STUDY ON ANTIBROWNING AGENTS AND THEIR EFFECT ON ACTIVITY OF POLYPHENOL OXIDASE (PPO) IN FRUITS AND VEGETABLES

A PROJECT REPORT

Submitted by

DEEPIKA.P

Register No: 1020203002

in partial fulfillment for the award of the degree

of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY,

(An Autonomous Institution affiliated to Anna University Of Technology, Coimbatore)

COIMBATORE-49

APRIL 2012

KUMARAGURU COLLEGE OF TECHNOLOGY

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DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitiled

STUDY ON ANTIBROWNING AGENTS AND THEIR EFFECT ON ACTIVITY OF POLYPHENOL OXIDASE (PPO) IN FRUITS AND VEGETABLES

is the bonafide record of project work done by

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of M.Tech (Biotechnology) during the year 2011-2012

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Project Guide **Head of the Department**

Submitted for the project Viva-Voce examination held on .....

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DECLARATION

I affirm that the project work titled "**STUDY ON ANTIBROWNING AGENTS AND THEIR EFFECT ON ACTIVITY OF POLYPHENOL OXIDASE (PPO) IN FRUITS AND VEGETABLES**" being submitted in partial fulfilment for the award of M.Tech (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

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ACKNOWLEDGEMENT

I wish to express my sincere thanks to **Dr.S.Ramachandran**, Principal, Kumaraguru College of Technology, Coimbatore, **Dr. A. Manickam** Head of the Department, Biotechnology, Kumaraguru College of Technology. Coimbatore.

I wish to express my warm and sincere gratitude to my guide **Dr.Vinohar Stephen Rapheal**, Associate Professor, Department of Biotechnology, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

I would like to extend my special thanks to **Dr.J.Aravind**, Assistant Professor (SRG), **Dr.T.Sathish Kumar,** Assistant Professor (SRG), **Mr.M.Shanmuga Prakash**, Assistant Professor (SRG), **Mr.S.Sivamani**, Assistant Professor (SRG) , Department of Biotechnology, for their expertise, understanding and patience, and for the assistance they provided at all levels of the research project.

I am grateful to the teaching and non-teaching staff members of the Department of Biotechnology for their kind and patient help throughout the project.

Above all, I am thankful to the Almighty for his blessings without which nothing would have been possible.

Deepika.P

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Abstract....

ABSTRACT

Polyphenol oxidase (PPO) belonging to the class phenolase or catecholase, is a copper containing mono oxygenase enzyme. It catalyzes the ortho hydroxylation of mono phenols to o-diphenols (cresolase activity) and oxidation of o-diphenols to o-quinones (catecholase activity) which are molecular oxygen dependent. The quinone products can then polymerize and react with amino acid groups of cellular proteins, resulting in black or brown pigment deposits. PPO is the major cause of enzymatic browning in higher plants. Activities of polyphenol oxidase (PPO) was compared between different species of plants, and banana peel PPO was chosen for further study. Immobilizations in calcium alginate, polyacrylamide gel and gelatin were carried out and stability and reusability of immobilized PPO was studied. It was seen that reusability of PPO was upto 4 reaction cycles and was more stable in immobilized form. Inhibitory effect of various antibrowning agents such as cinnamic acid, salicylhydroxamic acid (SHAM), L-ascorbic acid, L-cysteine, ferulic acid, sodium azide, sodium benzoate, benzoic acid, citric acid and potassium metabisulphite on enzyme activity were studied and found to be in a dose dependent manner. Inhibition kinetics of antibrowning agents was studied and the IC50 values were determined. The efficiency of a combination of inhibitors were tested on banana peel PPO. The most effective agents were determined as potassium metabisulphite, L-ascorbic acid and citric acid. Response surface methodology (RSM) was used to evaluate the potency of potassium metabisulphite, L-ascorbic acid and citric acid combination for the control of enzymic browning. The potassium metabisulphite, L-ascorbic acid, and citric acid combination provided better results than the individual compounds. The optimum combination was determined as 0.04mM potassium metabisulphite, 0.1mm L-ascorbic acid and 8mM citric acid. Various antibrowning methods including blanching, microwaving, optimum antibrowning agent combination application and combination of the above methods were employed. Microwaving and blanching along with inhibitor treatment and inhibitors sole treatment was found efficient for controlling enzymic browning in fruit and vegetable juices.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
1D	One dimensional
2D	Two dimensional
AEAC	Ascorbic acid equivalent antioxidant capacity
a_w	Reduced water activity
B3LYP	*Becke-3-Lee-Yang-Parr*
BA	Benzoic acid
BBD	Box-Behnken design
BSA	Bovine Serum Albumin
c.f.	Competitive flora
C.V	Cumulative variation
CFP	N-p-coumaroyl- N□ -feruloyl putrescine
CNSL	Cashew nut shell liquid
$CuSO_4.5H_2O$	Coppersulphate pentahydrate
DCP	N,N□-dicoumaroyl-putrescine
DFP	N, N□ -diferuloyl-putrescine
DFT	Discrete fourier transform
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
DVB	Divinyl benzene
EDTA	Ethylene diamine tetraacetic acid
E_h	Reduced redox potential
ELISA	Enzyme-linked immunosorbent assay
EO	Essential oils
F	High temperature
GMA	Glycidyl methacrylate

LIST OF ABBREVIATIONS (cont...)

GRAS	Generally regarded as safe
H_2O_2	Hydrogen peroxide
HHP	High hydrostatic pressure
IC_{50}	Half inhibitory concentration
IR	Infrared
$kcal.mol^{-1}$	kilocalories/mole
K_i	Inhibition constant
K_m	Michaelis Menton constant
KOG	Kaempferol-3-O-(2□□-O-galloyl-β-D-glucopyranoside)
k_{opi}	Operational inactivation rate constant
L-DOPA	L-3,4 Dihydroxyphenylalanine
MAE	Microwave assisted extraction
MePAPh	4-[(4-Methylphenyl) azo]-phenol
mM	Millimolar
MMA	Methyl methacrylate
MPa	Megapascal
MT	Mushroom tyrosinase
MW_r	Molecular weight
NaCl	Sodium chloride
$NaCO_3$	Sodium carbonate
NaOH	Sodium hydroxide
NMR	*Nuclear Magnetic Resonance*
Ns	Nanosecond
PA	Phthalic acid
PAGE	PolyAcrylamide Gel Electrophoresis
pH	Increased acidity

LIST OF ABBREVIATIONS (cont...)

POD	Peroxidase
PPO	Polyphenol Oxidase
Pres.	Preservatives
Pt	Platinum
PVATh	Pyrrole with vinyl alcohol with thiophene side groups
PVPP	Polyvinylpolypyrollidone
QOG	Quercetin-3-O-(2□□-O-galloyl-β-D-glucopyranoside)
RSM	Response surface methodology
SARs	Structure–activity relationships
SB	Sodium benzoate
SE	Soxhlet extraction
SHAM	Salicylhydroxamic acid
SO_2	Sulphur dioxide
Std. Dev.	Standard deviation
T	Low temperature
TPC	Total phenolic content
U	Enzyme activity unit
U/mg	Enzyme unit/milligram protein (specific activity unit)
UV– VIS	Ultraviolet-Visible
V_{max}	Limiting velocity

Introduction....

CHAPTER 1

INTRODUCTION

1.1 GENERAL

Polyphenoloxidases (monophenol dihydroxyphenylalanine : oxidoreductase: E.C. 1.14.18.1; PPO) are widely distributed among prokaryotes and eukaryotes. This PPO is a copper containing metalloprotein that catalyzes the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenol monooxygenase, tyrosinase, cresolase) and the oxidation of o-dihydroxyphenols to o-quinones (E.C.1.10.3.2; diphenol oxygen oxidoreductase, diphenol oxidase, or catecholase) (Fig. 1) (Sathyanarayana *et al*., 2011). Due to a strong electrophilic nature, o-quinones undergo autooxidation, polymerization with nucleophilic constituents of proteins and other secondary reactions, all of which contribute to browning observed in various tissues and food products (Anderson *et al*., 2003). Structural and comparative studies have identified conserved regions in which histidine residues bind 4 copper atoms located at 2 main sites that are involved in catalytic activity.

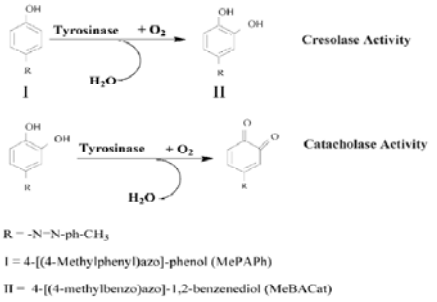

Fig 1.1: Cresolase (mono-oxygenase) and catecholase (oxidase) activities of tyrosinase. "I"and "II" represent the synthetic substrates used for assaying the cresolase and catecholase activities , respectively (Haghbeen *et al*., 2004)

The browning of fruits and vegetables has been of interest to research for years, partially due to its possible role in the plants defence mechanisms, as well as the economic concerns of the food industry resulting from the discolouration and loss of nutritional content. The process of browning involves the enzyme - catalysed synthesis of a dark pigment named melanin (Zhang, 2006). During melanin formation, tyrosinase catalyses the first two reactions: the conversion of tyrosine to dihydroxyphenylalanine (dopa), and the oxidation of dopa to *o*-dopaquinone (Fig.2).

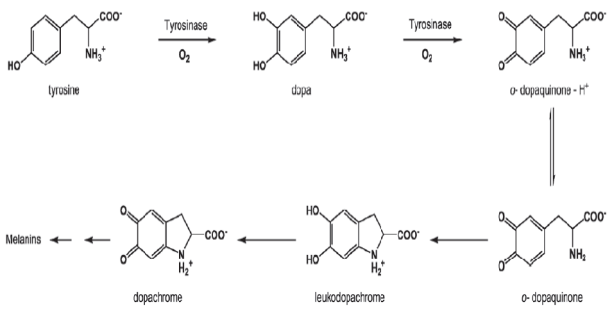

Fig 1.2: The mechanism of melanin synthesis

1.2 IMMOBILIZATION OF POLYPHENOL OXIDASE (PPO)

A biocatalyst is termed as immobilized if its mobility has been restricted by chemical or physical means, while retaining the catalytic activity. The environments where the enzymes are present affect the enzyme in several ways:

a) Partitioning effect
b) Diffusion limitation
c) Conformational changes
d) Steric restrictions
e) Inactivation

PPO has been widely studied in various fruits and vegetables such as potato tuber, peach, apple, apricot, banana, grape, pear, olive, strawberry, plum, kiwi and mango. Tyrosinases (monophenol, L-DOPA:oxygen oxidoreductase) are polyphenol oxidases (PPOs) that belong to a group of non-blue copper proteins. The other important group of PPOs are the blue-copper proteins, named laccases (EC 1.10.3.2), because their first description was in the lacquer tree. Laccases are multicopper proteins characterized by the presence of 3 different types of copper in the molecule, whereas tyrosinases only have a pair of type III coppers (Messerschmidt *et al*., 1990).

The activity of PPO varies between fruits and vegetables (Nunez- Delicado *et al*., 2005). PPO extracted from most plants and fruits is capable of oxidizing *o*-diphenol, while mushroom PPO can catalyze both mono- and *o*-diphenol oxidation (Cash *et al.*, 1976). Because PPO-catalyzed oxidative reaction in fruits and vegetables could negatively affect the appearance, its presence in fresh products could reduce their shelf life and consumer acceptance, and therefore, their economical value (Wang *et al*., 2007).

PPO action mechanism is based on its capacity to oxidize phenolic compounds. In the fruits cells, the enzyme is located inside plastids while potential substrates are stored in vacuoles, and this separation in different organelles limits enzymatic browning. Only in degenerating or senescent tissue does it occur free in the cytoplasm (Wuyts *et al*., 2006). In post-harvest evolution, fruits undergo some physiological changes that lead to a decreased ability to eliminate active oxygen. Thus, membranes become more affected by oxidative activity, which results in enhanced lipid peroxidation, reduced membrane fluidity, and increased membrane permeability. After these transformations, or when the tissue is damaged, the rupture of plastids leads to the enzyme coming into contact with these phenolic compounds (Mayer *et al*., 1979; Jiang *et al*., 2004). The wide range of polyphenol oxidase substrate specificity has led to many methods being proposed to measure its activity: radiometric, electrometric, chronometric, and especially spectrophotometric, which are fast and affordable by most laboratories (García-Molina *et al*., 2007; Falguera *et al*., 2010).

Enzymatic browning is considered one of the main causes of post-harvest fruit deterioration, along with pigment degradation and peroxidase activity. In fact, PPO activity is still the major practical limitation to fruit handling, storage, and processing (Jiang *et al*., 2004). In addition, browning processes affect the nutritional quality and appearance, reduce consumer's acceptability, and cause significant economic impact, both to food producers and to food processing industry. In this way, tropical and subtropical fruits and vegetables are the most susceptible to these reactions (Queiroz *et al*., 2008).

1.2.1 REASONS FOR ENZYME IMMOBILIZATION

Immobilized enzymes offer a considerable operational advantage over free enzymes. Immobilization makes it possible to achieve and maintain a high enzymatic activity in a small volume. Therefore, there is a reduction in the reaction time. Reusability, enhanced stability, possibility of batch or continuous operational modes, rapid termination of reactions, controlled product formation, possible greater efficiency in consecutive multistep reactions are advantages of enzyme immobilization. Furthermore, efficient separation of the biocatalyst from the products is possible via immobilization, which is very advantageous in industrial point of view. The use of enzyme repeatedly and continuously can be achieved by means of immobilization. Another aspect of immobilization process is being highly practical for automated analysis.

1.2.2 METHODS OF IMMOBILIZATION

A classification based on the method of immobilization covers the most important known form of immobilized biocatalysts. Immobilization methods are divided into two main categories: binding and physical retention methods

- Binding
  - Binding to carriers
  - Adsorption
  - Ionic Binding
  - Covalent Binding
- Crosslinking
  - Co-crosslinking
- Physical Retention
  - Matrix Entrapment
  - Gel Entrapment
  - Fiber Entrapment
  - Membrane Enclosure
  - Microencapsulation
  - Liposome Technique
  - Membrane Reactors

1.3 EFFECT OF ANTIBROWNING AGENTS

Enzymatic browning of raw commodities may result from physiological injury; senescence; pre- or postharvest bruising; disruption of the fruit or vegetable flesh by peeling, coring, slicing, or juicing; tissue disruption from freeze–thaw cycling; and tissue disruption by bacterial growth. The occurrence of enzymatic browning can limit the shelf-life of fresh-cut fruits and salad vegetables, fresh mushrooms, prepeeled potatoes, and other fresh products of commercial importance (Huxsoll *et al*., 1989). This problem has held back the development and commercialization of fresh-cut fruits such as sliced apples. Enzymatic browning also may be a problem with some dehydrated and frozen fruits and vegetables (Shewfelt, 1986; Hall, 1989). Enzymatic browning is usually controlled by blanching (McCord and Kilara, 1983; Hall, 1989; Ma *et al*., 1992); acidification; and application of sulfites or sulfite substitutes such as ascorbic acid or cysteine and other reducing agents (Fig 3). These substitutes are generally less effective than sulfites.

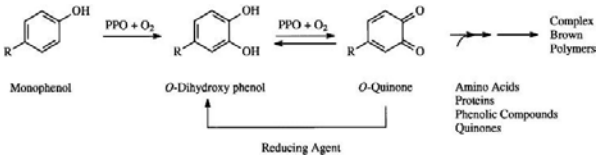

Fig 1.3: Enzymatic browning reaction, showing action of reducing agents as browning inhibitors (From Sapers, 1993)

PPO is relatively heat labile, and PPO-catalyzed reactions can be inhibited by acids, halides, phenolic acids, sulfites, chelating agents, reducing agents such as ascorbic acid and dithiothreitol, quinone couplers such as cysteine, and substrate binding compounds such as polyvinylpolypyrollidone (PVPP) and β-cyclodextrin. With some of these compounds, the inhibitory effect is directly on PPO, but in most cases the effect is to inhibit the browning reaction by removing substrates or blocking further reaction of intermediates.

The most widespread agents used for control of browning are sulfiting agents (Ozoglu *et al*., 2002). Due to adverse health effects, several studies have been devoted to the nonsulfite antibrowning agents such as reducing agents (ascorbic acid and analogs, glutathione, L-cysteine), enzyme inhibitors (aromatic carboxylic acids, substituted resorcinols, anions, peptides), chelating agents (phosphates, EDTA, organic acids), acidulants (citric acid, phosphoric acid), complexing agents (cyclodextrins) and enzymes (Labuza *et al*., 1992; Lambrecht, 1995; Martinez and Whitaker, 1995; McEvily *et al*., 1992; Molnar-Perl and Friedman, 1990a, 1990b; Monsalve-Gonzalez *et al*., 1993; Moon et al., 1999; Nicolas *et al*., 1994; Vamos-Vigyazo, 1995; Walker, 1995).

Walker and Wilson (1975) investigated inhibition of apple PPO by a number of phenolic acids. Walker (1976) reported that cinnamic acid at concentrations greater than 0.5 mM prevented browning of Granny Smith apple juice for 7 hours. Kojic acid [5-hydroxy-2-(hydroxymethyl)-□-pyrone] has been considered for use as an alternative to sulphite (Shi *et al*., 2005). This compound can reduce quinones to polyphenols, thereby preventing browning by the same mechanism as ascorbic acid (Chen *et al*., 1991). Tong and Hicks (1991, 1993) reported that carrageenans and other sulfated polysaccharides showed browning inhibitor activity in apple juice and diced apples; citric acid acted synergistically with these compounds in inhibiting browning. The mechanism of browning inhibition by the sulfated polysaccharides is not known. Xu *et al*., (1993) reported that maltodextrin inhibited browning of ground apple. Natural PPO inhibitors have been found in honey (Oszmianski and Lee, 1990), pineapple (Lozano de Gonzalez *et al*., 1993; Wen and Wrolstad, 1998), fig latex (McEvily, 1991) and a large number of botanical products (Choi *et al*., 1997).

1.4 RESPONSE SURFACE METHODOLOGY (RSM)

Enzymic browning control by agents can be classified into three categories, depending on whether they mainly affect reaction products, substrates or polyphenol oxidase. o-Quinones are reactive primary products and they can be either reduced back to o-diphenols or trapped as colourless addition compounds. However, secondary products resulting mainly from the oxidative polymerization of o-quinones often give highly coloured compounds that become less reactive as the browning reaction proceeds. Therefore, many of the compounds that act on o-quinones were investigated. Ascorbic acid is most frequently used for browning control of food products. Among sulfur-containing agents, L-cysteine is an effective compound to prevent enzymic browning. Aromatic carboxylic acids are inhibitors of polyphenol oxidase due to their structural similarities with the phenolic substrates and are reported to be effective antibrowning agents for providing long-term inhibition (McEvily *et al*., 1992; Nicolas *et al*., 1994; Sapers *et al*., 1989).

These differences in the mechanism of inhibition may allow the use of combinations of antibrowning agents that may result in enhancement of inhibition. Most combinations of antibrowning agents are ascorbic acid-based compositions (Pizzocarno *et al*., 1993). Mixtures of ascorbic and cyclodextrins were reported to be effective in the inhibition of apple juice browning (Hicks *et al*., 1990). It was found that cinnamate and benzoate enhanced the effectiveness of ascorbic acid and ascorbic acid derivatives as browning inhibitors in apple juice. Combinations of ascorbic acid with an acidic polyphosphate (ascorbic acid-2-phosphate and -triphosphate) were found highly to be effective in apple juice (Sapers *et al*., 1989).

1.5 APPLICATION OF ANTIBROWNING METHODS

The enzymatic browning reaction is the most severe when the structure of food has been altered or damaged by processing, and is usually an undesirable phenomenon that results in a decreased value and reduced acceptance by consumers. For this reason, extensive research has been done on ways to prevent or control enzymatic browning (Eissa *et al*., 2008). The raw juice can be protected from microbiological degradation for a few days by storage in a refrigerator, or may be protected indefinitely by pasteurization or by the use of permitted preservatives. Such juice is nearly always turbid, brown in colour and tends to sediment on storage (Lea, 1994). While application of conventional chemical preservatives such as benzoic and sorbic acids and sulfite to fruit juices is an alternative, the practice is not common as indeed such preserved fruit juices and the chemicals are often imported. The potential application of hurdles such as cold storage, mild heating and H_2O_2 disinfection for preservation were investigated.

1.5.1 HURDLE TECHNOLOGY

Since many years foods have been preserved by traditional methods such as adding chemical preservatives, canning, freezing, drying, chilling, fermentation, etc. Most traditional methods of food preservation provide sufficient safety by effectively killing or preventing the growth of pathogenic and spoilage microorganisms. However, when they applied alone almost all of them cause some changes in the sensory attributes of food such as texture, flavor and color. Also, the use of chemical preservatives at high concentrations causes some health concerns and reduces the consumer acceptance of foods. Thus, in recent years many efforts have been spent to develop some alternative preservation technologies that provide sufficient microbial safety, maintain the sensory attributes and minimize health concerns of consumers. Hurdle technology has appeared as a result of these intensive studies.

Factors used for food preservation are called '*hurdles*' and there are numerous hurdles that have been applied for food preservation. Potential hurdles for use in the preservation of foods can be divided into physical, physicochemical, microbially derived and miscellaneous hurdles (Leistner and Gorris, 1995) (Table 1).

Table 1.1: Examples of hurdles used to preserve foods (adapted from Ohlsson and Bengtsson, (2002)

Type of hurdle	**Examples**
Physical hurdles	Aseptic packaging, electromagnetic energy (microwave, radio frequency, pulsed magnetic fields, high electric fields), high temperatures (blanching, pasteurization, sterilization, evaporation, extrusion, baking, frying), ionic radiation, low temperature (chilling freezing), modified atmospheres, packaging films (including active packaging, edible coatings), photodynamic inactivation, ultra-high pressures, ultrasonication, ultraviolet radiation
Physico-chemical hurdles	Carbon dioxide, ethanol, lactic acid, lactoperoxidase, low pH, low redox potential, low water activity, Maillard reaction products, organic acids, oxygen, ozone, phenols, phosphates, salt, smoking, sodium nitrite/nitrate, sodium or potassium sulphite, spices and herbs, surface treatment agents
Microbially derived hurdles	Antibiotics, bacteriocins, competitive flora, protective cultures

In this technology carefully selected and combined preservative factors are applied to obtain the indicated benefits. There are more than 60 potential hurdles that may be used in this technology (Leistner, 2000). However, the most important hurdles used in food preservation are heating, water activity (aw), acidity (pH), redox potential, refrigeration and competitive microorganisms (e.g., lactic acid bacteria) (Table 2). The other hurdles include; oxygen tension (low or high), modified atmosphere (carbon dioxide, nitrogen, oxygen), pressure (high or low), radiation (UV, microwaves, irradiation), ohmic heating, pulsed electric fields, pulsed light, ultrasonication and new packaging (e.g., selective permeable films, advanced edible coating) methods.

Table 1.2: Most important hurdles for food preservation (adapted from Leistner, 1995)

Symbol	Parameter	Application
F	High temperature	Heating
T	Low temperature	Chilling, freezing
a_w	Reduced water activity	Drying, curing, conserving
pH	Increased acidity	Acid addition or formation
Eh	Reduced redox potential	Removal of oxygen or addition of ascorbate
Pres.	Preservatives	Sorbate, sulfite, nitrite
c.f.	Competitive flora	Microbial fermentations

Some hurdles are very effective and they may influence both the microbiological safety and flavor of foods positively when used properly. However, the same hurdles, when their intensity is increased too much, may cause a negative effect on the foods. Thus, considering the safety and quality, it is very critical to keep hurdles at the optimum range. The kind of hurdle differs according to the type of food. One or set of hurdles may be used to obtain high quality and food safety by keeping the normal population of the microorganisms under control.

***Blanching*:** Blanching is a unit operation prior to freezing, canning, or drying in which fruits or vegetables are heated for the purpose of inactivating enzymes; modifying texture; preserving color, flavor, and nutritional value; and removing trapped air. Hot water and steam are the most commonly used heating media for blanching in industry, but microwave and hot gas blanching have also been studied (Jose *et al*., 2004). Blanching is an old and well-established practice in the food industry. Early technological improvements focused on increasing product quality. Later, process efficiency in terms of product throughput, energy efficiency, and waste effluent reduction has been the main concern. Targeting the right enzyme indicator would reduce blanching time and tackle all these priorities: improving product quality (increasing retention of nutrients and other freshlike quality attributes), reducing energy consumption, and reducing waste production (Queiroz *et al*., 2008).

***Microwaving*:** Microwave heating is an alternative method for liquid food pasteurization. Compared to conventional heating methods, microwaves are able to heat products internally, have greater penetration depth and faster heating rates that would potentially improve retention of thermolabile constituents in the food (Deng *et al*., 2003; Heddleson *et al*., 1994) Microwave energy induces thermal effects over microorganisms and enzymes similar to those of conventional heating mechanisms (Canumir *et al*., 2002). Matsui *et al*., (2007) submitted solutions simulating the chemical constituents of coconut water to a batch process in a microwave oven and observed that PPO activity in water and in sugar solution was reduced after treatment. In salt solution, PPO stability was significantly affected and the contact between salt and enzyme promoted a drastic reduction of the initial activity. At temperatures above 90°C, the combined effects of salts and microwave energy reduced enzymatic activity to undetectable levels.

Objectives....

CHAPTER 2

OBJECTIVES

- To screen various sources for polyphenol oxidase (PPO) and to partially purify PPO by ammonium sulphate precipitation and study its activity.
- To study the effect of inhibitors acting as antibrowning agents on PPO activity.
- To optimize the best combination of antibrowning agents by response surface methodology (RSM).
- To test the efficiency of optimized combination of antibrowning agents applied with other antibrowning methods in fruits and vegetables.

Review of literature....

CHAPTER 3

REVIEW OF LITERATURE

3.1. GENERAL

Aniszewski *et al*., (2008) reviewed that polyphenol oxidase (PPO) includes tyrosinase or monophenol monooxygenase or cresolase (EC 1.14.18.1), laccase (EC 1.10.3.1), and catecholase (EC 1.10.3.2) that are distributed widely in nature whose mechanism and biological functions are not well known. Copper is present in trace amounts in living organisms to facilitate electron transfer. Spectroscopic studies revealed three types of copper containing proteins in that tyrosinase is a type three protein.

Land *et al*., (2008) stated that the reactivity of the *ortho*-quinone products of tyrosinase oxidation of catechols prompted the suggestion that the inactivation was the result of covalent binding to the enzyme and binding to tyrosinase has been demonstrated using radiolabelled substrates. However, the *ortho*-quinone product is also generated by oxidation of monohydric phenols and inactivation is not a feature of cresolase activity. Therefore a direct attack by the quinone product does not explain the inactivation of the enzyme.

Mayer (2006) reviewed that polyphenol oxidases or tyrosinases (PPO) are enzymes with a dinuclear copper centre, which are able to insert oxygen in a position ortho- to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. Molecular oxygen is used in the reaction. The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved. The enzyme seems to be of almost universal distribution in animals, plants, fungi and bacteria.

Seo *et al*., (2003) stated that mushroom tyrosinase has become popular because it is readily available and useful in a number of applications. The browning of mushrooms, fruits and vegetables is the result of the oxidation of phenolic compounds by tyrosinase when cells are broken or damaged. In fungi such as mushrooms, the role of tyrosinase and subsequent melanin production is related to reproductive organ differentiation and spore formation as well as protection of tissues after injury.

Yang *et al*., (2001) stated that the main PPO in banana peel is catechol oxidase (EC 1.10.3.1, o-diphenol: oxygen oxidoreductase). The enzyme showed two bands after staining with Coomassie brilliant blue on a PAGE gel, one very clear and the other faint.

3.2 LOCALIZATION OF POLYPHENOL OXIDASE (PPO)

Czaninski and Catesson (1974) studied enzyme histochemical localization using catechol for PPO activity staining. The principle of this reaction involves the development of an insoluble, electron dense reaction product from a synthetic substrate. A dense deposition of the PPO reaction product is readily visualized within the xylem cell walls and the parenchyma cell wall.

Czaninski and Catesson, (1974) experimented that in potato tuber, a heavy PPO reaction was observed in the thylakoids, vesicles and in the stroma when the specimen was stained with DOPA (dihydroxyphenylalanine).

Mayer and Harel (1978) stated that PPO in some higher plants is considered as a plastid enzyme localized in a diverse series of plastids such as the chloroplast and in the thylakoid membrane.

Obukowicz and Kennedy (1981) found that PPO enzymes have often been localized to the chloroplasts, where they are associated with the internal thylakoid membranes. They are also found in the cytoplasm and in vesicles between the plasmalemma and cell wall. Free phenolics are present mainly in the vacuole, but are synthesized in the cytoplasm (Vamos-Vigyazo, 1981; Walker and Ferrar, 1998), and perhaps may also become deposited in the cell walls.

Swain *et al.,* (1966); Kenten, (1958); Mayer and Friend, (1960); Moore and Flurkey, (1990) reported that polyphenol oxidase (PPO) is frequently a latent enzyme, which can be activated in vitro by a number of different factors and treatments such as detergents, proteases (Tolbert, 1973; King and Flurkey, 1987), low and high pH levels (Kenten, 1957) and exposure to fatty acids in the incubation mixtures (Golbeck and Cammarata,1981).

3.3 CATALYTIC PROPERTY OF POLYPHENOL OXIDASE (PPO):

Seigbahn (2003) studied the reaction mechanism for PPO using the hybrid DFT method B3LYP. Since no X-ray structure yet exists for PPO, the structural model used in the calculations was set up on the basis of similarities to other enzymes such as catechol oxidase and hemocyanin. Sequence similarities suggest that the copper dimer complex of tyrosinase has at least five histidines, but raises the possibility that the sixth ligand could be something else than a histidine. Stability of the enzyme was checked by storing the enzyme preparation at 4°C and by measuring their activity at 30°C periodically. Increase in storage time decreases the activity (Yang *et al*., 2006).

3.4 PARTIAL PURIFICATION OF POLYPHENOL OXIDASE (PPO)

Kwon *et al*., (1996) purified glycosylated PPO from potato by ammonium sulphate fractionization, sephadex G-100, and concanavalin A Sepharose column chromatography.

Seo *et al.*, (2003) reviewed that polyphenol oxidase (PPO) is found throughout the phylogenic tree and has been extracted from different sources such as fungi, fruits, and mammalian melanoma tumors. There are considerable reports indicating the great potential of this enzyme for food, medicine and agricultural industries as well as analytical and environmental purposes. PPO is used as biosensor as well as a key enzyme in the clinical studies, detection, determination and detoxification of phenolic compounds from industrial wastewater. For this purpose, simple procedure for purification of this enzyme is necessary (Haghbeen *et al*., 2004).

3.5 IMMOBILIZATION OF POLYPHENOL OXIDASE (PPO)

Arica *et al*., (2004) studied the immobilisation of tyrosinase onto modified poly(methyl methacrylate–glycidyl methacrylate–divinyl benzene), poly(MMA–GMA–DVB), microbeads. The activity yield of the immobilised tyrosinase on the spacer-arm-attached poly(MMA–GMA–DVB) microbeads was 68%, and this was 51% for the enzyme, which was immobilised on the aminated microbeads. Both immobilised tyrosinase preparation has resistance to temperature inactivation as compared to that of the free form. The operational inactivation rate constant (k_{opi}) of the immobilised tyrosinase was 1.25×10^{-5} min^{-1}.

Biegunski *et al*., (2006) prepared a conducting, polymeric film of poly(indole-5 carboxylic acid) by electrochemical polymerization for covalent immobilization of an enzyme belonging to the family of phenoloxidases-tyrosinase. The polymer was characterized by cyclic voltammetry, UV– VIS and Raman spectroscopy in a buffer solution. Immobilization of tyrosinase was confirmed by surface enhanced resonance Raman scattering spectra (SERRS) and by cyclic voltammetry as well. Tyrosinase was shown to retain its biological activity when being immobilized on the polymer surface. As proved by the electrochemical and spectroelectrochemical (UV–VIS) experiments, tyrosinase covalently bonded to the polymer matrix effectively catalyzes oxidation of catechol.

Duran *et al*., (2002) stated that polyphenol oxidase (PPO) enzyme can be immobilized suitably for various applications, including synthetic and analytical purposes, bioremediation, wastewater treatment, and must and wine stabilization. The oxygenated form consists of two tetragonal Cu (II) atoms, each coordinated by two strong equatorial and one weaker axial NH is ligands

Tembe *et al*., (2008) immobilized tyrosinase from a plant source *Amorphophallus companulatus* on eggshell membrane using glutaraldehyde. Membrane bound enzyme exhibited consistent activity in the temperature range 20–45 °C. Shelf life of immobilized tyrosinase system was found to be more than 6 months when stored in phosphate buffer at 4 °C. An electrochemical biosensor for dopamine was developed by mounting the tyrosinase immobilized eggshell membrane on the surface of glassy carbon electrode.

Yildiz *et al*., (2007) immobilized alcohol oxidase and tyrosinase by constant potential electrolysis at room temperature in a typical three-electrode cell containing PVATh coated platinum foil (1 cm^2) as working, bare Pt as counter and Ag/Ag^+ as reference electrodes. After electrolysis, enzyme electrodes were washed with distilled water in order to remove both excess supporting electrolyte and unbound enzyme and kept in citrate buffer at 4 ◦C when not in use.

Zamora *et al*., (2007) immobilized mushroom tyrosinase from an extract onto glass beads covered with the cross-linked totally cinnamoylated derivates of d-sorbitol (sorbitol cinnamate) and glycerine (glycerine cinnamate). The enantiomers l-tyrosine, dl-tyrosine, d-tyrosine,l-dopa, dl-dopa, d-dopa, l-α-methyldopa, dl-α-methyldopa, l-isoprenaline, dl-isoprenaline, l-adrenaline, dl-adrenaline, l-noradrenaline, and d-noradrenaline were assayed with tyrosinase immobilized on a chiral support (sorbitol cinnamate), whereas l-tyrosine, dl-tyrosine, d-tyrosine, l-dopa, dl-dopa, d-dopa, l-α-methyldopa, and dl-α-methyldopa were assayed with tyrosinase immobilized on a nonchiral support (glycerine cinnamate). No difference was observed in the stereospecificity of tyrosinase immobilized on a chiral (sorbitol cinnamate) or nonchiral (glycerine cinnamate) support.

3.6. EFFECT OF ANTIBROWNING AGENTS

Akhtar *et al*., (2011) determined the antioxidant potential as well as tyrosinase inhibitory activity of citrus fruit waste. Fruit peels of 4 species of genus citrus viz. *Citrus sinensis* (Malta), *C. reticulate* (Orange), *C. paradisi* (Grape fruit) and *C. aurantifolia* (Lemon), were analyzed for these bio-reactive properties. Tyrosinase inhibition capacity was evaluated by using kojic acid as standard tyrosinase inhibitor. Alcoholic fractions of orange, malta, and lemon showed 90, 87, and 69% tyrosinase inhibition respectively, while no tyrosinase inhibition was found in any fraction of grape fruit.

Arung *et al*., (2006) investigated 44 Indonesian medicinal plants belonging to 24 families. Among those plants, the extract of *Artocarpus heterophyllus* was one of the strongest inhibitors of tyrosinase activity. By activity-guided fractionation of *A. heterophyllus* wood extract, they isolated artocarpanone, which inhibited both mushroom tyrosinase activity and melanin production in B16 melanoma cells. This compound is a strong candidate as a remedy for hyperpigmentation in human skin.

Babendure *et al*., (2011) stated that tyrosinase is a copper-containing enzyme whose activity may be inhibited by a variety of natural and synthetic compounds. Natural inhibitors of mushroom tyrosinase include ascorbic acid, green tea, aloe vera, and some sulfur compounds.

Chan *et al.,* (2008) screened leaves of 26 ginger species belonging to nine genera and three tribes fir their total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC). For 14 species, TPC and AEAC of rhizomes were also assessed. Of the five species of *Etlingera*, leaves of *E. elatior* displayed the strongest tyrosinase inhibition activity, followed by leaves of *Etlingera fulgens* and *E. maingayi.* Values of their inhibition activity were significantly higher than or comparable to the positive control. Besides promising tyrosinase inhibition ability, leaves of these three *Etlingera* species also have high antioxidant activity and antibacterial properties.

Choi *et al*., (2007) evaluated the antioxidant activity of three major polyamine conjugates, N,N□-dicoumaroyl-putrescine (DCP), N-p-coumaroyl- N□ -feruloyl putrescine (CFP), and N, N□ -diferuloyl-putrescine (DFP) isolated from corn bran, and their related hydroxycinnamic acids, p-coumaric acid and ferulic acid by three antioxidant in vitro assay systems, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide and hydroxyl radicals generated by enzymatic and nonenzymatic reactions. DCP (IC_{50} = 181.73 μM) showed potent tyrosinase inhibitory activity toward L-tyrosine as the substrate, whereas DFP (IC_{50} = 733.64 μM) significantly inhibited melanin synthesis in B16 melanoma cells.

Choi *et al*., (2008) evaluated the methanol extracts of Cheonggukjang, fermented for various times for their free-radical-scavenging and tyrosinase inhibitory activities and the underlying mechanisms were elucidated The tyrosinase-inhibition activity increased with the length of fermentation. Therefore, the antioxidative and tyrosinase-inhibition activities exhibited by methanol-extracted Cheonggukjang samples may be attributable to the contents of total phenolic compounds.

Chun *et al*., (2011) evaluated the total polyphenol content and antityrosinase capacity of mulberry medicine extract for its development and utilization. Total polyphenols were extracted from mulberry medicine with 70% alcohol solution. The content of total polyphenols was determined by ultraviolet spectrophotometer and the capacity of antityrosinase was evaluated by using the enzyme-linked immunosorbent assay (ELISA) method. Results show that the content of polyphenols was up to 8.668 mg/g and tyrosinase half inhibitory concentration (IC_{50}) was 12.12 mg/ml. Kinetic study indicated that the mechanism of tyrosinase inhibition was mixed type inhibition.

Fais *et al*., (2009) resynthesized coumarin-resveratrol hybrids 1-8 to investigate the structure-activity relationships and the IC_{50} values of these compounds were measured. The results showed that these compounds exhibited tyrosinase inhibitory activity. Compound 3-(3',4',5'-trihydroxyphenyl)-6,8-dihydroxycoumarin (8) is the most potent compound (0.27 mM), more than umbelliferone (0.42 mM), used as reference compound. The kinetic studies revealed that compound 8 caused non-competitive tyrosinase inhibition.

Fuller *et al*., (2009) examined the effects of various inhibitors on crude, commercial and partially purified commercial mushroom tyrosinase by comparing IC_{50} values. Kojic acid, salicylhydroxamic acid, tropolone, methimazole, and ammonium tetrathiomolybdate had relatively similar IC_{50} values for the crude, commercial and partially purified enzyme. 4-Hexylresorcinol seemed to have a somewhat higher IC_{50} value using crude extracts, compared to commercial or purified tyrosinase. Some inhibitors (NaCl, esculetin, biphenol, phloridzin) showed variations in IC_{50} values between the enzyme samples. In contrast, hydroquinone, lysozyme, Zn^{2+}, and anisaldehyde showed little or no inhibition in concentration ranges reported to be effective inhibitors. Organic solvents (DMSO and ethanol) had IC_{50} values that were similar for some of the tyrosinase samples. Depending of the source of tyrosinase and choice of inhibitor, variations in IC_{50} values were observed.

Gheibi *et al*., (2009) studied the catecholase and cresolase activities of mushroom tyrosinase (MT) in presence of some n-alkyl carboxylic acid derivatives. Catecholase activity of MT achieved its optimal activity in presence of 1.0, 1.25, 2.0, 2.2 and 3.2 mM of pyruvic acid, acrylic acid, propanoic acid, 2-oxo-butanoic acid, and 2-oxo-octanoic acid, respectively. Contrarily, the cresolase activity of MT was inhibited by all type of the above acids. Propanoic acid caused an uncompetitive mode of inhibition (K_i = 0.14 mM), however, the pyruvic, acrylic, 2-oxo-butanoic and 2-oxooctanoic acids showed a competitive manner of inhibition with the inhibition constants (K_i) of 0.36, 0.6, 3.6 and 4.5 mM, respectively. So, it seems that, there is a physical difference in the docking of mono- and o-diphenols to the tyrosinase active site.

Khan (2007) discovered and reported a large number of mild to potent inhibitors of several classes, such as phenolics, terpenes, steroids, chalcones, flavonoids, alkaloids, long-chain fatty acids, coumarins, sildenafil analogs, bipiperidines, biscoumarins, oxadiazole, tetraketones, etc. The structure–activity relationships (SARs) of different classes of synthetic tyrosinase inhibitors was also discussed

Kim *et al.,* (2006) investigated that flavonoids, a group of naturally occurring antioxidants and metal chelators, can be used as tyrosinase inhibitors due to their formation of copper-flavonoid complexes. In addition, large differences in the tyrosinase inhibitory activities and chelating capacities according to the location of the hydroxyl group(s) in combination with the A and B rings in the flavonoids were confirmed. Accordingly, the

major conclusions from this work are as follows: (i) The tyrosinase inhibitory activity is not only dependent on the number of hydroxyl groups in the flavonoids, (ii) the enzyme is primarily quenched by the hydroxyl group(s) of A and B rings on the ether side of the flavonoids, and (iii) the tyrosinase inhibitory activity of 7,8,3¢,4¢-tetrahydroxyflavone is supported by a virtual model of docking with the mushroom tyrosinase, which depicts the quenching of the enzyme.

Kubo *et al*., (1998) characterized anisaldehyde in the seeds of *Pimpinella anisum* L. (Umbelliferae), also known as aniseed, which was found to inhibit the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase (EC 1.14.18.1) with an ID_{50} of 43 μg/mL (0.32 mM). The inhibition kinetics analyzed by a Lineweaver-Burk plot established anisaldehyde to be a noncompetitive inhibitor for this oxidation.

Kubo *et al*., (2007) searched for tyrosinase inhibitors from plants using L-3,4-dihydroxyphenylalanine (*L*-DOPA) as a substrate and quercetin was found to be partially oxidized to the corresponding *o*-quinone under catalysis by mushroom tyrosinase (EC 1.14.18.1). Simultaneously, *L*-DOPA was also oxidized to dopaquinone and both *o*-quinones were further oxidized, respectively. The remaining quercetin partially formed adducts with dopaquinone through a Michael type addition. In general, flavonols form adducts with dopaquinone as long as their 3-hydroxyl group is free. Quercetin enhanced melanin production per cell in cultured murine B16-F10 melanoma cells, but this effect may be due in part to melanocytotoxicity. The concentration leading to 50% viable cells lost was established as 20 μM and almost complete lethality was observed at 80 μM.

Lee *et al*., (2002) investigated the inhibitory effects of onion extract on browning of potato. The addition of heated onion extract to potato exhibited a marked inhibitory effect on potato polyphenol oxidase and the formation of a brown colour. Inhibitory effects of onion extract was dependent on its heating temperature. The addition of both glycine and glucose increased the inhibitory effect of the onion extract towards potato polyphenol oxidase.

Ley (2001) synthesized and evaluated several benzaldoximes, benzaldehyde-O-ethyloximes, and acetophenonoximes as tyrosinase inhibitors by an assay based on tyrosinase catalyzed L-DOPA oxidation. Benzaldoxime itself is only a weak inhibitor, its derivatives with one or two hydroxy or methoxy moieties in para and meta positions depress tyrosinase activity. Acetophenonoximes and trisubstituted benzaldoximes show no inhibitory activity. The IC_{50} of 3,4-dihydroxybenzaldehyde-O-ethyloxime (0.3 ± 0.1 mmol l^{-1}) is of the same magnitude as tropolone (0.13 ± 0.08 mmol l^{-1}), one of the best tyrosinase inhibitors known so far.

Lin *et al*., (2011) evaluated rosmarinic acid (confirmed as the major compound in *R. serra*), methyl rosmarinate and pedalitin isolated from *R. serra* for their inhibitory effects and mechanisms on tyrosinase and α -glucosidase. The inhibitory effects on both tyrosinase and α-glucosidase were in decreasing order, pedalitin > methyl rosmarinate > rosmarinic acid. The IC_{50} values for the tyrosinase and α -glucosidase activity inhibited by pedalitin were 0.28 and 0.29 mM, respectively. Both rosmarinic acid and methyl rosmarinate were considered as noncompetitive inhibitors of tyrosinase, while pedalitin was suggested to be a mixed-type inhibitor of tyrosinase.

Mojica *et al*., (2005) studied that tyrosinase can be inhibited by analogues of its substrate, tyrosine. Cashew nut shell liquid (CNSL) extract from cashew (*Anacardium occidentale*) contains several natural phenols that can serve as analogue of tyrosine. This study tested the tyrosinase inhibitory activity of CNSL against mushroom tyrosinase and compared it with two other tyrosinase inhibitors, benzoic acid and cyanide. In addition to this, an assay downscaled to microtiter format for measuring spectrophotometric tyrosinase activity assay was also optimized. This optimized method was found to have comparable results with the standard method and it is more advantageous in terms of cost and length of time of analysis. The data showed that CNSL can significantly inhibit tyrosinase activity to a concentration as low as 0.005 mg/mL.

Rao *et al.,* (2009) isolated 5 known secondary metabolites, namely 6, 8-iprenylumbelliferone (**1**), bergaptan (**2**), isopimpinellin **(3),** tritriacontanol (**4**), and isoquercetrin (**5**) from the leaves of *Chloroxylon swietenia* DC, a folklore medicinal plant. Three compounds were isolated for the first time from this genus. The structures of these compounds were elucidated by UV, IR, 1D and 2D NMR techniques. Different fractions from the leaves of *C. swietenia* were investigated for their tyrosinase inhibition activity. The crude extract and its fractions showed tyrosinase inhibition activity. The hexane and n-butanol fractions showed more activity than chloroform and ethyl acetate fractions.

Saboury *et al*., (2008) investigated the effect of ethyl xanthate(I) and propyl xanthate(II) on the kinetics of hydroxylation by mushroom tyrosinase (MT) at 20ºC in 10mM phosphate buffer solution, pH 6.8. 4-[(4-Methylphenyl) azo]-phenol (MePAPh) was used as a synthetic substrate for the enzyme for cresolase reaction. The results show that ethyl xanthate and propyl xanthate can activate or inhibit the cresolase activity of mushroom tyrosinase depending on the concentration of these effectors. Both I and II act uncompetitive at relatively high concentrations (20-50M). However, both I and II act as activators at relatively low concentrations (0-11.5 M). The enzyme has two distinct sites for both effectors. The first one is a high-affinity activation site and the other is a low-affinity inhibition site.

Jeon *et al*., (2005) measured the inhibitory effects on the activity of the enzyme by preincubating the enzyme with each inhibitor for 2 min and initiating the reaction by addition of 3 mM L-dopa at 37 °C. The concentration of inhibitor giving 50% inhibition (IC_{50}) was determined from plot of residual activity against inhibitor concentration. In this study, IC_{50} values of six inhibitors were determined, that were widely used as skin whitening agents.

Si *et al*., (2012) simulated docking (binding energies for AutoDock Vina: -9.1 kcal/mol) and performed a molecular dynamics simulation to verify docking results between tyrosinase and rutin. The docking results suggest that rutin mostly interacts with histidine residues located in the active site. A 10 ns molecular dynamics simulation showed that one copper ion at the tyrosinase active site was responsible for the interaction with rutin. Kinetic analyses showed that rutin-mediated inactivation followed a first-order reaction and mono- and biphasic rate constants occurred with rutin. The inhibition was a typical competitive type with K_I 1.10 ± 0.25 mM.

Xue *et al.,* (2011) isolated compounds such as hyperoside, isoquercitrin, trifolin, astragalin, chrysontemin, quercetin-3-O-(2□□-O-galloyl-β-D-glucopyranoside) (QOG), and kaempferol-3-O-(2□□-O-galloyl-β-D-glucopyranoside) (KOG). Their inhibitory activity was tested against tyrosinase for the oxidation of L-DOPA, and only chrysontemin showed inhibitory activity. To investigate the differences of their inhibitory effects, the tyrosinase inhibitory activities of their aglycons, cyanidin, quercetin, and kaempferol, were also tested. As a result, it was confirmed that the most influential moiety for tyrosinase inhibition was the 3□,4□-dihydroxy groups of the catechol moiety. Moreover, the tyrosinase inhibitory activity of chrysontemin, which was identified in persimmon leaves for the first time, is supported by a simulated model of chrysontemin docking into mushroom tyrosinase.

Yang *et al*., (2000) purified polyphenol oxidase (EC 1.10.3.1, PPO) in the pulp of banana (*Musa sapientum* L.) to 636-fold with a recovery of 3.0%, using dopamine as substrate. The enzyme activity was completely inhibited by L-ascorbic acid, cysteine, sodium diethyldithiocarbamate, and potassium cyanide. Under a low buffer capacity, the enzyme was also strongly inhibited by citric acid and acetic acid at 10 mM.

Yin *et al*., (2011) studied tyrosinase inhibition due to its medicinal applications such as hyperpigmentation. For probing effective inhibitors of tyrosinase, a combination of computational prediction and enzymatic assay via kinetics was important. They predicted the 3D structure of tyrosinase, used a docking algorithm to simulate binding between tyrosinase and phthalic acid (PA), and studied the reversible inhibition of tyrosinase by PA. PA inhibited tyrosinase in a mixed-type manner with a Ki = 65.84±1.10mM. Measurements of intrinsic and ANS-binding fluorescences showed that PA induced changes in the active site structure via indirect binding.

Zarivi (2003) presented the purification, the molecular properties and the reversible inhibition of *Tuber melanosporum* tyrosinase by dimethyl-sulfide and bis[methylthio]methane, the main flavour compounds of black and whitish truffles. The MW_r is 39,000. L-3,4-dihydroxyphenylalanine and L-tyrosine stain corresponding bands as expected for a true tyrosinase. Phenylthiourea, diethyldithiocarbamate and mimosine inhibit L-tyrosine and L-3,4-dihydroxyphenylalanine oxidation.

3.7 RESPONSE SURFACE METHODOLOGY

Ozoglu *et al*., (2002) subjected golden delicious apple juice to enzymic browning in the presence of the selected antibrowning agents: ascorbic acid, isoascorbic acid, L-cysteine, sorbic acid, benzoic acid, cinnamic acid and β-cyclodextrin. The most effective agents were determined as L-cysteine, cinnamic acid and ascorbic acid. Response surface methodology (RSM) was used to evaluate the potency of the L-cysteine, ascorbic acid and cinnamic acid combination for the control of enzymic browning. The ascorbic acid, L-cysteine and cinnamic acid combination provided better results than the individual compounds. The optimum combination was determined as 0.49 mM ascorbic acid, 0.42 mM L-cysteine and 0.05 mM cinnamic acid in the cloudy apple juice stored for 2 h at 25 ±1°C.

3.8 APPLICATION OF ANTIBROWNING METHODS

Anthon *et al*., (2002) determined the kinetic parameters for the thermal inactivation of several enzymes in carrot and potato homogenates. In carrots the most heat-resistant activity was polygalacturonase, followed by peroxidase and pectinmethylesterase. In potatoes peroxidase was the most resistant, followed by pectin methylesterase, polyphenol oxidase, and lipoxygenase.

Jose *et al*., (2004) reviewed blanching principles and equipment, effects of blanching on product quality, blanching indicators, and energy and waste considerations. Blanching removes trapped air (e.g., in broccoli florets) and metabolic gases within vegetable cells and replaces them with water, forming a semicontinuous water phase that favors a more uniform crystal growth during freezing. Targeting the right enzyme indicator would reduce blanching time and tackle all these priorities: improving product quality (increasing retention of nutrients and other freshlike quality attributes), reducing energy consumption, and reducing waste production.

Eissa *et al*., (2008) investigated five essential oils (EO) extracted from lemon grass (*Cymbopogon citratus*), basil (*Ocimum basilicum*), rosemary (*Rosmariinus officinalis*), sage (*Salvia officinalis*), and clove (*Eugenia aromatica*), for their inhibitory effect against polyphenoloxidase (PPO) enzymatic browning, microbial activity, as well as effect on food spoilage and mycotoxin producing fungi, *Aspergillus flavus* and *Aspergillus dchraceus*. Results showed that the EO from lemon grass, clove and rosemary were the most effective and prevented the growth and mycotoxin formation of the two fungi on apple juice. Also, the results showed that apple juices treated with essential oil (EO) extract from lemon grass, clove and rosemary had a positive effect towards the inhibition of PPO activity and reducing browning as compared to untreated, basil and sage treated juices, at room temperature (25°C) and at refrigerator (4°C), then increased shelf life of apple juice up to 4 weeks.

Hernandez *et al*., (1999) employed microwave for inactivation of PPO than conventional hot water treatment which can be translated into increased benefit and enhanced quality products for food industry. The short exposure time required for complete inactivation of aqueous solution of PPO irradiated with microwaves within monomode cavities is very important to reduce the browning rates of mushroom extracts, and could lead to a much greater rate of product profitability when treating whole processed mushrooms.

Kanimozhi *et al*., (2010) studied the application of microwave as an extraction tool for extraction of phytoconstituents present in *Artemisia pallens* wall (compositae) and determination of tyrosinase inhibitory activity which serves as an useful target in the treatment of hyper pigmentation skin disorder. The percentage yield of extract obtained by microwave assisted extraction (MAE) of plant was found to be highly significant when compared to soxhlet extraction (SE) method. The tyrosinase inhibitory activity of MAE was found to be highly significant ($p< 0.0001$) when compared with that obtained by SE method.

Lamikanra *et al*., (2007) determined the effect of mild heat fruit pretreatment on some properties of fresh-cut cantaloupe melon during storage. Whole fruit, previously held at 4ºC, was immersed in heated water (60ºC) with and without dissolved calcium lactate (1%). Reduced lipase activity occurred in heat treated fruit during storage at 10ºC, while the fruit that was cut 24 h after treatment had a reduced peroxidase activity, unlike fruit that was processed immediately after heating. Textural measurements showed increased hardness, chewiness and cohesiveness, but springiness decreased in heat-treated fruit. Lipase activity was, however, higher in fruit heated in calcium solutions. Results indicated the potential improvement of shelf life of cut cantaloupe melon by mild heat pre-treatment of the fruit, and that the addition of calcium to the treatment water did not further improve product quality.

Lee (2004) reviewed the concept of combining preservative factors for food preservation called 'hurdle technology'. In hurdle technology, combination treatments are applied because it is expected that the use of combined preservative factors will have greater effectiveness at inactivating microorganisms than the use of any single factor. However, recent studies show that the combination of preservation factors can have unexpected antimicrobial activity.

Mizobutsi *et al*., (2010) evaluated the influence of pH and temperature on peroxidase and polyphenoloxidase activities, in a partially purified preparation of pericarp of the litchi cultivar Bengal. Fruits were harvested at the ripe stage and polyphenoloxidase was partially purified by sequential saturation in 80% ammonium sulfate. Pre-incubation of the enzyme extract for 45 min at pH 2.5 or 9.5 completely inactivated the enzymes, with the highest degree of efficiency at pH 2.5. Polyphenoloxidase activity was highest at 20°C and remained active for a period of 120 min at 40 and 50°C and was inactivated after 10 min at 60ºC. Due to the high temperature of inactivation of the peroxidase and polyphenoloxidase activities, the enzymes can be inactivated more easily in fruits using acid or alkaline solutions.

Palou *et al*., (1999) evaluated the effects of blanching and high hydrostatic pressure (HHP) treatments on natural flora evolution, polyphenoloxidase (PPO) activity and color of banana puree adjusted to pH 3.4 and water activity (a_w) of 0.97 during 15 days storage at 25°C. Blanching time was found to affect ($p<0.05$) puree color. HHP treatments retained the initial color of the banana purees. Longer browning induction times and slower browning rates were observed when a longer blanching time was combined with a 689 MPa pressure treatment. A residual PPO activity < 5% was observed in the puree when a 7 min blanch was followed by HHP treatment at 689 MPa for 10 min.

Queiroz *et al.,* (2008) reviewed about antibrowning methods and alternatives for chemical preservatives in food processing. Alternatives to heat treatment, such as high hydrostatic pressure, irradiation, pulsed electric fields, etc. have been studied to control enzymatic browning with minimal changes in sensory and nutritional characteristics.

Rico *et al*., (2007) reviewed a need to find alternatives for preservation of freshcut fruit and vegetables in order to improve the efficacy of washing treatments. Alternatives or modified methods have been proposed, as antioxidants, irradiation, ozone, organics acids, modified atmosphere packaging, whey permeate, etc.; however, none have yet gained widespread acceptance by the industry.

Yemenicioglu *et al*., (1999) studied the heat inactivation kinetics of taro polyphenol oxidase (PPO) and peroxidase (POD) in the temperature range of 50°–80°C which followed the first–order kinetic model. In the range of 60°–70°C, heat stable isoenzymes accounted for 33–34% of POD and 67–72% of PPO. E_a and z values of heat stable isoenzymes were, respectively, 19.4 kcal.mol^{-1} and 25.9°C for POD, and 21 kcal.mol^{-1} and 25.5°C for PPO. The pH optimum was 5.9 for POD and 6.5 for PPO. POD was densely located on the surface of taro tubers whereas PPO was located more to the center. Taro PPO possessed both catechol oxidase and phloroglucinol oxidase activities but no laccase activity. Inhibition of PPO by EDTA, SO_2, NaCl and ascorbic acid was also determined.

Materials and Methods....

CHAPTER 4

MATERIALS AND METHODS

4.1 COLLECTION OF SAMPLES

Samples for screening of Polyphenol Oxidase (PPO) such as apple, banana, brinjal, guava, mushroom, papaya and potato were purchased from the local market, Coimbatore, Tamilnadu.

4.2 CHEMICALS

Analytical grade chemicals were purchased from Sigma, Himedia, Loba, Fisher and Sdfine

TABLE 4.2.1: List of chemicals and its purpose

PURPOSE	CHEMICALS
For extraction buffer	Polyvinyl pyrollidone (PVP), Triton X-100, sodium phosphate buffer (0.2M, pH 6.5) (Wuyts *et al*., 2006)
Substrates	L- DOPA, catechol, pyrogallol, phenol, tyrosine (prepared with sodium phosphate buffer, 0.05M, pH 7) (Wuyts *et al*., 2006)
For protein estimation	Folinciocalteau reagent, sodium carbonate, sodium hydroxide, potassium sodium tartarate, copper sulphate, bovine serum albumin (BSA)
For immobilization	gelatin, formaldehyde, polyacrylamide, sodium alginate, calcium chloride
PPO inhibitors	potassium metabisulphite, sodium azide, L-ascorbic acid, ferulic acid, citric acid, salicylhydroxamic acid (SHAM), sodium benzoate (SB), L-cysteine, benzoic acid (BA), cinnamic acid
General chemicals	ammonium sulphate, ethanol, double distilled water

4.3 EQUIPMENTS

Spectrophotometer, pH meter, waterbath, microwave oven

4.4 METHODOLOGY

4.4.1 ISOLATION OF POLYPHENOL OXIDASE (PPO)

PPO was isolated from apple, banana peel, banana pulp, brinjal, guava, mushroom, potato and papaya peel using extraction buffer containing 0.2M phosphate buffer (pH 6.5), 0.25% triton X 100 and 1% polyvinyl pyrollidone (PVP). The samples were homogenized in 1:10 ratio (sample: extraction buffer), filtered through four layers of cheese cloth and the crude enzyme obtained was stored at 4ºC as 1ml aliquots.

4.4.2 AMMONIUM SULPHATE PRECIPITATION

To the crude enzyme 30% ammonium sulphate was added and dissolved by gentle mixing. It was then centrifuged at 4ºC. The pellet was dissolved in 5ml phosphate buffer and to the supernatant 60% ammonium sulphate was added, mixed and centrifuged at 4ºC. Supenatant was discarded and pellet was redissolved in buffer and stored as enzyme fraction.

4.4.3 ENZYME ACTIVITY ASSAY

Presence of PPO was confirmed through enzyme assay involving its specific substrates and from the rate of quinone formation. Quinone resulted in reddish brown colour development.
PPO activity was assayed by measuring the rate of increase in absorbance at 475nm wavelength (dependent on the specific quinone product of the different substrates) at 25 °C. The reaction mixture contained 1 ml substrate in 0.05 M sodium phosphate buffer (pH 7.0), 1ml sodium phosphate buffer (0.2M, pH 6.5) and 0.9ml double distilled water and 0.1ml enzyme. The reference contained 1ml substrate,1ml sodium phosphate buffer (0.2M, pH 6.5) and 1ml double distilled water . The linear portion of the activity curve was used to express enzyme activity (Wuyts *et al*., 2006).

4.4.4 PROTEIN ESTIMATION

The protein concentration was estimated through Lowry's method. The method is as follows:

Reagent A: 2% $NaCO_3$ in 0.1N NaOH

Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% potassium sodium tartarate

Reagent C: Mix 50ml reagent A with 1ml reagent B, prior to use (alkaline copper sulphate)

***Reagent D*:** Folinciocalteau reagent

Stock: Weigh 50mg of BSA and dissolve in distilled water. Make upto 50ml in standard flask

Working standard solution: Dilute 10ml of stock to 50ml with distilled water in a standard flask (1ml of solution contains 200µg protein).

***Procedure*:**

i.) Measure 0.2, 0.4, 0.6, 0.8, 1ml of working standard solution and 0.1ml of test solution keeping 1ml of water as blank.
ii.) Make up the volume in all tubes to 1ml with distilled water.
iii.) 5ml reagent C was added in all tubes and incubated for 10mins.
iv.) 0.5ml of reagent D was added in all tubes and incubated for 30mins
v.) Blue colour developed was read at 660nm.

4.4.5 IMMOBILIZATION OF POLYPHENOL OXIDASE (PPO)

PPO enzyme was immobilized in sodium alginate, gelatin and polyacrylamide matrices in varying concentrations. The activity of immobilized enzyme was measured by using L-DOPA as substrate. Stability and reusability of immobilized enzyme was measured at different time intervals.

4.4.6 EFFECT OF ANTIBROWNING AGENTS

Antibrowning agents such as potassium metabisulphite, sodium azide, L-ascorbic acid, ferulic acid, citric acid, salicylhydroxamic acid (SHAM), sodium benzoate (SB), L-cysteine, benzoic acid (BA), cinnamic acid were added in different concentrations and activity of PPO was studied at 475nm according to Wuyts *et al*., (2006). The approximate IC_{50} concentrations were determined.

4.4.7 RESPONSE SURFACE METHODOLOGY (RSM)

Design expert version 8.0.7.1 was used to optimize the best combination of antibrowning agents by Box-Behnken design (BBD).

4.4.8 EFFECT OF ANTIBROWNING AGENTS – VISUAL OBSERVATION

Optimized antibrowning agent combination was sprayed on freshly cut fruits (apple, banana) and vegetables (brinjal, potato) and browning due to polyphenol oxidase (PPO) was observed with time.

4.4.9 APPLICATION OF ANTIBROWNING METHODS IN FRUITS AND VEGETABLES

Fruits (apple, banana) and vegetables (brinjal, potato) were grinded in a mortar and pestle and the juice was filtered through four layers of cheese cloth. They were than subjected to various antibrowning treatments (Hernandez *et al.,* 1999).

***No treatment*:** Juices were assayed for PPO activity without any treatment.

***Inhibitor treatment*:** Juices were subjected to optimized inhibitor combination and was assayed for reduction in PPO activity.

***Microwave treatment*:** The juices were treated in a microwave at 180W for 30secs and immediately assayed to PPO activity.

***Water Blanching*:** The juices were preincubated at 60ºC in a waterbath for 10mins and was immediately immersed in ice cold water. This juice was taken as PPO enzyme source and was assayed according to Wuyts *et al*., (2006).

Microwaved + Inhibitor treatment: Microwaved juices were added with optimized antibrowning agents combination and were assayed for PPO activity.

***Water Blanching + Inhibitor treatment*:** Water blanched juices were added with optimized antibrowning agents combination and were assayed for PPO activity.

***Microwave treatment + Water Blanching*:** Microwaved juices were immediately immersed in ice cold water and assayed for PPO activity.

Results and Discussion....

TABLE 5.1: COMPARISON OF POLYPHENOL OXIDASE (PPO) ACTIVITY FROM VARIOUS SOURCES

S.NO	SPECIES	TOTAL ACTIVITY (U)	SPECIFIC ACTIVITY (U/mg)
1	Apple	10.50	8.30
2	Banana peel	221.55	**169.11**
3	Banana pulp	180.85	50.04
4	Brinjal	204.05	85.23
5	Guava	NA	NA
6	Mushroom	46.55	82.35
7	Papaya peel	145.85	14.32
8	Potato	455.00	166.49

Screening was done for polyphenol oxidase (PPO) enzyme in eight different sources including plant (vegetable and fruits) and fungi. Activity (total and specific activity) of PPO was measured in all the sources and the source which had the maximum specific activity was chosen for further studies. Wuyts *et al*., (2006) reported PPO activity of 285U in banana pulp with an extraction buffer of pH 6.5 and 0.3M dopamine as substrate. According to the observation it was found that banana peel exhibited maximum specific activity of 169.11U/mg with an extraction buffer of pH 6.5 and 6.67mM L-DOPA as substrate. Hence banana peel PPO was chosen for further studies.

TABLE 5.2: ACTIVITY OF POLYPHENOL OXIDASE (PPO) IN AMMONIUM SULPHATE PRECIPITATED FRACTIONS

S.NO.	SPECIES	ACTIVITIES IN AMMONIUM SULPHATE PRECIPITATED FRACTIONS			
		TOTAL ACTIVITY (U)		SPECIFIC ACTIVITY (U/mg)	
		30%	60%	30%	60%
1	Apple	3.30	NA	3.80	NA
2	Banana peel	65.00	9.15	**366.80**	49.60
3	Banana pulp	43.33	28.33	68.18	73.17
4	Brinjal	87.50	3.50	52.15	96.89
5	Guava	2.65	NA	1.43	NA
6	Mushroom	15.00	6.65	12.77	24.54
7	Papaya peel	15.83	11.65	11.99	8.89
8	Potato	81.50	70.80	149.47	359.97

The screened sources were further purified by ammonium sulphate precipitation (30-60% saturation) at 4ºC. Activity (total and specific activity) for PPO were calculated. The source which exhibited maximum specific activity in partially purified fraction was chosen for further studies. Gouzi *et al.,* (2007) reported mushroom PPO specific activity of 40.1U/mg with catechol as substrate at 60% saturation. According to the observation it was seen that banana peel had maximum specific activity of 366.80U/mg in 30% ammonium sulphate saturation and hence was chosen for further studies.

TABLE 5.3: PERCENT IMMOBILIZATION OF ENZYME IN DIFFERENT MATRICES

S.NO	SPECIES	% IMMOBILIZATION IN DIFFERENT MATRIX TYPES								
		POLYACRYLAMIDE			GELATIN			SODIUM ALGINATE		
		A	B	C	A	B	C	A	B	C
1	Banana peel	100			100			98-99		
2	Mushroom	100			100			92-97		
3	Potato	100			100			99-100		

A,B,C denotes concentration of enzyme (in terms of protein concentration)

A= 45.80µg/ml

B= 91.60µg/ml

C= 137.4µg/ml

Immobilization of polyphenol oxidase (PPO) isolated from banana peel, mushroom and potato purified through ammonium sulphate precipitation were immobilized in different matrices such as polyacrylamide, gelatin and sodium alginate. Tembe *et al*., (2008) reported immobilization of PPO from a plant source *Amorphophallus companulatus* on eggshell membrane using glutaraldehyde. Among the approaches used for immobilization, activation of eggshell membrane by glutaraldehyde followed by enzyme adsorption on activated support could stabilize the enzyme and was found to be effective. In our present study, the percent immobilization of PPO was calculated. It was found that 100% immobilization was achieved in polyacrylamide and gelatin but the percent immobilization in sodium alginate ranged between 92-100%. This was calculated by assaying the calcium chloride solution in which the beads of sodium alginate were stored.

TABLE 5.4: PERCENT ACTIVITY OF POLYPHENOL OXIDASE (PPO) IN DIFFERENT MATRICES

S.NO	SPECIES	% ACTIVITY OF PPO IN DIFFERENT MATRICES								
		POLYACRYLAMIDE			GELATIN			SODIUM ALGINATE		
		A	B	C	A	B	C	A	B	C
1	Banana peel	25.94	77.88	71.53	88.9	74.01	78.35	98.18	88.90	86.28
2	Mushroom	69.06	35.2	72.27	83.6	4.28	87.44	98.74	88.70	97.74
3	Potato	68.99	31.94	69.39	68.6	87.3	77.37	98.50	96.61	95.34

A,B,C denotes concentration of enzyme (in terms of protein concentration)

A= 45.80µg/ml

B= 91.60µg/ml

C= 137.4µg/ml

Biegunski *et al*., (2006) reported that PPO retained its biological activity when being immobilized on a polymer surface. Percent activity of polyphenol oxidase (PPO) immobilized in different matrices were calculated. It was found that activity of PPO increased in certain cases with respect to that of the enzyme not immobilized, indicating that enzyme had maximum activity in the immobilized form. Various concentrations of enzyme was used for immobilization and the activity increase was compared for each concentration. Accordingly, the efficiency of each matrix was also measured. It was found that sodium alginate was found more efficient compared to polyacrylamide and gelatin.

TABLE 5.5: PERCENT REUSABILITY AND STABILITY OF POLYPHENOL OXIDASE (PPO) IN DIFFERENT MATRICES

S.NO	BANANA PEEL	% STABITY & REUSABILITY OF PPO IN DIFFERENT MATRICES								
		POLYACRYLAMIDE			GELATIN			SODIUM ALGINATE		
		A	B	C	A	B	C	A	B	C
1	Reusability	74.41	85.46	82.56	60.4	45.71	49.80	99.49	91.65	98.60
2	Stability	73.07	78.90	89.98	90.1	86.09	99.80	99.52	87.51	84.22

A,B,C denotes concentration of enzyme (in terms of protein concentration)

A= 45.80µg/ml

B= 91.60µg/ml

C= 137.4µg/ml

Percent reusability and stability of PPO immobilized in various matrices were calculated. For instance, banana peel PPO was checked for its stability and reusability for a duration of 15 days and 4 reaction cycles respectively. Sodium alginate immobilized PPO was very stable compared to polyacrylamide and gelatin immobilized one. Its reusability also proved to be superior than that of gelatin and polyacrylamide. Polyacrylamide also proved an efficient matrix than that of gelatin, but less efficient than sodium alginate.

TABLE 5.6: EFFECT OF ANTIBROWNING AGENTS

TABLE 5.6.1: Effect of Potassium Metabisulphite on Banana peel PPO

S.NO	CONCENTRATION IN mM	LAG PERIOD (sec)	AVERAGE % INHIBITION
1	0.025	60	20.48 ± 0.9
2	0.05	90	38.08 ± 1.3
3	0.075	240	46.41 ± 0.4
4	0.1	270	56.22 ± 2.5
5	0.15	900	85.08 ± 1.6
6	0.2	-	100
7	0.3	-	100

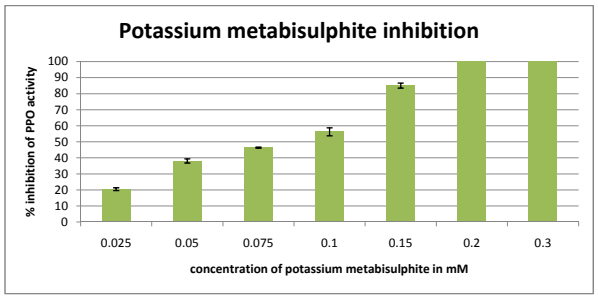

Fig 5.6.1: Effect of Potassium Metabisulphite on Banana peel PPO

Prabha *et al.,* (1982) reported potassium metabisulphite, a reducing agent to effectively inhibit polyphenol oxidase (PPO) at 0.1mM levels. In our study, it was observed that 56% inhibition of PPO activity was achieved at 0.1mM concentration. Also it was observed that a lag period in exhibiting of PPO activity was observed in increasing levels of potassium metabisulphite concentration. Higher the concentration of potassium metabisulphite, longer the lag period. At concentrations above 0.2mM, complete inhibition of PPO was observed.

TABLE 5.6.2: Effect of Sodium azide on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	0.5	47.11 ± 1.4
2	1.0	51.94
3	1.5	60.61 ± 1.4
4	2.0	63.47
5	2.5	68.28 ± 1.3
6	3.0	73.09 ± 2.7

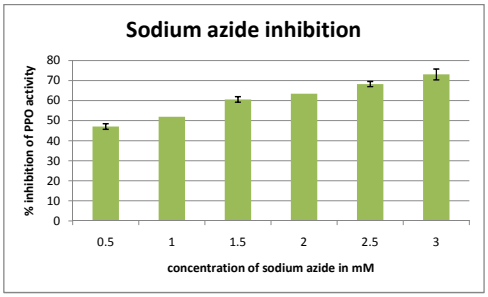

Fig 5.6.2: Effect of Sodium azide on Banana peel PPO

Azide can form complexes with many copper enzymes, inhibiting their activity (Shi *et al.*, 2002). Dogan *et al*. (2005) showed that the inhibition of polyphenol oxidase extracted from the artichoke (*Cynara scolymus* L.) by sodium azide was competitive inhibition. Gouzi *et al*., (2007) demonstrated the effect of sodium azide as an inhibitor of PPO using pyrogallol as substrate. The Lineweaver-Burk double reciprocal plot showed that the inhibitor changed the K_m value but not the V_{max} value. In our present study, approximately 1mM concentration was required to inhibit 50% PPO activity and inhibition was dose dependent.

TABLE 5.6.3: Effect of L-Ascorbic acid on Banana peel PPO

S.NO	CONCENTRATION IN mM	LAG PERIOD (sec)	AVERAGE % INHIBITION
1	0.1	60s	22.49 ± 3.3
2	0.2	210s	43.78 ± 2.9
3	0.3	360s	47.89 ± 2.5
4	0.4	510s	62.38 ± 1.7
5	0.5	780s	78.98 ± 3.8
6	0.6	1950s	98.58 ± 0.6

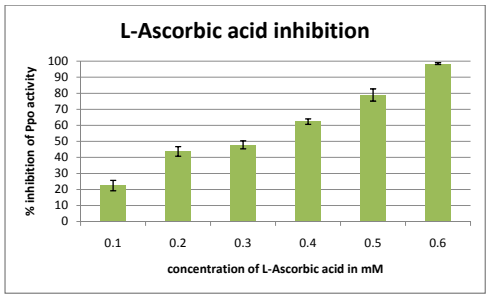

Fig 5.6.3: Effect of L- Ascorbic acid on Banana peel PPO

Goldhirsh *et al.,* (1984) reported that L-ascorbic acid is active at the level of PPO products as it reduces quinones back to the phenolic substrates, whereby it gets oxidised. Wuyts *et al*., (2006) reported that high concentrations of ascorbic acid may also inactivate PPO and complete inhibition was achieved at 1mM concentration. In our study, it was observed that at concentrations above 0.6mM, complete inhibition of PPO was observed. Also it was observed that a lag period in exhibiting of PPO activity was observed in increasing levels of L-Ascorbic acid concentration. Higher the concentration of L-Ascorbic acid, longer the lag period.

TABLE 5.6.4: Effect of Ferulic acid on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	0.5	14.08 ± 2.7
2	1.0	19.83 ± 1.1
3	1.5	22.85 ± 1.7
4	2.0	29.39 ± 2
5	2.5	34.25 ± 2.7
6	3.0	38.25 ± 2.7
7	3.5	43.45 ± 2.5
8	4.0	54.25 ± 1.3

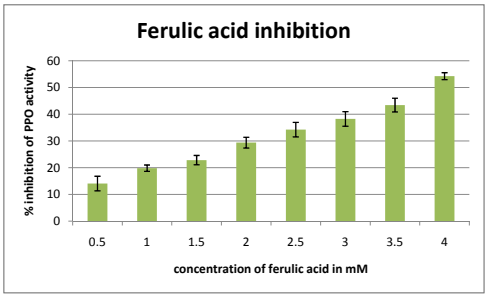

Fig 5.6.4: Effect of Ferulic acid on Banana peel PPO

Prabha *et al.,* (1982) reported that ferulic acid inhibits PPO activity at 1mM concentration. In our study, it was observed that PPO activity inhibition was achieved from 0.5mM concentration and higher concentrations led to higher percent inhibition of PPO activity.

TABLE 5.6.5: Effect of Citric acid on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	1	2.45 ± 0.07
2	5	14.04 ± 2.9
3	10	26.05 ± 1.3
4	15	33.52 ± 2.3
5	20	38.62 ± 0.2
6	21	47.24 ± 0.08
7	22	52.87 ± 1.7
8	23	58.03 ± 1.3
9	24	63.46 ± 0.4
10	25	65.75 ± 5.3

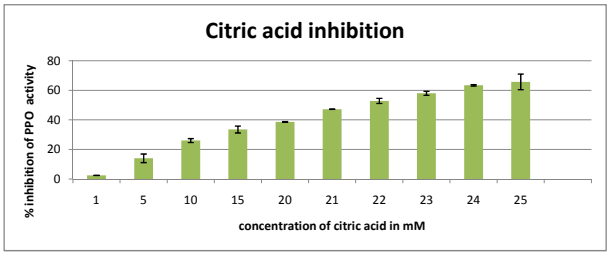

Fig 5.6.5: Effect of Citric acid on Banana peel PPO

Wuyts *et al.,* (2006) reported that citric acid inhibits PPO enzyme activity by lowering the pH of the extraction buffer. Citric acid was not a very potent inhibitor of root PPO, but it may be due to the pH-buffering capacity of the 0.05 M sodium phosphate buffer. The IC_{50} was 22mM and complete inhibition of PPO activity was achieved at 44mM concentration. Yang *et al.*, (2000, 2001) showed that under low buffer capacity citric acid (10 mM) can lower the pH to 3.1 and inhibit banana pulp and peel PPO activity. In our present study, nearly 53% inhibition was achieved at 22mM concentration and inhibition percent increased as concentration of citric acid was increased.

TABLE 5.6.6: Effect of Salicylhydroxamic acid (SHAM) on Banana peel PPO

S.NO	CONCENTRATION IN µM	AVERAGE % INHIBITION
1	1	22.27 ± 2.3
2	2	28.88 ± 1.7
3	4	31.25
4	8	31.93 ± 2.3
5	10	46.65 ± 3.1
6	16	56.50 ± 4.6
7	20	61.13 ± 1.5
8	30	73.00
9	32	72.79 ± 1.5
10	40	75.56
11	50	81.10 ± 1.5
12	60	81.10 ± 1.5
13	64	82.38 ± 3.2
14	70	84.40 ±
15	80	86.67 ±
16	90	88.89 ±
17	128	89.26 ± 2.2

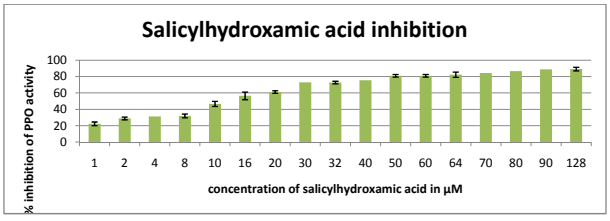

Fig 5.6.6: Effect of Salicylhydroxamic acid (SHAM) on Banana peel PPO

Paranjpe *et al.,* (2003) observed that approximately 12µM concentration SHAM competitively inhibited PPO activity by 50%, L-DOPA being the substrate. In our present study, it was observed that IC_{50} range of PPO was between 10-16µM. Inhibition pattern increased as concentration was of salicylhydroxamic acid (SHAM) was increased.

TABLE 5.6.7: Effect of Sodium benzoate (SB) on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	4	25.05 ± 0.1
2	5	27.65 ± 2.0
3	6	31.84 ± 2.6
4	7	36.83 ± 1.6
5	8	39.11 ± 1.4
6	9	42.51 ± 1.8
7	10	44.14 ± 1.4
8	11	48.17 ± 3.5
9	12	48.23 ± 1.9
10	15	50.01 ± 1.2
11	20	53.44 ± 0.9
12	25	71.08 ± 1.5

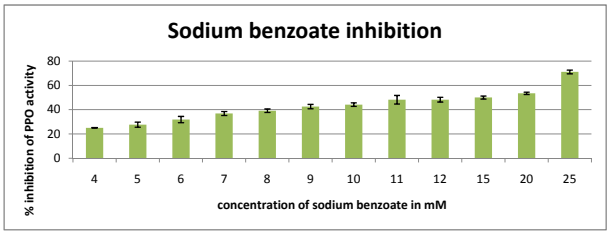

Fig 5.6.7: Effect of Sodium benzoate (SB) on Banana peel PPO

Weemaes *et al*., (1999) reported that the antibrowning agents appeared capable of modulating the pressure stability of the sensitive enzyme fraction. Addition of 10 mM sodium benzoate to the PPO enzyme solution resulted in a marked stabilization towards pressure of the avocado PPO fraction. In our present study, it was observed that at 15mM concentration, PPO activity was reduced by 50%, L-DOPA being the substrate.

TABLE 5.6.8: Effect of L-Cysteine on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	0.05	28.82 ± 2.6
2	0.1	29.50 ± 0.9
3	0.2	33.38 ± 2.4
4	0.3	36.58 ± 3.3
5	0.4	42.49 ± 2.2
6	0.5	47.81 ± 2.8
7	0.6	50.35 ± 3.8

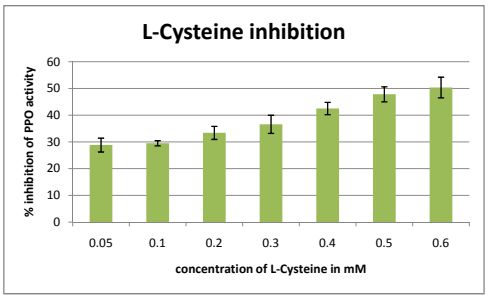

Fig 5.6.8: Effect of L-Cysteine on Banana peel PPO

Wuyts *et al*., (2006) reported that amino acids (L-cysteine) inhibits polymerization of quinones by reducing quinones back to phenols or by forming complexes with quinones and preventing polymerization, while substrate continues to be consumed. PPO activity was completely inhibited at 1mM concentration and IC_{50} was 0.322mM. In our present study, it was observed that IC_{50} was nearly 0.6mM and inhibition increased as concentration of L-cysteine increased.

TABLE 5.6.9: Effect of Benzoic acid (BA) on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	4	33.36 ± 3.1
2	5	34.47 ± 1.6
3	6	39.33 ± 14.1
4	7	42.60 ± 2.5
5	8	54.45 ± 1.6
6	9	60.00
7	10	64.40
8	11	66.67
9	12	67.80 ± 7.8

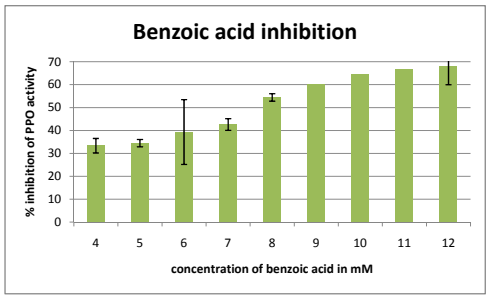

Fig 5.6.9: Effect of Benzoic acid (BA) on Banana peel PPO

Liu *et al*., (2003) reported that benzoic acid had inhibition effects on the diphenolase activity of mushroom PPO. Menon *et al*., (1990) reported that benzoic acid inhibited α, β and isozymes of *A. bisporus* PPO competitively for cresolase reaction, but showed partial uncompetitive inhibition for α and β isozymes and a simple competitive inhibition for isozyme in catecholase reaction. In our present study, it was observed that benzoic acid had inhibitory effects on diphenolase activity of banana peel PPO.

TABLE 5.6.10: Effect of Cinnamic acid on Banana peel PPO

S.NO	CONCENTRATION IN mM	AVERAGE % INHIBITION
1	0.1	29.39 ± 1.7
2	0.5	47.34 ± 4.5
3	1.0	55.50 ± 0.9
4	1.5	72.75 ± 3.7
5	2.0	82.42 ± 3.1
6	2.5	87.56 ± 3.7

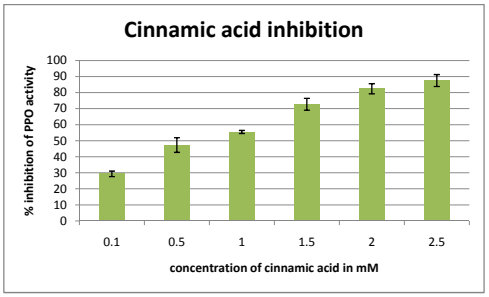

Fig 5.6.10: Effect of Cinnamic acid on Banana peel PPO

Shi *et al*., (2005) reported that cinnamic acid strongly inhibited the diphenolase activity of mushroom PPO and the inhibition was reversible. The IC_{50} value was estimated to be 2.10mM, respectively. Kinetic analyses showed that the inhibition type of cinnamic acid was noncompetitive. In our present study, IC_{50} of cinnamic acid was between 0.5-1mM and inhibition was dose dependent. Higher the concentration of cinnamic acid, higher was the inhibition of banana peel PPO activity.

TABLE 5.7: COMPARISON OF INHIBITION TYPE OF SOME COMPOUNDS ON THE DIPHENOLASE ACTIVITY OF PPO

S.NO	INHIBITOR	TYPE OF INHIBITION	IC_{50}	REFERENCE
1	Potassium metabisulphite	competitive	-	Prabha *et al*., 1982
2	Sodium azide	Mixed	3.20±0.13 mM	Gouzi *et al*., 2010
3	L-Ascorbic acid	Competitive	0.437mM	Wuyts *et al*.,2006
4	Ferulic acid	Competitive	1mM	Prabha *et al*., 1982
5	Citric acid	Competitive	22mM	Wuyts *et al*.,2006
6	Salicylhydroxamic acid (SHAM)	Competitive	12µM	Paranjpe *et al*., 2003
7	Sodium benzoate	Competitive	-	Weemaes *et al*., 1999
8	L-Cysteine	Competitive	0.322mM	Wuyts *et al*.,2006
9	Benzoic acid	Competitive	0.64mM	Kubo *et al*., 1988
10	Cinnamic acid	Non competitive	2.10mM	Shi *et al*.,2005

TABLE 5.8: SPECIFICITY OF BANANA PEEL POLYPHENOL OXIDASE

S.NO	PHENOLIC COMPOUND*	WAVELENGTH (nm)	ENZYME ACTIVITY (U)
1	Catechol (diphenol)	420	9
2	L-DOPA (diphenol)	475	7.67
3	Pyrogallol (triphenol)	420	1.5
4	Tyrosine (monophenol)	420	0
5	Phenol (monophenol)	390	0

* phenolics were added at 20mM concentration (6.67mM in reaction mixture)

The substrate specificity of the banana peel PPO is shown in table 5.8. Catechol and L-DOPA had an oxidation rate higher than 75% that of pyrogallol, The enzyme was inactive towards phenol and tyrosine.

TABLE 5.9: RANKING OF ANTIBROWNING AGENTS BASED ON EFFECTIVENESS AND COST

S.NO	BASED ON EFFECTIVENESS	BASED ON COST (sigma price)	BASED ON EFFECTIVENESS & COST
1	Salicylhydroxamic acid (SHAM)	Potassium metabisulphite	**Potassium metabisulphite**
2	Potassium metabisulphite	Citric acid	Sodium azide
3	L-Ascorbic acid	Sodium benzoate	**L-Ascorbic acid**
4	Sodium azide	Benzoic acid	Ferulic acid
5	Cinnamic acid	Ferulic acid	**Citric acid**
6	L-Cysteine	Sodium azide	Salicylhydroxamic acid (SHAM)
7	Ferulic acid	L-Ascorbic acid	Sodium benzoate
8	Benzoic acid	L-Cysteine	L-Cysteine
9	Sodium benzoate	Salicylhydroxamic acid (SHAM)	Benzoic acid
10	Citric acid	Cinnamic acid	Cinnamic acid

Table 5.9 ranks antibrowning agents based on cost and effectiveness. Effectiveness was based on minimum concentration required for maximum inhibition of PPO activity, in which salicylhydroxamic acid (SHAM) was most effective at 16µM concentration (approx. IC_{50}), whereas citric acid had an approximate IC_{50} of 21mM. Cost is an important criteria which is categorized by Sigma pricelist. Potassium metabisulphite costed Rs.5/g, whereas cinnamic acid costed about Rs.571/g, hence stands last in ranking. Giving both criteria equal importance, ranking was given for antibrowning agents in which potassium metabisulphite, L-Ascorbic acid and citric acid were chosen (based on toxicity and availability) for further studies.

TABLE 5.10: PERMISSIBLE LIMITS OF ANTIBROWNING AGENTS

S.NO	INHIBITOR NAME	PERMISSIBLE/ALLOWABLE LIMITS	
1	Potassium metabisulphite	10-2000mg/kg	(0.044-8.99mM)
2	L-Ascorbic acid	0.2-0.3g/kg (GRAS)	(1.135-1.703mM)
3	Citric acid	0.5%	(23.79mM)

TABLE 5.11: CHOSEN IC50 LEVELS OF ANTIBROWNING AGENTS

S.NO	INHIBITOR NAME	PERMISSIBLE/ALLOWABLE LIMITS (mM)	CHOSEN LIMIT (mM)
1	Potassium metabisulphite	0.044-8.99	0.075
2	L-Ascorbic acid	1.135-1.703	0.3
3	Citric acid	23.79	21

TABLE 5.12: CONCENTRATION LEVELS FOR ANTIBROWNING AGENTS COMBINATION

S.NO	CONCENTRATION LEVELS	% INHIBITION OF PPO ACTIVITY
1	IC_{50}	100
2	$IC_{50}/2$	99.99
3	$IC_{50}/3$	55.12
4	$IC_{50}/4$	36.66
5	$IC_{50}/6$	26.56
6	$IC_{50}/8$	16.10

Table 5.11 shows the permissible limits of antibrowning agents along with the chosen experimented approximate IC_{50} levels, which lie within the allowable limits.

Table 5.12 shows various combinations of chosen levels of antibrowning agents and their respective percent inhibition of banana peel PPO activity. Nearly 50% inhibition was achieved in $IC_{50}/3$ level and hence optimization was done further using response surface methodology (RSM) for this concentration limit.

TABLE 5.13: RESPONSE SURFACE METHODOLOGY (RSM)

TABLE 5.13.1: BOX-BEHNKEN DESIGN (BBD) LAYOUT FOR OPTIMIZATION OF ANTIBROWNING AGENT CONCENTRATION

Std	Run	Factor 1	Factor 2	Factor 3	Response 1
		A:KBS	B:AA	C:CA	% inhibition
-	-	mM	mM	mM	%
11	1	0.03	0.05	8	67.44
10	2	0.03	0.15	6	62.79
16	3	0.03	0.1	7	32.55
6	4	0.04	0.1	6	37.2
7	5	0.02	0.1	8	81.39
17	6	0.03	0.1	7	32.55
12	7	0.03	0.15	8	72.09
5	8	0.02	0.1	6	0
14	9	0.03	0.1	7	32.55
8	10	**0.04**	**0.1**	**8**	**86.04**
2	11	0.04	0.05	7	62.79
4	12	0.04	0.15	7	58.13
1	13	0.02	0.05	7	34.88
15	14	0.03	0.1	7	32.55
13	15	0.03	0.1	7	32.55
9	16	0.03	0.05	6	30.22
3	17	0.02	0.15	7	27.9

TABLE 5.13.2: ANOVA FOR RESPONSE SURFACE QUADRATIC MODEL

Response 1 : % inhibition						
ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	7297.528	9	810.8364	4.700488	0.0268	**significant**
A-KBS	1249.75	1	1249.75	7.244907	0.0310	
B-AA	81.79205	1	81.79205	0.474155	0.5132	
C-CA	3905.07	1	3905.07	22.63802	0.0021	
AB	1.3456	1	1.3456	0.007801	0.9321	
AC	264.8756	1	264.8756	1.535506	0.2552	
BC	194.8816	1	194.8816	1.129745	0.3231	
A^2	43.08211	1	43.08211	0.249751	0.6326	
B^2	436.0255	1	436.0255	2.527677	0.1559	
C^2	999.7035	1	999.7035	5.795366	0.0470	
Residual	1207.503	7	172.5005			
Lack of Fit	1207.503	3	402.5012			
Pure Error	0	4	0			
Cor Total	8505.031	16				

The Model F-value of 4.70 implies the model is significant. There is only a 2.68% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, C, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

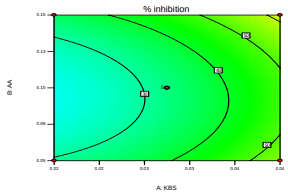

Fig 5.13.1(a): Contour plot for antibrowing agents (potassium metabisulphite and L-ascorbic acid) Optimization

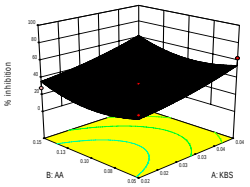

Fig 5.13.1(b): Surface plot for anti-browning agents (potassium metabisulphite and L-ascorbic acid) optimization

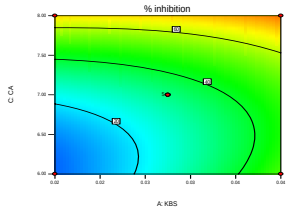

Fig 5.13.2(a): Contour plot for antibrowing agents (potassium metabisulphite and citric acid) optimization

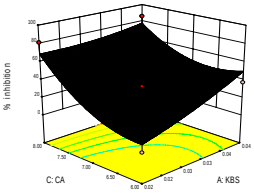

Fig 5.13.2(b): Surface plot for anti-browning agents (potassium metabisulphite and citric acid) optimization

Note:

KBS - potassium metabisulphite ; AA- L-Ascorbic acid; C- Citric acid

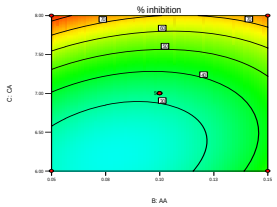

Fig 5.13.3(a): Contour plot for antibrowing agents (L-ascorbic acid and citric acid) optimization

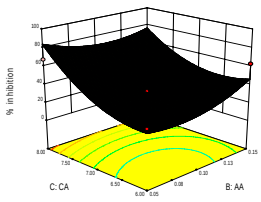

Fig 5.13.3(b): Surface plot for anti-browning agents (L-ascorbic acid and citric acid) optimization

Note:

KBS - potassium metabisulphite ; AA- L-Ascorbic acid; C- Citric acid

Fig. 5.13.1, 5.13.2 and 5.13.3 (a, b) represents some examples for contour and response surface plots for percent inhibition of banana peel polyphenol oxidase (PPO) activity by antibrowning agents such as potassium metabisulphite, L-ascorbic acid and citric acid. It was observed from surface plots that inhibition of PPO was attributed mainly by citric acid followed by potassium metabisulphite and then by L-ascorbic acid respectively.

TABLE 5.13.3: MODEL SUMMARY

Std. Dev.	13.13394	R-Squared	0.858025
Mean	46.09529	Adj R-Squared	0.675485
C.V. %	28.49303	Pred R-Squared	-1.2716

A negative "Pred R-Squared" implies that the overall mean is a better predictor of the response than the current model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 7.503 indicates an adequate signal. This model can be used to navigate the design space.

Ozoglu *et al*., (2002) used response surface methodology (RSM) to evaluate the potency of the L-cysteine, ascorbic acid and cinnamic acid combination for the control of enzymic browning. The ascorbic acid, L-cysteine and cinnamic acid combination provided better results than the individual compounds. The optimum combination was determined as 0.49 mM ascorbic acid, 0.42 mM L-cysteine and 0.05 mM cinnamic acid in the cloudy apple juice stored for 2 h at 25±1°C. In our present study, potassium metabisulphite, L-Ascorbic acid and citric acid provided better results than individual compounds (based on effectiveness and cost) and optimum concentrations were 0.04, 0.1 and 8mM for potassium metabisulphite, L-ascorbic acid and citric acid respectively. Hence this combination of antibrowning agents was used further for applications in fruits and vegetables.

EFFECT OF ANTIBROWNING AGENTS ON FRUITS AND VEGETABLES- VISUAL OBSERVATION

Control Test

Fig 5.13.4: Effect of antibrowning agent on apple (after 3 hours)

Control Test

Fig 5.13.5: Effect of antibrowning agent on banana (after 3 hours)

Control Test

Fig 5.13.6: Effect of antibrowning agent on brinjal (after 3 hours)

Control Test

Fig 5.13.7: Effect of antibrowning agent on potato (after 3 hours)

TABLE 5.14 :APPLICATION OF ANTIBROWNING METHODS IN FRUITS AND VEGETABLES

S.NO	ANTIBROWNING METHOD	% INHIBITION OF PPO ACTIVITY			
		APPLE	BANANA	POTATO	BRINJAL
1	No treatment	-	-	-	-
2	With inhibitor	94.95	100	100	100
3	Microwave treated (180W, 30sec)	94.98	92.40	46.20	81.20
4	Water blanched (60°C,10mins)	80	78.60	58.49	31.09
5	Microwave treated+inhibitor	100	100	100	100
6	Water blanched+inhibitor	100	100	94.33	100
7	Microwave treated+blanched	84.98	83.60	65.64	81.20

Table 5.14 shows application of various antibrowning methods involving microwaving with power input of 180W for 30sec, water blanching (pretreatment at 60ºC for 10minutes), inhibitor treatment (antibrowning agents optimized combination) and combination of the above said methods. It was seen that inhibitor sole treatment and microwaving and water blanching with inhibitor treatment of fruits and vegetable juices proved efficient than other antibrowning methods which was observed from the percent inhibition of polyphenol oxidase (PPO) activity .

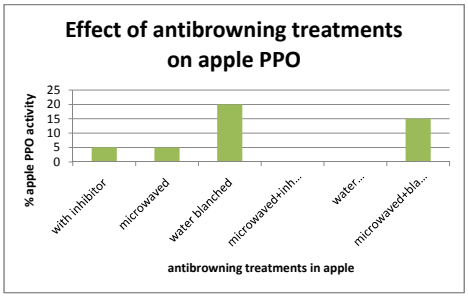

Fig 5.14.1: Effect of antibrowning treatments on apple PPO

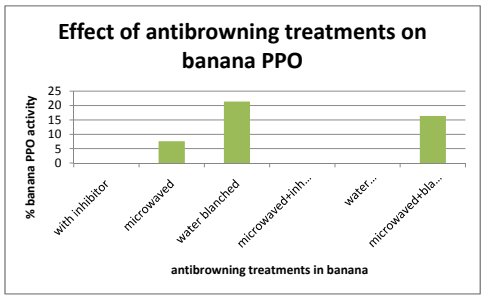

Fig 5.14.2: Effect of antibrowning treatments on banana PPO

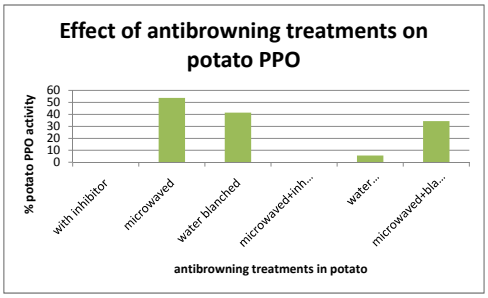

Fig 5.14.3: Effect of antibrowning treatments on potato PPO

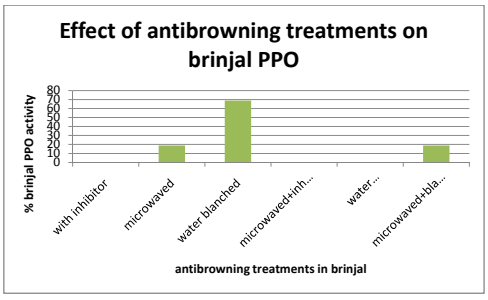

Fig 5.14.4: Effect of antibrowning treatments on brinjal PPO

Figures 5.14.1, 5.14.2, 5.14.3,5.14.4 show the percent activity exhibited by PPO in fruit and vegetable juices after being subjected to various antibrowning treatment methods. From the figures, it is observed that inhibitor addition enhances the antibrowning effect of other treatment methods.

Conclusion....

CHAPTER 6

CONCLUSIONS

Banana peel had highest polyphenol oxidase (PPO) activity. Specific activity of banana peel was highest in 30% ammonium sulphate precipitated fraction which was 366.80U/mg.

Percent immobilization in sodium alginate, polyacrylamide and gelatin was around 90-100%. Enzyme retained 50-99% activity after 14 days and immobilized enzyme retained 50% activity after 4 reaction cycles.

Increasing concentrations of inhibitors acting as antibrowning agents had increased effect on inhibiting polyphenol oxidase (PPO) activity and was dose dependent. PPO was more specific to diphenol substrates than monophenols. Potassium metabisulphite, L-ascorbic acid and citric acid were more effective in inhibition of PPO and was also cost effective. Concentration of antibrowning agents employed for combinations fell within the permissible limits and showed effective inhibition upto 86%.

From the response surface methodology (RSM), it was observed that citric acid contributed more for inhibition followed by potassium metabisulphite and L-ascorbic acid respectively.

Other methods to inhibit enzymes like water blanching and microwaving treatment were compared to the enzyme inhibitors. It was found that addition of inhibitor enhanced the inhibition of other treatments. Therefore, further studies can be carried out to develop efficient methods to prevent browning of fruits and vegetables involving PPO inhibitors.

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CHAPTER 7

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