the role of POD in browning of Juglangs nigra. Sandhu et al., 2007 found POD as a biochemical maker in potato at maturity level. Flurkey et al., 2008 studied the proteolytic process of PPO in fungi and plants. Nokthai et al., 2010 studied the molecular modeling of POD and PPO.

Both PPO and POD are used in bioremediation of phenols from polluted water (Mukherjee et al., 2013). Enzymatic browning by PPO and POD and their characterization was investigated by Zhang et al., 2015. Niphadkar et al., 2015 purified PPO from waste potato peel by Aqueous two phase partitioning (ATPs) and three phase partitioning (TPP) technique. Molecular weight of purified PPO and POD monomer was found to be 20-36 kDa. Esmaeili et al., 2017 purified PPO by DEAE-Sephadex A_{25} in *Crocus sativus* and also showed the correlation between PPO and phenolic contents during dormancy and sprouting. The optimum temperature of PPO and POD was found at 20ºC-45ºC whereas optimum pH was 5-7 followed by the decreased in activity with the increase in temperature and pH (Yadav et al., 2017).

2.9.1 Origin of the problem

PPO and POD are known to be the effective enzymes against oxidative stress and are found in most known fruits and vegetables in multiple forms. Their actions are mainly concerned with the enzymatic browning of fresh and frozen horticulture products that leads to the formation of brown pigments. Hence, this lower downs the nutritive value as well as the market value of the product. The problems of both enzymes are: they catalyze more than one reaction and react with the variety of substrates. The studies till date don't seem sufficient to provide the basic understanding of these enzymes. The physiological role of both enzymes is still unclear (VamVigyazo et al; 1981). Storage of potato tubers at different temperature affect the metabolism and alter the phytochemical properties of enzymes. Various aspects of ROS metabolism and defense machinery during storage conditions of potato are still unclear. Particularly there is very little report available on Indian potato varieties. Keeping in view, this study focused on understanding polyphenol oxidase and peroxidase in Indian potato cultivars with following objectives.

2.9.2 Objectives of the study

- To grow disease-free micropropagated potato plantlets under field condition and harvesting various organs at different stages of growth
- Isolation and estimation of Polyphenol oxidase and Peroxidase activities in the potato organs and also under different conditions
- Partial Purification and characterization of the enzymes by three phase partitioning (TPP)

3.1 Plant materials

PPO and POD was estimated in the five Indian cultivars namely, Kufri-Chipsona-1(CS-1), Kufri-Chipsona-2 (CS-2), Kufri Pukhraj (PR), Kufri Jyoti (KJ), Kufri Ashoka (AS) along with the reference cultivar Desiree (DE) procured from CPRI Shimla and relatively maintained in the growth room of TIET. There potato plantlets were first acclimatized and then transferred to field in the mid November till mid March. Harvesting of plants was done at various time intervals to collect the organs at different period of growth for the estimation of enzyme activity.

3.2 Other materials and reagents

Various chemicals, reagents and another materials used in the study were purchased such from Siga-aldrich India Pvt.Ltd, and HiMedia Laboratories Mumbai.

Catechol, Guaiacol, Polyvinylpyrrolidone (PVP), Hydrogen peroxide, Di-sodium hydrogen phosphate and Potassium dihydrogen phosphate.

3.3 Harvesting of plant materials

Harvesting of different organs was done under different time interval in field conditions. Different organs of potato i.e. tubers, leaves and stems were collected and washed thoroughly with water to remove dirt and other material After air drying, all the organs were shade frizzed in liquid nitrogen to arrest their metabolic stages and stored at -80ºC for future experiments (Thygesen et al., 1995).

3.4 Preparation of the enzymes extracts

All steps of enzyme extraction were carried out at 4°C. 200 mg of sample was taken in motor and pestle. Crushed them into a paste and extracted with 5.0 mL 0.1M phosphate buffer (pH 7.0) followed by adding a pinch of polyvinylpyrrolidone (PVP) and mixed well. The homogenate was filtered and the filtrate was centrifuged at 2,500 rpm for 20 min at 4ºC (Arnnok et al., 2010). The supernatant was collected and stored at -20ºC for determination of enzyme activities.

3.5 Assay of the enzymatic antioxidants

3.5.1 PPO assay: PPO activity was determined spectrophotometrically by taking absorbance at the wavelength of 410 nm. Reaction mixture was prepared by adding 1.95 mL of 0.1M phosphate buffer, 1.0 mL of 0.1M catechol as a substrate and 50 µL of enzyme extract in a 1.0 cm path length cuvette and mixed thoroughly. The absorbance was recorded continuously for 2.0 min, activity was expressed in unit where, one unit of activity is defined as the amount of enzyme which catalyzes the decomposition of 1.0 µmol catechol as calculated from the extinction coefficient of 100 $M^{-1}cm^{-1}$ at 410 nm.

3.5.2 POD assay: Reaction mixture was prepared by adding 0.15mL of 4% guaiacol, 0.15mL of 1% H_2O_2 as a substrate, 2.66 ml of 0.1 M phosphate buffer pH 7 and 40 µL of enzyme extract in a 1 cm of path length cuvette and the increase in absorbance was recorded at 470 nm for 2 min. The specific activity was expressed in unit where, one unit of activity is defined as the amount of enzyme which catalyzes the decomposition of 1 µmol H_2O_2 as calculated from the extinction coefficient of 2.66 $mM^{-1}cm^{-1}$ at 470 nm (Arnnok et al; 2010).

3.6 Protein estimation by Folin-Lowry method

Lowry method was used for protein estimation from different plant organs like leaves, stem and tubers. In a protein, phenolic compound in amino acids namely, tyrosine and tryptophan produces a blue purple complex, which absorbs at 660 nm. Protein estimation was done using Bovin Serum Albumin (BSA) as a standard.

The following solutions were used for determination of protein.

Solution A: 2% Sodium bicarbonate (Na_2CO_3) in 0.1N NaOH

Solution B: 1% Na-K Tartrate in H_2O

Solution C: 0.5% $CuSO_4.5H_2O$ in distilled water.

The preparation of reagent I was done by mixing 48 mL of A, 1mL of B and 1 mL of C reagent and reagent II contains 1 part of Folin-Phenol [2N]: 1 part of water.

3.6.1 Sample preparation

200 mg of potato sample was taken and extracted with 5mL of 0.1 M phosphate buffer. Added a pinch of PVP and centrifuged at 2500 rpm for 20 minutes at 4ºC. Supernatant was filtered and stored at -20ºC for future use.

3.6.2 Estimation of protein

100 µL of sample of protein was taken in different test tubes from each of the six varieties of potato. To make the volume upto 1000 µL, 900 µL of distilled water was added followed by the addition of 5 mL reagent I. The tubes were then incubated in dark for 10 min followed by the addition of 500 µL of folin reagent. The tubes were then vortexed and incubated in dark for about 30 min. The absorbance was taken at the wavelength of 660 nm and the standard graph was plotted. The amount of protein was estimated from different plant organs i.e. leaves, stems and tuber according to standard curve equation.

3.7 Partial purification of polyphenol oxidases by Three Phase Partitioning method (TPP)

TPP is a modest and efficient bio-separation technique. It is used for the separation of protein directly from crude suspension. This technique works by extracting the biomolecules into the three phases. TPP involves the addition of ammonium sulphate in crude extract followed by the addition of t-butanol that facilitates the separations of phases into an upper organic t-butanol phase, lower aqueous phase and middle layer of concentrated proteins (Niphadkar et al., 2015). This technique works by removing the contaminant from middle protein layer subjected to the aqueous phase. Depending on the nature of reagents and molecular weight of protein, the target layer gets partitioned into either of phases (Vetal et al., 2015).

3.7.1 Reagents required

Ammonium sulphate: The concentration of Ammonium sulphate affects the purification of enzymes in TPP. This reagent is undergoes protein-protein interactions and salting out by precipitating the protein. Different ammonium sulphate concentrations varied 20-80% was used in TPP. As the ammonium sulphate concentration increased, it promoted water dissipation from the layer around the proteins. With the results, hydrophobic particles of proteins were formed and strongly bonded with other. This phenomenon leads to coagulation of protein.

t-Butanol: t-Butanol has branched chain structure and higher molecular size. Due to its structure and size it doesn't penetrate inside folded protein structure and doesn't cause denaturation of protein-butanol increases partitioning of enzyme by crowding and kosmotropic effects at 20º-32°C temperature ranges. All these properties increase the three phase partitioning.

3.7.2 Preparation of crude enzyme extract for PPO

Washed the different potato cultivars with distilled water. Approximately, 2g of tuber were weighed and homogenized in 20 mL of 0.2M phosphate buffer of pH 7.0. The homogenate was centrifuged at 10,000 rpm for 20 min at 30ºC. The supernatant was collected after centrifugation and stored at -20ºC for the determination of polyphenol oxidase activity and protein content.

3.7.3 Three phase partitioning (TPP) of PPO

TPP for PPO was performed in a 50 mL glass reactor in which 1mL of crude extract was taken and dissolved with different concentrations of ammonium sulphate i.e. 0–20, 0–40, 0-60, and 0–80% (w/v) at room temperature followed by addition of t-butanol from ratio 1:0.5-1:2 (v/v). The mixture was stirred mechanically at 400 rpm for 30 min. This reaction mixture was centrifuged at 8000 rpm for 20 min to attain three distinct phases. The upper organic layer of t-butanol was carefully removed but the middle protein layer and aqueous bottom layer were collected which contains the fraction of TPP. The sample was dialyzed with 1.0 mL of 0.2 M phosphate buffer and 100 µL of NaOH. Finally, the sample was stored at -20°C and used for enzymatic activity of polyphenol oxidase and protein content.

3.7.4 Measurement of PPO activity and protein content

It was done spectrophotometrically and protein content was estimated by Folin-Lowery method using bovine serum albumin (BSA) as a standard.

3.7.5 Preparation of the enzyme extract for POD

Washed the different potato cultivars with distilled water. Approximately, 2g of tuber were weighed and homogenized in 20 mL of 0.1M phosphate buffer of pH 7.0. The homogenate was centrifuged at 10,000 rpm for 20 min at 30ºC. The supernatant was collected after centrifugation and stored at -20ºC for the determination of peroxidase activity and protein content.

3.7.6 Three phase partitioning (TPP) of POD

TPP for POD was performed in a 50 mL glass reactor in which 1ml of crude extract was taken and dissolved with different concentrations of ammonium sulphate i.e. 0–20, 0–30, 0-40, 0-50 and 0–60% at room temperature followed by the addition of t-butanol ratios i.e. 1:0.5-1:2. The mixture was stirred mechanically at 200 rpm for 100 min. This reaction mixture was centrifuged

at 5000 rpm for 20 min to attain three distinct phases. The upper organic layer of t-butanol was carefully removed but the middle protein layer and aqueous bottom layer were collected which contains the fraction of TPP. The sample was dialyzed with 1.0 mL of 0.1 M phosphate buffer and 100µL of NaOH. Finally, the sample was stored at -20°C and used for enzymatic activity of peroxidase and protein content.

3.7.7 Measurement of POD activity and protein content

It was done spectrophotometrically and protein content was estimated by Folin-Lowery method using bovine serum albumin (BSA) as a standard.

3.8 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is used to detect the proteins in complex compounds. The relative movment of proteins through polyacrylamide gel depends on the charge density, mass and shape of the molecule. SDS denatures protein, causing multimeric proteins to dissociate into their subunits. SDS-PAGE gel have two parts one stacking gel and other is resolving gel as shown in Table (3) and (4). For this study 12% stacking gel was used as reported in literature (Vetal et al., 2015).

3.8.1 Methodology

The constituents of the resolving gel was added in a test tube and poured in the casting apparatus followed by over layering with n-butanol.

After 30 min, when the gel was polymerized, n-butanol was decanted and gel was washed by distilled water to remove the impurities.

The comb was placed over casting apparatus and then stacking gel was prepared according to table 4. Then gel was immediately poured over the polymerized resolving gel. After polymerization, the comb was removed carefully.

The fully polymerized gel was submerged in 1X SDS-PAGE running buffer. The protein samples were mixed with coomassie blue R-250 dye and boiled for 3-5 min in a boiling water bath Gel was run at 100 volts and 90 mA for 2 hrs. After this, gel was stained in staining solution for 1 hour and then destained in destaining solution for 2-3 hrs. The protein bands were visualized clearly and photographed.

Table 3: Composition of separating gel

Components	Percentage(12%)
Distilled water	3.35 mL
Tris buffer (1.5M, pH 8.8)	2.5 mL
Bis-acrylamide	2.0 mL
10% SDS	100 µL
10% APS	50 µL
TEMED	15 µL

Table 4: Composition of stacking gel

Components	Percentage(4%)
Distilled water	1.68 mL
Tris buffer (0.5M, pH 6.8)	1.25 mL
Bis-acrylamide	2.0 mL
10% SDS	50 µL
10% APS	25 µL
TEMED	15 µL

Table 5: Composition of staining solution

Components	Percentage
Methanol	30 %
Glacial acetic acid	10 %
Coomassie Brilliant Blue-R250	0.15 %

Table 6: Composition of destaining solution

Components	Percentage
Methanol	30%
Glacial acetic acid	10%

4.1 Plant growth and harvesting of the potato organs

Different varieties of potato were grown in the field of TIET, Patiala and various organs like leaves, stems, and tubers were collected and initially stored at ambient conditions as shown in Fig. 6 & 7. All the cultivar-specific plant materials were stored at different experimental temperatures.

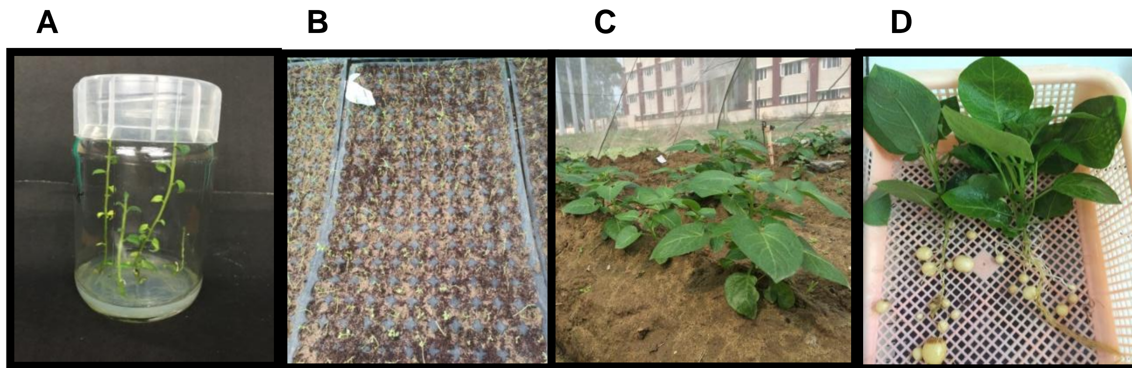


Fig. 6: Steps of plant grown in lab to field conditions; **A** Culture maintained in MS medium in lab; **B** Acclimatization and hardening of plantlets; **C** Field growth; **D** Collection of plants

Fig 7: **A** Plants at early stage (Stem, Leaf and Tuber collected in January), **B** Plants at mid-maturation (Stem, Leaf and tuber collected in early March) and **C** Mature plants (Stem, Leaf and Tuber collected in late March)

4.2 Estimation of protein in different potato cultivars

Different organs of potato cultivars i.e. leaves, stems and tubers were crushed in liquid nitrogen and homogenized in phosphate buffer (pH 7.0) as mentioned in Materials and methods.

Standard curve for proteins was made using BSA (Bovine serum albumin) at different concentrations and absorbance was taken at 660 nm as shown in Table 7 and Fig 9.

Specific activity (per mg protein) and total activity (per gram of tissues) were calculated.

Table 7: BSA at different concentration and absorbance 660 nm

Concentration (µg)	A_{660} Value
Blank	0
20	0.037
40	0.067
60	0.123
80	0.146
100	0.184
120	0.223
140	0.249
160	0.303
180	0.353
200	0.378

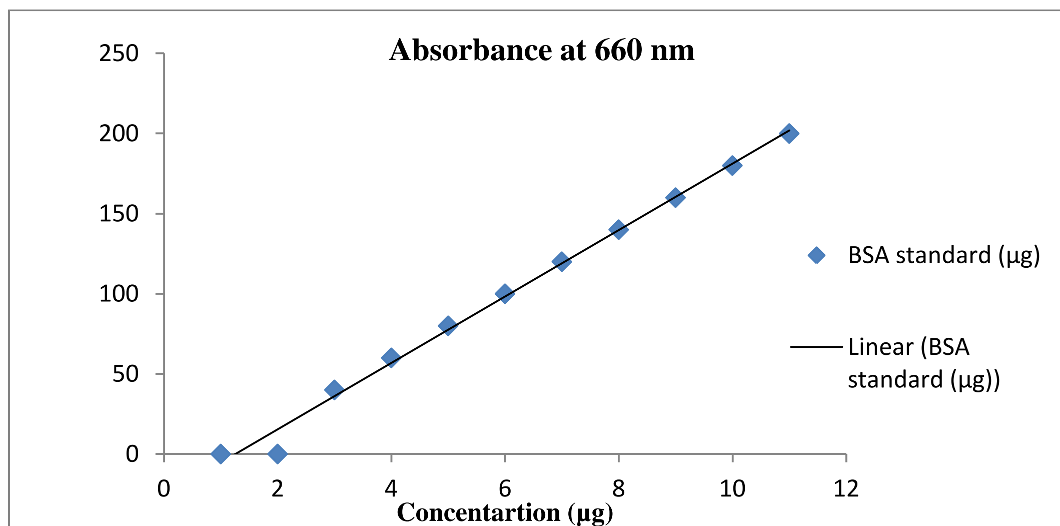


Fig 9: Standard curve of BSA

4.3 Estimation of PPO activity from various potato cultivars

The PPO was estimated from three organs namely, leaves, stems and tubers at different stages of growth. PPO was estimated by using catechol as a substrate by using the method as mentioned above. Catechol degradation was recorded for 2 minutes. Specific activity of PPO was expressed in U/mg of protein.

4.3.1 PPO activity in leaves: As shown in Table 8, it was observed that specific activity of PPO in leaves was highest in DE (3200 U/ mg protein) during young stage, (5290 U/ mg protein) growing stage and (64545 U/ mg protein) mature stage. It was lowest in AS (1213 U/ mg protein) during young stage, PR (1690 U/ mg protein) growing stage and PR (1840 U /mg protein) mature stage. From the data, it was observed that specific activity of PPO increased two folds towards maturation. These differences could be due to different stress conditions with regard to genetic makeup. As PPO is directly correlated with ROS, hence higher activity might be the resultant of the leaf efforts to combat both abiotic and biotic stress. This high activity could be used as a desirable trait in crop breeding process (Boeckx et al, 2015).

Table 8: Comparison of PPO activity in the leaves of the potato cultivars at different stages of growth under field condition (the experimental data is presented as mean of three independent leaf extracts)

Potato Cultivars	PPO activity in leaves (U/mg of protein)		
	Young	Growing	Mature
Kufri Chipsona-1	1850	1900	2877
Kufri Chipsona-2	1900	3280	3290
Kufri Pukhraj	1581	1690	1840
Kufri Ashoka	1213	2677	3686
Kufri Jyoti	1471	2409	2400
Desiree	3200	5290	6545

4.3.2 PPO activity in stem: As presented in Table 9, it was observed that specific activity of PPO in stem was highest in DE (2190 U/ mg protein) during young stage, (2635U/ mg protein)

growing stage and (3636U/ mg protein) mature stage. It was lowest in CS-1 (1060 U/ mg protein) during young stage, (1690 U/ mg protein) growing stage and CS-2 (3170 U /mg protein) in mature stage**.** From the data, it was observed that specific activity of PPO increased thrice from young stage to mature. The differences could be due to different stress conditions with regard to genetic makeup. It might be correlated to that mature stems show more enzymatic browning due to accumulation of high amount of polyphenols towards maturity.

Table 9: Comparison of PPO activity in the stem of the potato cultivars at different stages of growth under field condition (the experimental data is presented as mean of three independent stem extracts)

Potato Cultivars	PPO activity in stems (U/mg of protein)		
	Young	Growing	Mature
Kufri Chipsona-1	1060	1690	3500
Kufri Chipsona-2	1967	2607	3170
Kufri Pukhraj	1494	2112	3527
Kufri Ashoka	1590	1914	3274
Kufri Jyoti	1417	2514	3255
Desiree	2190	2635	3636

4.3.3 PPO activity in tuber: As shown in Table 10, it was observed that specific activity of PPO in tuber was highest in PR (2660 U/mg protein) during young stage, (2635 U/ mg protein) growing stage and (3505 U/mg protein) and AS (47140 U/mg protein) mature stage. It was lowest in DE (1063 U/mg protein) during young stage, (1363 U/mg protein) during growing stage and (12790 U/mg protein) during mature stage**.** Approximately, 10 fold increased was observed in the tubers from the growing stages towards maturity. Increased levels of PPO might be correlated with increased levels of POD in mature tubers as both enzymes work coherently.

Table 10: Comparison of PPO activity in the tubers of the potato cultivars at different stages of growth under field condition (the experimental data is presented as mean of three independent tuber extracts)

Potato Cultivars	PPO activity in tubers (U/mg of protein)		
	Young	Growing	Mature
Kufri Chipsona-1	2463	3025	32400
Kufri Chipsona-2	2035	1436	13267
Kufri Pukhraj	2660	3505	20610
Kufri Ashoka	1745	2742	47140
Kufri Jyoti	1349	1739	26910
Desiree	1063	1363	12790

4.4 Estimation of POD activity from various potato cultivars

The POD was estimated from three organs namely, leaves, stems and tubers at different stages of growth. POD was estimated by using hydrogen peroxide as a substrate by using the method as mentioned above. Hydrogen peroxide (H_2O_2) degradation was recorded for 2 minutes. Specific activity of POD was expressed in U/mg of protein.

4.4.1 POD activity in leaves: As represented in Table 11, it was observed that specific activity of POD in leaves was highest in AS (127.1U/ mg protein) during young stage, (109.2U/ mg protein) growing stage and CS-1 (200.5 U/ mg protein) mature stage. It was lowest in CS-2 (54.6 U/ mg protein) during young stage, PR (61.7 U/ mg protein) growing stage and DE (97.5U /mg protein) mature stage**.** From the data, it was observed that specific activity of PPO increased from young stage to mature. POD activity was maximum in the mature stage in comparison with other three stages of growth, whereas the young stage showed minimum activity, and growing stage showed intermediate levels. The three fold increase was observed towards maturity, that might be due to maturity leaves on stage of senescence has to face more stress and hence the increased levels of ROS metabolizing enzyme. Such types of studies are also reported by Cechin et al., 2010.

Table 11: Comparison of POD activity in the leaves of the potato cultivars at different stages of growth under field condition (the experimental data is presented as mean of three independent leaf extracts)

Potato Cultivars	POD activity in leaves (U/mg protein)		
	Young	Growing	Mature
Kufri Chipsona-1	65.05	47.08	200.5
Kufri Chipsona-2	54.6	61.7	134.3
Kufri Pukhraj	102.8	106.8	194.5
Kufri Ashoka	127.1	109.2	150.9
Kufri Jyoti	58.6	71.3	180.5
Desiree	80.1	82.3	97.5

4.4.2 POD activity in stem: As presented in Table 12, it was observed that specific activity of POD in stem was highest in CS-1(168.0 U/ mg protein) during young stage, (291.6 U/ mg protein) growing stage and CS-2 (410.6 U/ mg protein) during mature stage. It was lowest in PR (45.9 U/ mg protein) during young stage, DE (81.3U/ mg protein) growing stage and DE (176.5 U/mg protein) mature stage. The decrease in activity may be due to low ROS metabolism in the fully differentiated mature stem organs.

Table 12: Comparison of POD activity in the stems of the potato cultivars at different stages of growth under field condition (the experimental data is presented as mean of three independent stem extracts)

Potato Cultivars	POD activity in stems (U/mg protein)		
	Young	Growing	Mature
Kufri Chipsona-1	129.8	129.8	410.6
Kufri Chipsona-2	168.0	291.6	307.5
Kufri Pukhraj	45.9	98.4	235.0
Kufri Ashoka	85.31	82.7	229.2
Kufri Jyoti	116.3	226.3	297.0
Desiree	59.9	81.3	176.5

4.4.3 POD activity in tuber: As shown in Table 13, it was observed that specific activity of POD in stem was highest in CS-2 (100.5 U/ mg protein) during young stage, CS-2 (148.8U/ mg protein) growing stage and CS-2 (200.4 U/ mg protein) mature stage. It was lowest in DE (23.5 U/ mg protein) during young stage, DE (63.3 U/ mg protein) growing stage and DE (191.5 U/mg protein) mature stage**.** Approximately 9 fold increase was found in cultivars DE followed by PR whereas AS with 5 fold increase was observed. However, in CS-1 and CS-2 only 2 fold increases was observed. As evident from the earlier studies; POD activity increases towards maturity of the tuber. Hence, it is a marker of maturity (Sandhu et al., 2007).

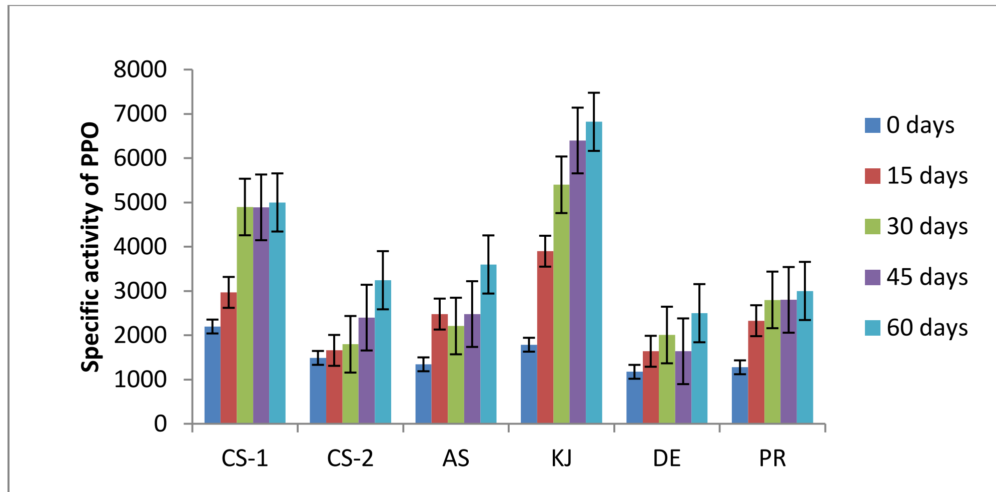
Table 13: Comparison of POD activity in the tubers of the potato cultivars at different stages of growth under field condition (the experimental data is presented as mean of three independent tuber extracts)

Potato Cultivars	POD activity in tubers (U/mg of protein)		
	Young	Growing	Mature
Kufri Chipsona-1	94.5	105.9	193
Kufri Chipsona-2	100.5	148.8	200.4
Kufri Pukhraj	40.0	91.1	203.9
Kufri Ashoka	42.6	83.3	250.9
Kufri Jyoti	67.9	93.4	182.2
Desiree	23.5	63.6	191.5

4.5 Effect of storage on PPO activity at different temperatures

In the case of tuber tissue, initial increase in the PPO activity and then decrease was observed. The significant increase in specific activity of PPO was observed from 0 to 60 days of storage. The specific activity ranged from 1640 to 6822 U/ mg protein at room temperature and from 2710 to 9871 U/mg protein at 4ºC and 1835 to 27181 at -20ºC from 0 to 60 days of storage. The specific activity of PPO fluctuated during the whole storage period but increased till the 60th day of storage as observed from Fig. 10. Prolonged storage at −20ºC was associated with more accumulation of the enzymes as compared to 4ºC and room temperature as reported earlier by Mahmood et al., 2009.

A



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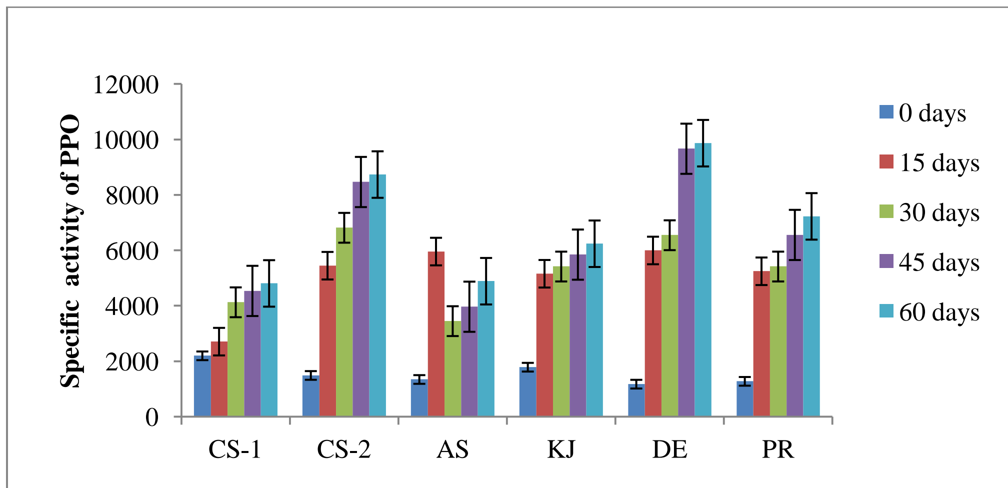

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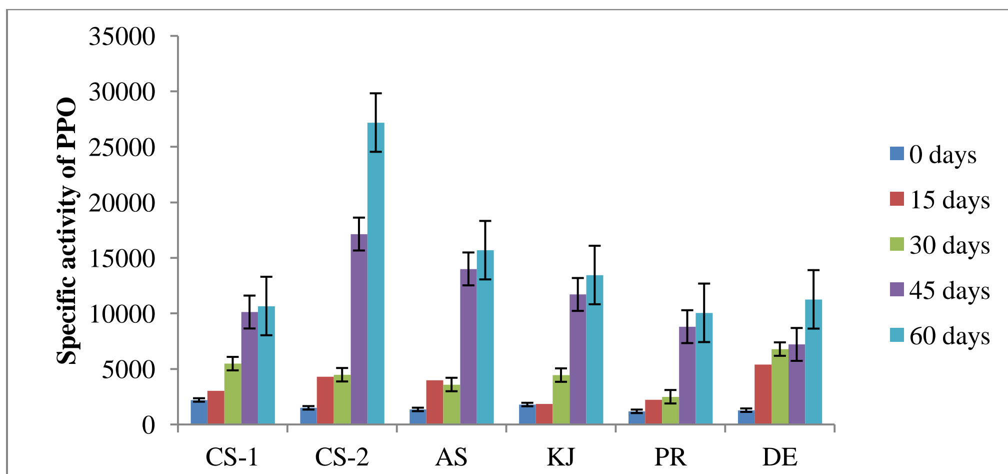
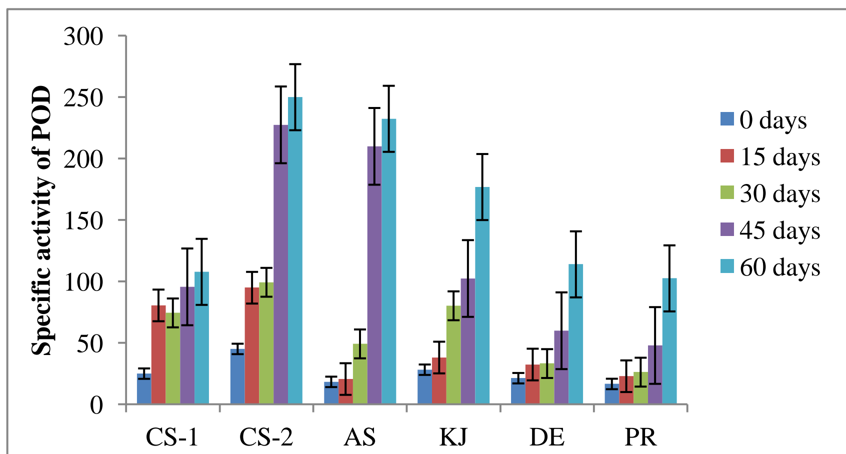


Fig 10: Specific activity of PPO at different storage temperatures. **A** room temperature, **B** 4ºC and **C** -20ºC.

4.5 Effect of storage on POD activity at different temperatures

In the case of tuber tissue, initial increase in the POD activity and then decrease was observed. The significant ncrease in specific activity of POD was observed from 0 to 60 days of storage. The specific activity ranged from 23 to 232 U/ mg protein at room temperature and from 17.4 to 296 U/mg protein at 4 ºC and 24.3 to 337 at -20 ºC from 0 to 60 days of storage. The specific activity of PPO fluctuated during the whole storage period but increased till the 60th day of storage as observed from Fig. 11. Prolonged storage at −20ºC was associated with more accumulation of the enzymes as compared to 4ºC and room temperature. (Mahmood et al., 2009).

A



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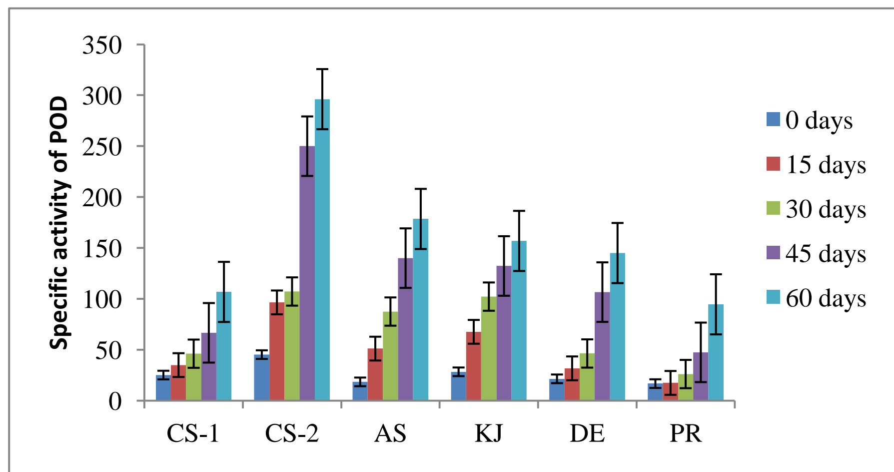

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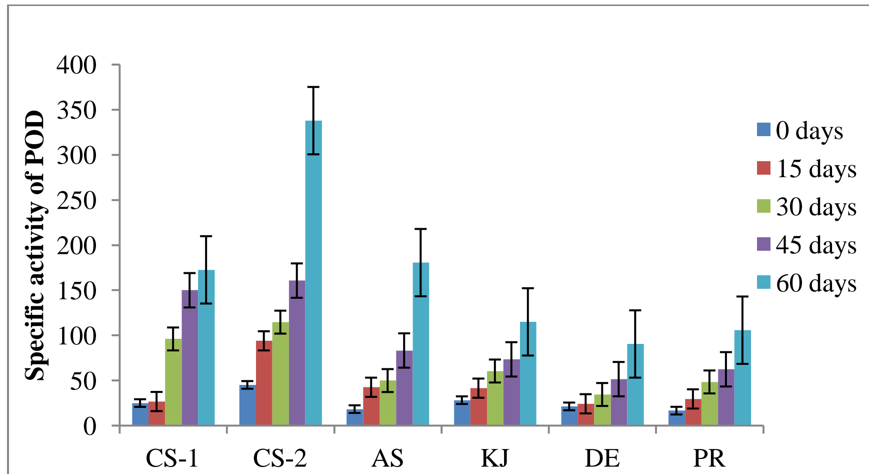

Fig 11: Specific activity of POD at different storage conditions. **A** room temperature, **B** 4ºC and **C** -20ºC.

4.5 Three phase partitioning (TPP)

As evident from literature (Vetal et al., 2015), TPP is easy, most reliable, modest and convenient method of protein purification. The focus was on mainly two parameters such as ammonium sulphate concentrations and crude to t-butanol ratios. 40% ammonium sulphate concentration and 1:1 crude to t-butanol ratio worked effectively for PPO where, the enzyme was purified up to 1.64 fold (with 96% recovery) and 2.73 fold (with 86% recovery) for the cultivars CS-1 and DE, respectively. Whereas 50% ammonium sulphate concentration and 1:0.67 crude to t-butanol ratio worked well for POD. In this case, the enzyme was purified up to 3.21 fold (with 95% recovery) and 1.98 fold (with 134% recovery) for the cultivars CS-1 and DE, respectively.

Table 14: Purification and recovery profile of Polyphenol oxidase (PPO) from mature organs of the potato cultivars by three phase partitioning TPP

Potato Cultivars (Tuber)	Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
CS-1	Crude extract	330	0.159	2075	1.00	100
	Interfacial phase of TPP	320	0.094	3404	1.64	96
DE	Crude extract	220	0.154	1479	1.00	100
	Interfacial phase of TPP	190	0.042	4047	2.73	86

Table 15: Purification and recovery profile of Peroxidase (POD) from mature organs of the potato cultivars by three phase partitioning (TPP)

Potato Cultivars (Tuber)	Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
CS-1	Crude extract	6.5	0.159	80.6	1.00	100
	Interfacial phase of TPP	6.2	0.094	259.2	3.21	95
DE	Crude extract	5.2	0.154	92.7	1.00	100
	Interfacial phase of TPP	7.0	0.042	184.2	1.98	134

-

4.5.1 Effect of Ammonium Sulphate concentration on PPO partitioning

As shown in Fig. 12, it was found that maximum purification of enzyme (PPO) was attained in 40% ammonium sulphate concentration. At this concentration, the enzyme was purified up to 1.64 fold (with 96% recovery) and 2.73 fold (with 86% recovery) for the cultivars CS-1 and DE, respectively. With the increase in concentration of ammonium sulphate up to (0-80%), partitioning and purity of enzyme decreases. This could be due to protein precipitation and its irreversible denaturation at higher ammonium sulphate concentration. Therefore, lowest purification fold and activity recovery was found higher at ammonium sulphate concentration (80%). This is in concordance with the studies of Niphadkar et al., 2015.

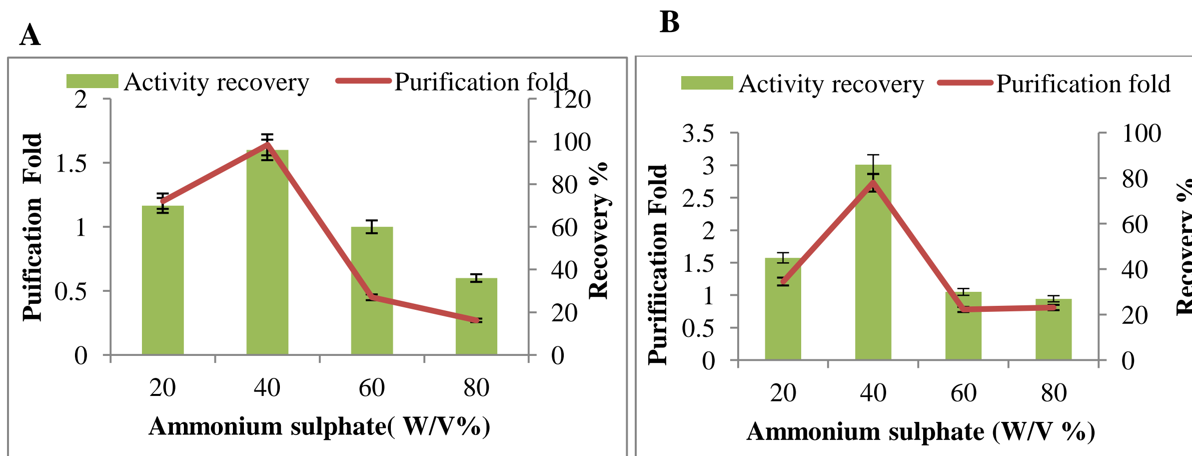

Fig 12: Effect of Ammonium sulphate concentration on purification of PPO. **A** CS-1 and **B** DE.

4.5.2 Effect of crude to t-butanol ratio on PPO partitioning

From Fig. 13, it was observed that t-butanol plays important role in enzyme partitioning. Small amount of t-butanol is unable to partition the protein properly whereas higher amount of t-butanol leads to denaturation of protein as in the studies done by Niphadkar et al., 2015. Therefore, at 1:1 ratio, the enzyme was purified up to 1.64 fold (with 96% recovery) and 2.73 fold (with 86% recovery) for the cultivars CS-1 and DE, respectively.

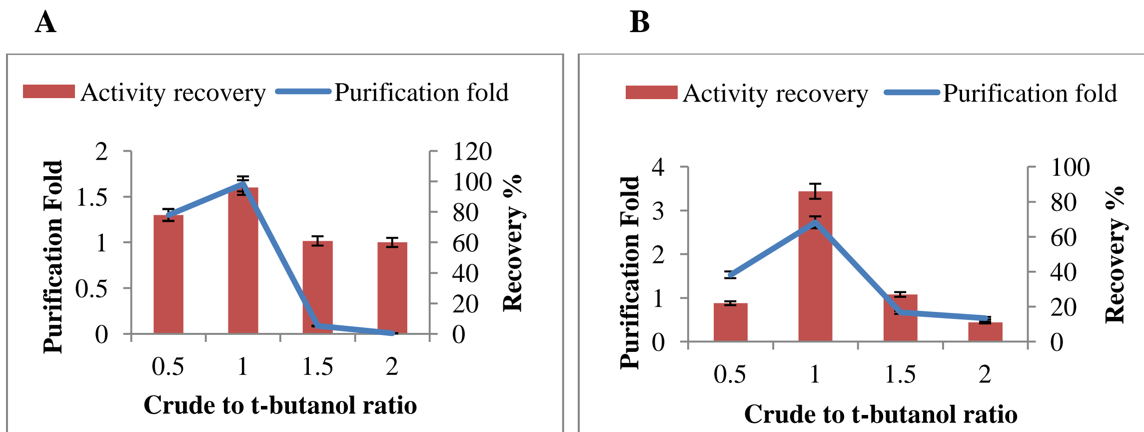

Fig 13: Effect of crude to t-butanol ratio on purification of PPO. **A** CS-1 and **B** DE

4.5.3 Effect of Ammonium Sulphate concentration on POD partitioning

As shown Fig 14, it was found that maximum purification of enzyme (POD) was attained in 50% concentration of ammonium sulphate. In this case, the enzyme was purified up to 3.21 fold (with 95% recovery) and 1.98 fold (with 134% recovery) for the cultivars CS-1 and DE, respectively.

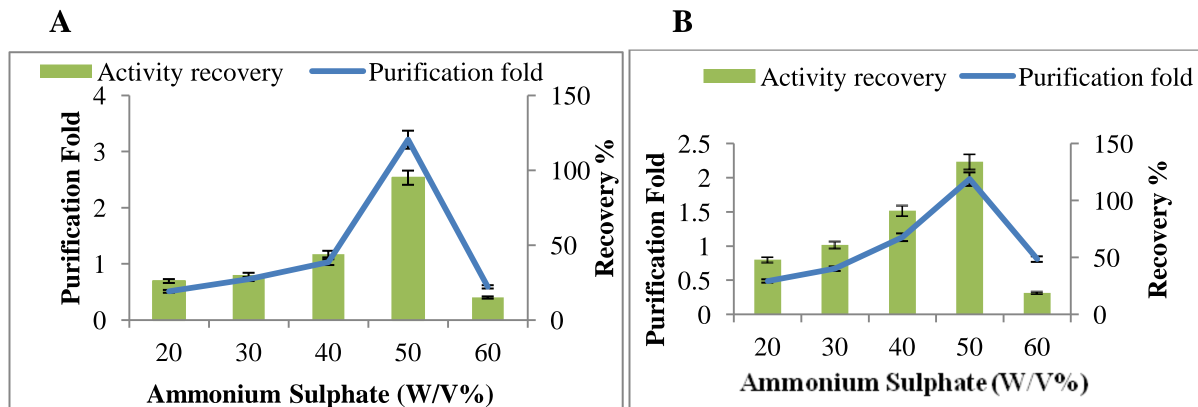

Fig 14: Effect of Ammonium Sulphate concentration on purification of POD. **A** CS-1 and **B** DE

4.5.4 Effect of crude to t-butanol ratio on POD partitioning

Fig. 15, showed that t-butanol plays important role in enzyme partitioning Small amount of t-butanol is unable to partition the protein properly whereas higher amount of t-butanol leads to

denaturation of protein (Vetal et al., 2015). At 1:0.67 ratio, the enzyme was purified up to 3.21 fold (with 95% recovery) and 1.98 fold (with 134% recovery) for the cultivars CS-1 and DE, respectively.

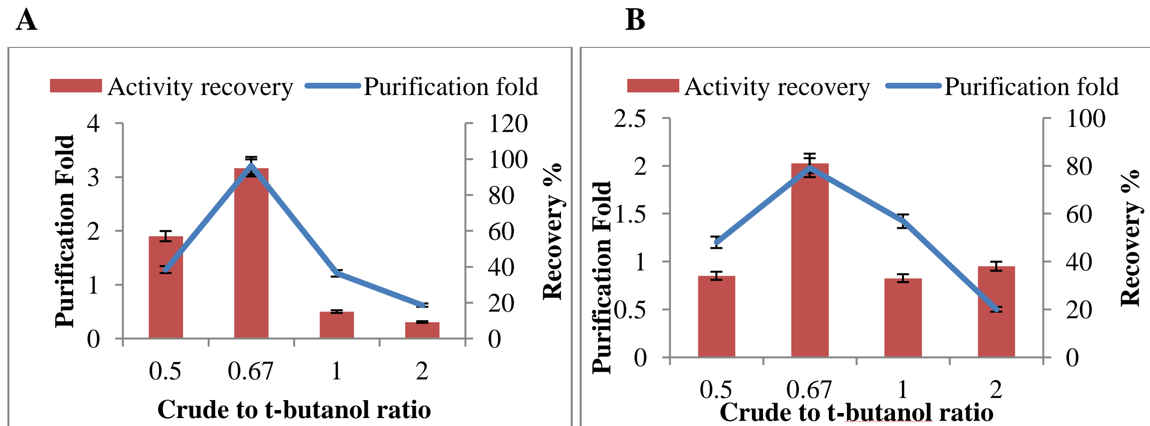

Fig 15: Effect of crude to t- butanol ratio on purification of POD. **A** CS-1 and **B** DE

4.6 Denaturing Polyacrylamide gel electrophoresis

TPP- purified extract of PPO and POD were analyzed by denaturing SDS page. The purified PPO was found to be approximately 20 kDa whereas POD was found to be approximately 24 kDa and is in concordance with as reported in literature (Niphadkar et al., 2015; Vetal et al., 2015).

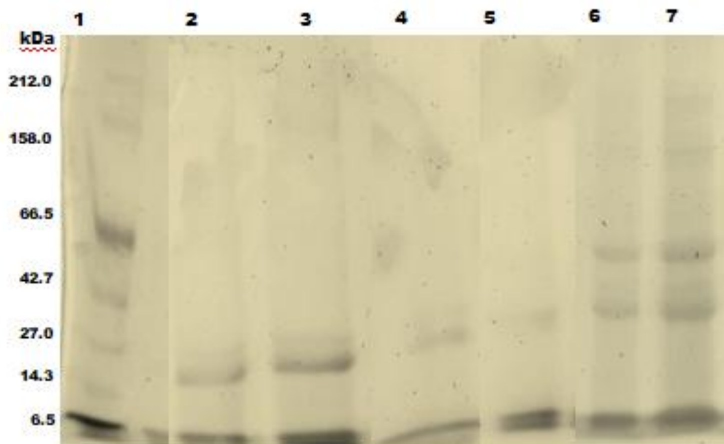

Fig. 16: Denaturing SDS-PAGE of TPP-purified PPO and POD from potato tubers. Lane 1, protein molecular weight markers; Lanes 2 & 3, purified PPO from potato tuber of CS-1 and DE, respectively; Lanes 4 & 5, purified POD from potato tuber of CS-1 and DE, respectively; Lanes 6 & 7, crude extracts from potato tuber of CS-1 and DE, respectively.

4.7 Conclusions

On the basis of experimental data the following conclusions were made:

PPO the main browning enzyme was found to be highest in the mature tubers.

POD, commonly regarded as 'marker of maturity', was found to be highest in mature tubers. Our observation was consistent with other reports.

Both PPO and POD were found to accumulate significantly in the tubers during storage at –20ºC. Prolonged storage (for a period of 60 days) at this temperature showed considerable variation in the enzyme activities probably due to their varying genotypes. This observation was consistent with earlier reports.

Three phase partitioning (TPP) method was found to be the effective and convenient for the purification of the aforesaid enzymes.

The parameters like 40% ammonium sulphate concentration and 1:1 crude to t-butanol ratio worked effectively during purification of PPO.

Likewise, in the case of POD there was a slight change in the parameters such as 50% ammonium sulphate concentration and 1:0.67 crude to t-butanol ratio were adopted.

Overall, such data could be useful to clearly distinguish between the potato cultivars in terms of the antioxidative capacities and eventually for the improvement of this non-grain food crop.

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