

**EXPRESSION OF POLYPHENOL OXIDASE IN
Yeast *Pichia pastoris***

THESIS

BY

KANIKA SHARMA

Submitted to



**CHAUDHARY SARWAN KUMAR
HIMACHAL PRADESH KRISHI VISHVAVIDYALAYA
PALAMPUR – 176 062 (H.P.) INDIA**

IN

Partial fulfillment of the requirements for the degree

OF

**MASTER OF SCIENCE IN AGRICULTURE
(AGRICULTURAL BIOTECHNOLOGY)
2007**

PALAMPUR

Oxidase in yeast - *Pichia*...

[illegible]

K. R. KRISHNAN VISHVA VIDYALAYA
LIBRARY, PALAMPUR (T.N.)
Accession No... 13831
Date 03.09.07
Source CSK HPKV, Palampur
In No.
Accessioned by *[Signature]* Checked by

589.23
S 33E

[Signature]
11/9/07

Dedicated To My Beloved Parents

Dedicated To My Beloved Parents

Dedicated To My Beloved Parents

Dedicated To My Beloved Parents

Acknowledgement

In this highly complex society, no work can be accomplished by a single individual but it needs inspiration and sincere guidance of intellectuals, as well as the grace of God. With limitless humility, I would like to praise and thank the Lord, Almighty who bestowed me with the favorable circumstances to go through this crucial juncture. Every effort is motivated by ambition and all ambitions have inspiration behind. I owe this pride place to my Papa and Mummy, who believed in giving educational wings to their daughter. Their selfless persuasion, sacrifices, heartfelt blessings and firm faith have made this manuscript a feeble recompense to translate their dreams in to reality.

I take this opportunity to express my deep sense of gratitude to Dr. Vipin Hallan, Chairman of my Advisory Committee for his guidance, constant encouragement, constructive criticism and painstaking supervision during the course of the investigation. It would have been impossible for me to present this thesis without his outstanding supervision. His company and assurance at the time of crisis would be remembered life long. He always gave me the freedom to work that I cherished the most.

It is my proud privilege to express my gratitude and obligation to my esteemed co-supervisor Dr Sanjay Kumar, a molecular biologist of repute. It is more than mere thanks that I acknowledge his guidance.

Grateful thanks are also due to the worthy members of my Advisory Committee, Dr. P.S. Ahuja, Dr. Kamal Mohini and Dr. K. K. Katoch for their painstaking efforts, professional dexterity and inspiring guidance rendered to me from time to time. I also extend my sincere thanks to all my teachers at ACHBB Dr. R.K. Kapila, Dr. T. R. Sharma, Dr. K.D. Sharma, Dr. P. Plaha, Dr. R.S. Chouhan, and Dr. R. Rathore for their help and support.

I would like to thank the Programme Director ACHBB, Dr. S.R. Thakur, for his full support during my M.Sc. My heart felt thanks are extended to Dr. P. S. Ahuja, Director IHBT, Palampur for the moral support, permission to use the available facilities to venture in the fascinating world of biotech and for the faith, which he kept on me during the tenure of my work at IHBT.

I avail myself of this rare opportunity to express my thanks to ^{Dean COA and} Dr. Pradeep. K. Sharma, Dean, Postgraduate studies. I emphatically express my venerable thanks to the Department of Biotechnology, Government of India, New Delhi for providing financial assistance in form of scholarship.

I shall be failing my duty, if I do not thank, Head of floriculture division, IHBT, Dr. A. A. Zaidi for always boosting up mi morale and showing me the path of perseverance even in the hardest times.

I express my heartfelt thanks to Dr Raja Ram, Lakmir sir, Amit sir, Birender sir, Yogesh sir, Gaurav sir for their suggestions and encouragement whenever needed. I

acknowledge with cordial thanks for the help and cooperation of Ranjeet bhaiya for providing their assistance in their related aspects.

No words can ever meet the love and affection showered on me by Vanita di and Tanuja di which constantly pushed me to do my work all smilingly. Their encouragement, lively sentiments, benevolence led me accomplish the task with earnest efforts.

Words are incapable of translating my sincere feelings for my friend Vandna and Pankaj for always being there when I needed them the most. Diction is not enough to express my thanks to Sunny, Arti, Dinesh and all my juniors whose eternal affection along with direct and indirect help led me to achieve my destination successfully.

My eternal gratitude goes to my biggest strength my sister, Vaishali, Sumant jiju and my brother Karan for their inspiration, encouragement and moral support without which my aspirations would have been vanished.

Last but not the least, I admit that all the errors in the dissertation are mine.

*Place: Palampurⁱⁿ
Dated: July, the 19, 2007*

Kanika
(Kanika Sharma)

Dr. Vipin Hallan
Scientist

**Plant Virology Lab,
Floriculture Division,
Institute of Himalayan Bioresources, Technology,
CSIR,
Palampur-176062 (H.P.) INDIA.**

CERTIFICATE I

This is to certify that the thesis entitled "**Expression of polyphenol oxidase in yeast *Pichia pastoris***" submitted in partial fulfilment of the requirement for the award of the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a bonafide research work carried out by **Ms. Kanika Sharma (Admission No. A-2004-30-3)**, daughter of **Sh. L.R. Sharma** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

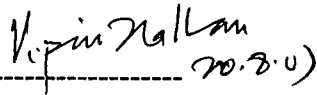
The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Palampur
Dated: the 19th July, 2007


(Dr. Vipin Hallan)
Chairman,
Advisory Committee

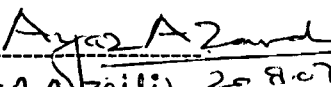
CERTIFICATE II

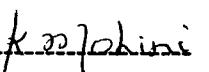
This is to certify that the thesis entitled "**Expression of polyphenol oxidase in yeast *Pichia pastoris***" submitted by **Ms. Kanika Sharma** (**Admission No. A- 2004- 30- 3**) daughter of Sh. L.R. Sharma to Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.


----- 20.8.07
(Dr. Vipin Hallan)
Chairman,
Advisory Committee

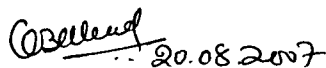

----- 20.08.07
External Examiner
Dr. G. S. Vohra

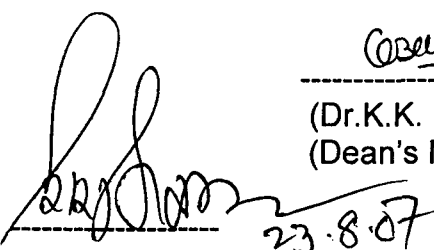

----- 20.8.07
(Dr. Sanjay Kumar)
Member


----- 20.8.07
(Dr. A. A. Zaidi)
Member



(Dr. Kamal Mohini)
Member


----- 20.08.2007
(Dr. K. K. Katoch)
(Dean's Nominee)


----- 23.8.07
(Dr. S. R. Thakur)
Programme Director
Advanced Centre of Hill Bioresources and Biotechnology
CSK HPKV, Palampur


----- 30.8.07
(Dr. Pradeep K. Sharma)
Dean
Postgraduate Studies

CONTENTS

CHAPTER	TITLE	PAGE NO.
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-22
III	MATERIALS AND METHODS	25-49
IV	RESULTS	50-69
V	DISCUSSION	70-78
VI	SUMMARY	79-80
	REFERENCES	81-98
	APPENDICES	99-107

LIST OF TABLES

Table No.	Title	Page No.
2.1	PPO reported in various plants	8
2.2	Various proteins expressed in <i>Pichia pastoris</i>	22
3.1	Various components of reaction mix for ligation in pGEM-T easy vector.	28

LIST OF FIGURES

Figure No.	Title	Page No.
1.1	A generalized reaction scheme catalysed by PPO	2
2.1	Generalized reaction scheme for catecholase activity	6
2.2	Domain structure of tyrosinase from different groups conserved Cu binding sites (CuA and CuB).	9
2.3	Schematic representation of binuclear copper site.	10
2.4	Generalized diagram of transit peptides that target proteins to the thylakoid lumen of chloroplasts.	11
3.1	Sequence of PPO gene specific primers.	25
3.1.1	Sequence of vector specific primers.	25
3.2	Diagrammatic representation of pGEM-T easy vector.	27
3.3	Expression vector pPIC9.	33
4.1	Gel photograph showing amplification of PPO.	50
4.2	Gel photograph showing restriction digestion of recombinant pGEM-T Easy vector with <i>Eco</i> RI	51
4.3	Gel photograph showing digestion of recombinant pGEM-T vector and pPIC9 vector with <i>Xho</i> I and <i>Not</i> I.	52
4.4	Colony PCR showing PPO insert in <i>Pichia</i> expression vector pPIC9.	53

Figure No.	Title	Page
4.5	Agarose gel electrophoresis of restriction digested plasmid DNA.	53
4.6	Nucleotide sequence of PPO cDNA cloned in expression vector pPIC9.	54
4.7	Gel photograph showing linearization of recombinant expression plasmid pPIC9 with <i>Sac</i> I, <i>Sal</i> I and <i>Bgl</i> II.	55
4.8	Gel photograph showing linearized recombinant expression plasmid eluted from agarose gel.	56
4.9	Photograph showing growth of <i>Pichia</i> transformant on His deficient media.	57
4.10	Screening of <i>Pichia pastoris</i> for methanol utilization phenotype.	58
4.11	Gel photograph showing PCR analysis of <i>Pichia</i> integrants using genomic DNA.	59
4.12	SDS PAGE analysis of supernatant of positive <i>Pichia</i> transformants of various time intervals.	60-61
4.13	SDS PAGE analysis of pellets from <i>Pichia</i> transformants taken at various time intervals.	62
4.14	SDS PAGE analysis of ammonium sulphate precipitated total protein.	63
4.15	Diagrammatic representation of PPO enzyme assay.	64
4.16	SDS PAGE showing His tag purified PPO.	66
4.17	Bar diagram showing comparison between specific activity of crude, negative control as well as purified sample	67-68
4.18	Native in gel activity analysis of purified PPO.	69

LIST OF PLATES

PlateNo.	Title	Page No.
2.1	<i>Camellia sinensis</i> : Source of Tea PPO	5
	<i>Pichia pastoris</i> : system for heterologous expression of proteins	

ABBREVIATIONS:

%	Percent
°C	Degree centigrade
Amp	Ampicillin
AOX	Alcohol oxidase
APS	Ammonium per sulphate
bp	Base pairs
BSA	Bovine Serum Albumin
BMGY	Buffered minimal glycerol
BMMY	Buffered minimal methanol
cDNA	Complimentary DNA
CS PPO	<i>Camellia sinensis</i> PPO
CVD	Cardiovascular diseases
DNA	Deoxyribo nucleic acid
dNTP	Deoxyribo nucleotide triphosphate
EC	Enzyme classification
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra-acetic acid
EGCG	Epigallocatechin gallate
Fig.	Figure
g	Gram
h	Hour
<i>HIS4</i>	Hitidinol dehydrogenase gene
His	Histidine
IHBT	Institute of Himalayan Bioresource Technology
i.e.	Idest (that is)
kbp	Kilobase pairs
kD	Kilo Dalton
LB	Luria bertani
MCS	Multiple cloning site
μl	Micro litre
Min	Minutes
mm	Milli liter
Mg	Milli gram
mM	Mili molar
MD	Minimal Dextrose
MM	Minimal Methanol
Mut ⁺	Methanol utilization positive phenotype
Mut ^s	Methanol utilization slow phenotype
OD	Optical density
P	page

pp	pages
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
<i>P.pastoris</i>	<i>Pichia pastoris</i>
PPO	Polyphenol oxidase
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolution per minute
SCP	Single celled protein
Sec	Second
SDS	Sodium dodecyl sulphate
<i>Taq</i>	<i>Thermus aquaticus</i>
TEMED	N,N,N',N' tetramethyl ethylenediamine
Tet	Tetracycline
TF	Theaflavin
TR	Thearubigin
U	Units
YPD	Yeast Peptone Dextrose

INTRODUCTION

1. INTRODUCTION

Polyphenol oxidase is a ubiquitous enzyme present in diverse species like bacteria, fungi, vertebrates, insects and all higher plants (Mayer, 1987). Polyphenol oxidase has been studied frequently since their discovery. It was first reported in 1895 by Bertrand (Bertrand, 1895; Bertrand, 1896), while studying mushroom *Russula nigricans*, the cut flesh of which turned red and then black on exposure of air (Seo *et al.*, 2003). Subsequent discovery of polyphenol oxidase in wheat bran in 1907 (Bertrand and Muttermilch, 1907), then called tyrosinase, had been the subject of intensive investigation. Kubowitz 1937 (Bonner, 1957) succeeded in preparing polyphenol oxidase with 50% purity from potato. Working on the same line Keilin and Mann (Bonner, 1957) prepared a pure PPO from *Agaricus campestris*. The physical and chemical nature of PPO is being studied not only for its most visible impact i.e. browning in fruits and vegetables, but also to understand its physiological role, structure, and its industrial applications for the benefit of human kind.

Polyphenol oxidases are the nuclear encoded copper containing metalloproteins which catalyze the hydroxylation of monophenols to o-diphenols (EC 1.14.18.1; commonly called as monophenol monooxygenase, tyrosinase, cresolase) and / or dehydrogenation of o-dihydroxyphenols to o-quinines (EC 1.10.3.1; commonly called as diphenol oxygen oxidoreductase, catecholase). The labile quinines formed in the PPO reaction may react either together to yield melanoid pigments, or react with amino acids

or proteins to form brown colored complexes called as “tannins” (Mason and Peterson, 1965; Walker, 1975; Nicholas *et al.*, 1994) or the melanin.

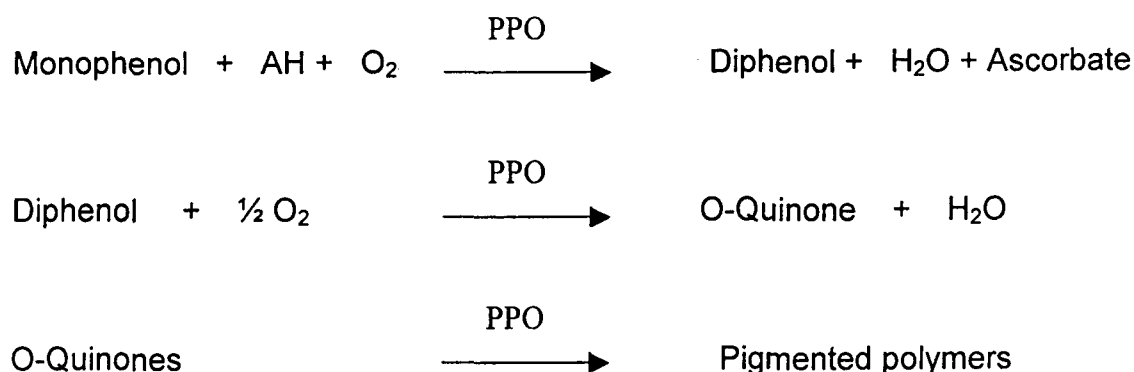


Fig 1.1: A generalized reaction scheme catalyzed by PPO (adapted from Stafford, 1974)

PPOs are ubiquitous among the angiosperms (Yu, 1992), but absent from the citrus family (Robb, 1984). In healthy green plants, it is exclusively present in the chloroplast on the thylakoid membrane with their cytochemical reaction products accumulating in the lumen of thylakoid (Golbeck *et al.*, 1981), or may exist in lumen as soluble polypeptide (Sommer *et al.*, 1994). Besides, it is also present in certain non-green vesicles such as amyloplasts in potatoes.

The physiological function of PPOs in plants is still unclear and is yet to be determined. Although PPO is thought to participate in various plant functions like plant defense against insects and plant pathogens (Mayer and Harel, 1979; Mayer 1987; Constabel *et al.*, 1995; Thipyapong *et al.*, 1995; Li and Steffens, 2002), it may function in pseudocyclic photophosphorylation and regulation of plastidic oxygen levels (Vaughn

and Duke, 1984; Trebst and Depka, 1995), its role has been investigated in respiration as it's a redox enzyme (Kubowitz, 1938; Boswell and Whitig, 1938 and its also involved in pigment formation in *Phytolacca americana* and *Antirrhinum majus* (Joy *et al.*, 1995; Nakayama *et al.*, 2000). Genes encoding PPO have been isolated and characterized from a wide range of plants like potato, tomato, grapes, apples, phytolacca, spinach, apricot, sugarcane and banana. These generally belong to multigene family (Cary *et al.*, 1992; Newman *et al.*, 1993; Hind *et al.*, 1995; Goldman *et al.*, 1998; Constable *et al.*, 2000; Haruta *et al.*, 2001., Stewart *et al.*, 2001; Gooding *et al.*, 2001). A high degree of sequence conservation has been found in all the investigated PPOs. All PPO genes encode mature proteins of molecular weight ranging from 52 kD – 62 kD with 8 to 12 kD transit peptides responsible for the transport of enzyme into the thylakoid lumen.

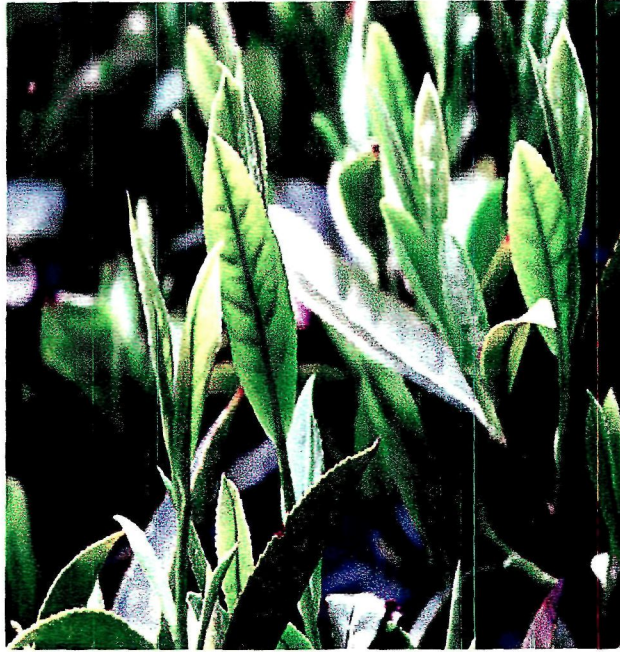
PPOs isolated from various sources have different substrate affinities. The substrate affinity is found to depend on the abundance and type of substrate present in a given source and whether PPO has monophenolase or diphenolase or both the activities. PPO from apple and mung bean has highest affinity for 4-methylcatechol. PPO from coffee and pear has greatest affinity for chlorogenic acid while banana has affinity for dopamine. Tea PPO has maximal affinity towards the catechins. Substrate affinity of tea PPO towards catechins and gallocatechins is known to play an important role in the synthesis of black tea pigments theaflavins (TF) and thearubigins (TR). These two pigments are antioxidants of high commercial demand apart from imparting the characteristic color, flavor and strength to black tea.

Though polyphenol oxidase is known for its deleterious effect in the browning of fruits and vegetables and other food products but PPO plays a central role in black tea

processing, where it catalyzes conversion of catechins into TF and TR which imparts characteristic color and strength to black tea. TF and TR so produced are antioxidants, anticarcinogenic, antibacterial and deodorizer properties and have great implications in human health. PPO imparts golden brown color in dried fruits as raisins, prunes, dates and figs (Marshall *et al.*, 2000). PPO has been used as a potential tool to determine genetic variations among various isolates in fungal species (Bruhn *et al.*, 1998). PPO has also been used to develop biosensors. PPO catalyses the oxidation of phenol to o-quinone by dioxygen, phenols, several biosensors based on PPO were elaborated for phenols detection (Russel and Byrton, 1999; Stanca *et al.*, 2003).

Expression of PPO in a suitable expression system provides an opportunity of its production allowing its use for medicinal and other industrial purposes. In the present thesis an effort was made to develop heterologous expression system of PPO in *Pichia pastoris* so that the enzyme is available independent of tea plant, which, otherwise, is the crop of commerce. Realising no reports on the expression of plant PPO protein in *Pichia pastoris* the following objectives were laid down in the present research programme:

1. To express polyphenol oxidase in yeast *Pichia pastoris*.
2. To purify and characterize the expressed protein.



***Camellia sinensis*: Source of Tea PPO.**



***Pichia pastoris*: System for the Heterologous Expression of PPO.**

REVIEW OF LITERAURE

2. REVIEW OF LITERATURE

2.1) Polyphenol oxidase in plants:

Polyphenol oxidases (PPO) are ubiquitous plastid associated plant enzymes and known for enzymatic browning in fruits and vegetables. PPO are nuclear encoded (Lax *et al.*, 1984, Walker and Ferrar, 1998) copper containing oxidoreductases enzymes (Kubowitz, 1938 as cited in Malkin and Malonstorm, 1970) having specificity for a variety of substrates.

There are two categories of PPO: one called as catechol oxidase, catecholase, ortho-diphenol oxidase (o-DPO), o-dihydroxyphenol oxidase, PPO and tyrosinase. This form is involved in oxidation of o-dihydroxy phenolics to the corresponding o-quinone (called 'catecholase' activity). Some of the forms of this enzyme from plants exhibit a monohydroxylating 'cresolase' activity (Zawistowski *et al.*, 1991). Other category of enzyme catalyses the oxidation of both o- and p-dihydroxy phenols but does not exhibit monohydroxylase activity. This enzyme is referred to as p-diphenol oxidase (p-DPO) and commonly called as laccase.

These two enzymes in the polyphenol oxidase category are referred as: catechol oxidase or o-diphenol: oxygen oxidoreductase (EC.1.10.3.1) and laccase or p-diphenol: oxygen oxidoreductase (EC 1.10.3.2) by the International Commission on enzymes. But later on both these enzymes were grouped together as monophenol oxygenase EC 1.14.18.1 (Mayer and Harel, 1979). As the substrate specificities for the enzymes were different the International nomenclature was revised again. Monophenol monooxygenase

(tyrosinase), which exhibits cresolase activity, was referred as EC 1.14.18.1, catecholase activity was renamed as diphenol oxygen: oxidoreductase (EC 1.10.3.2) and laccase activity as EC1.10.3.1 (Mayer, 1987; Walker and Ferrar, 1998).

Usually in plants PPO exhibit catecholase activity (EC1.10.3.2) so therefore plant PPOs are better called as catechol oxidases.

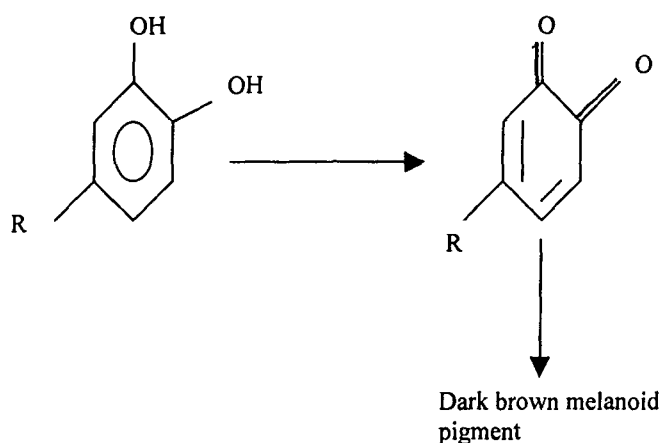


Fig. 2.1: Generalized reaction scheme for catecholase activity.

2.2) Occurrence:

Polyphenol oxidase in plants have been extensively studied since their initial discovery by Bertrand in 1895 in mushroom, *Russula nigricans*, (Bertrand, 1895), who initially proposed the name tyrosinase for this oxidase (Baker and Nelson, 1943). PPO has been shown to be almost universally present in varied and diverse number of species like bacteria, fungi, insects, vertebrates and plants (Mayer, 1987). All the angiosperms (Mayer and Harel, 1979) except citrus fruits (Robb, 1984) and some other higher plants contain polyphenol oxidase. Sherman *et al.*, 1991 performed a survey on the phylogenetic distribution of PPO. A wide spectrum of higher plants was included in this survey and spectrophotometric assays, PAGE enzymatic activity stains, and

cytochemistry analysis was performed to determine the presence of PPO in a given species. Among the algae *Coleochaete* and *Nitella* (charalean algae) showed polyphenol oxidase activity in trace amounts. PPO activity was not detected in any of the mosses. PPO was commonly present in *Anthoceros*, *Hepaticas* (Bryophytes), ferns and fern allies except in two species of *Riccia*. In gymnosperms only few species for example *Pinus*, *Zamia floribunda* and *Taxodium* spp. were reported to show PPO activity.

2.3) Location of Polyphenol oxidase:

Polyphenol oxidases are intracellular enzymes (Mayer, 1979). In green plants it is localized in chloroplasts on the thylakoid membrane (Vaughn and Duke, 1981; Golbeck *et al*, 1981; Vaughn *et al* 1988) or may exists in the lumen as soluble polypeptide (Sommer *et al.*, 1994; Sokolenko *et al.*, 1995; Kieselbach *et al.*, 1998). It's location within the thylakoids has been confirmed in *Aegopodium* (Vaughn *et al*, 1981). Further evidence of chloroplastic nature of PPO comes from work with the enzyme in *Vicia faba* (Flurky *et al.*, 1985). In other plastid types, PPO are present in various types of vesicles; either tubular (root leucoplast of carrot) or more oval-shaped e.g. those in cotton epidermal plastids (Vaughn, 1981) and leucoplasts of trichomal and epidermal cells in solanaceous species (Yu *et al*, 1992). Although nearly all plastid types have PPO, plastids in some tissues have no active PPO: bundle sheath plastids in NADP⁺- malic enzyme type C4 plants, guard cell plastids (Vaughn *et al.*, 1981) and hypocotyls cells of certain tissue layers in cotton (Muller and Bechman, 1978).

2.4) Sequence characteristics of plant polyphenol oxidases:

A number of *PPO* genes and / or cDNAs have been isolated and characterized as summarized in table 2.1. Coding sequence of PPO generally ranges from 1.8 kb to 2.2 kb encoding protein of 58 to 72 kD.

S.No.	Plant	Tissue source	Size of PPO (kb)	Protein (kDa)	Reference
1	Apricot (<i>Prunus armeniaca</i>)	Young fruit	2.2	67.1	Chevalier <i>et al.</i> , 1999
2	Banana(<i>Musa cavendeshii</i>)	Young fruit	2.07	-	Gooding <i>et al.</i> , 2001
3	Broad bean (<i>Vicia faba</i>)	Mature leaves	2.2	65	Cary <i>et al.</i> , 1992
4	Grapes(<i>Vitis vinifera</i>)	Immature berries	2.2	67	Rathjen <i>et al.</i> , 1992; Dry and Robinson,1994)
5	Spinach (<i>Spinacia oleracea</i>)	Leaves	2.5	64	Hind <i>et al.</i> ,1995
6	Tomato(<i>Lycopersicon esculentum</i>)	Leaves	-	58-60	Shahar <i>et al.</i> , 1992; Newman <i>et al.</i> , 1993
7	Sugarcane (<i>Saccharum officinarum</i>)	Stem	2.2	67	Bucheli <i>et al.</i> , 1996
8	Hybrid poplar(<i>Populus deltoids</i>)	Wounded leaves	2.1	54	Constabel <i>et al.</i> ,2000
9	Tobacco	Stigma/style	2.3	57.5	Goldman <i>et al.</i> , 1998
10	Potato	Young leaves	2.0	67	Hunt <i>et al.</i> , 1993
11	Alfalfa	-	3	-	Sullivan <i>et al.</i> , 2003

Table 2.1: Polyphenol oxidase reported in various plants.

2.5) Domains of PPO:

Basically polyphenol oxidase can be divided into three domains a) the central domain containing two copper binding site a) CuA and CuB b) N-terminal domain and c) transmembrane domain.

The central copper-binding domain is highly conserved domain within different PPO genes. This copper-containing domain not only does share sequence homology within plants but also within different taxas like with haemocyanins in mollusk and arthropods (Jackman *et al*, 1991). In addition other conserved domains can be identified for each group (Fig.2.2)

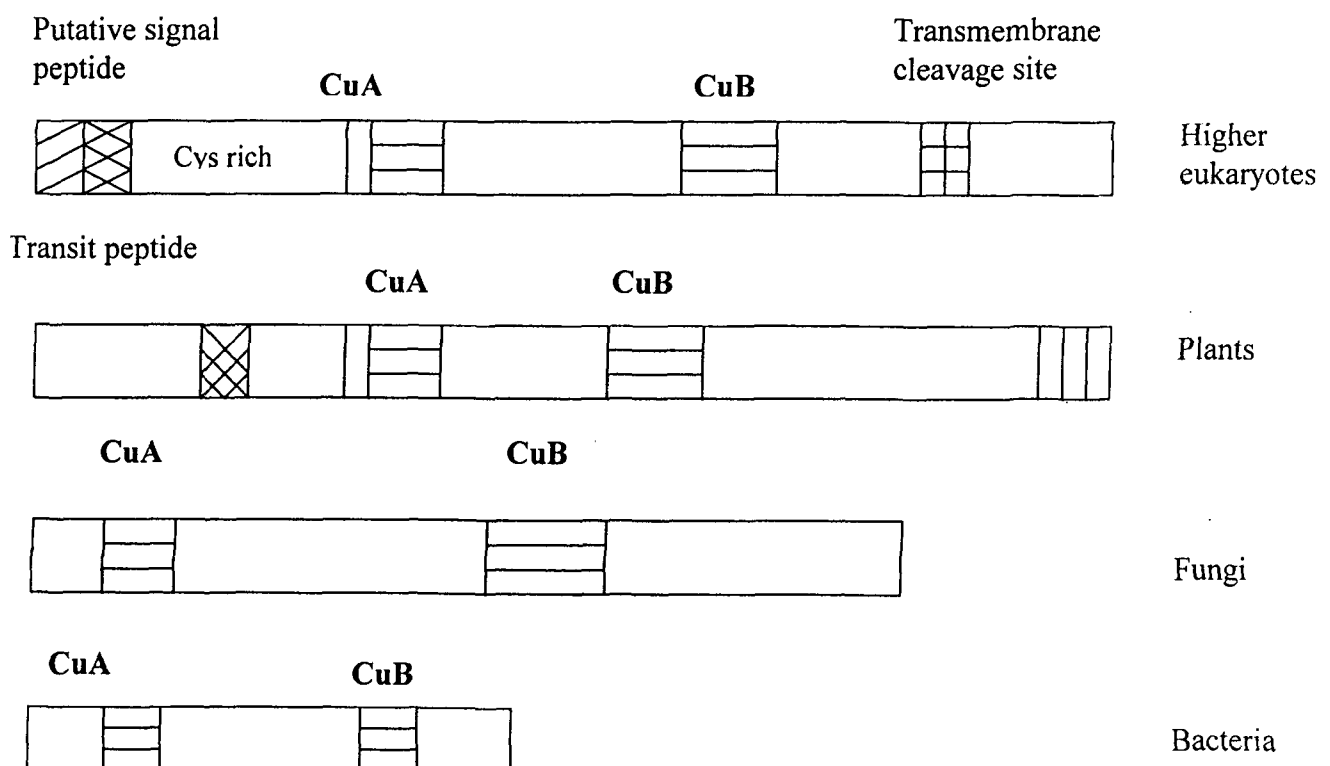


Fig 2.2: Domain structure of tyrosinases from different groups conserved Cu binding sites (CuA and CuB) are indicated.

2.6) Copper binding site: the central domain of PPO

All PPO sequences contain two copper binding sites CuA and CuB. A pair of copper ions constituting the active site of the enzyme is bound by conserved three histidine residues (Jackman *et al.*, 1991), which interact with both molecular oxygen and its phenolic substrate as shown in fig 2.3.

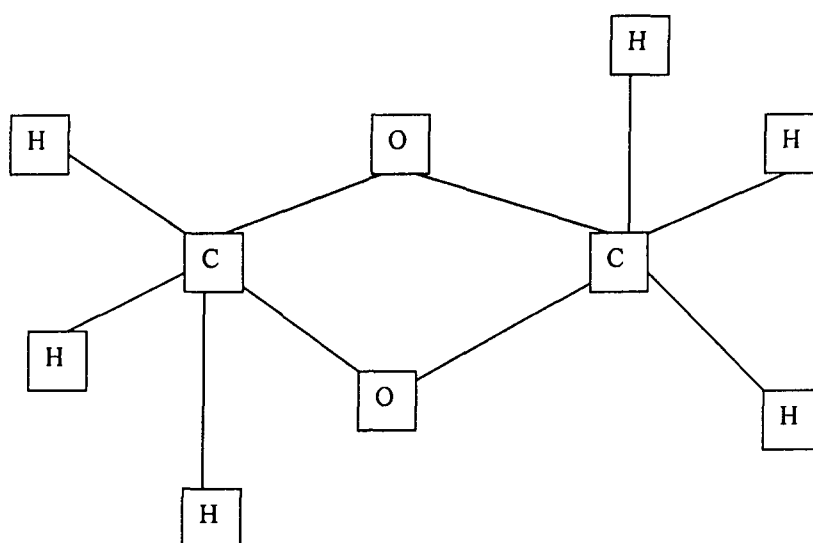


Fig 2.3: Schematic representation of binuclear copper site. C=Cu ion, O = oxygen, and H = His

The CuA region is more highly conserved than CuB region whereas the histidine residues are totally conserved in both the domains. Plant PPOs, in addition to the known copper binding domains among all species, have a third histidine rich domain at the C terminus of each sequence (Hunt *et al.*, 1993; Joy *et al.*, 1995). Van Gelder *et al.*, 1997 reported that apart from three histidines in each copper binding site, several amino acids are also conserved in CuA and CuB amino acids sequences aligned from plants, fungi, bacteria, amphibians and human.

2.5.2) N-terminal domain:

Higher plant PPOs possess transit peptides which direct the protein to chloroplast for further processing and post translational modifications. These transit peptides are characterized by high amounts of hydroxyl amino acids, serine (20%) and threonine and lack of negatively charged amino acid (de Boer *et al.*, 1991). In the amino terminal portion of transit peptide there are high proportions of hydrophilic amino acids, typically of chloroplast transit proteins. Before the site of cleavage of transit peptide, there is a hydrophobic domain of about 22 amino acids preceded by two arginine residues which is conserved in all PPO transit peptide sequences and is characteristic of lumen targeted peptides.

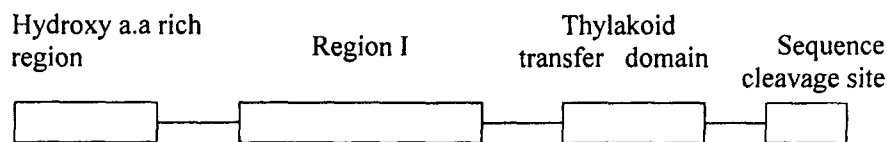


Fig 2.4: Generalized diagram of transit peptides that target proteins to the thylakoid lumen of chloroplasts.

2.5.3) Transmembrane domain:

In most of the higher plants PPO is membrane bound. Rodriguez and Flurkey, 1992 showed that detergent extraction of the tissue chloroplasts increase the activity and / or release of PPO from the membrane. Two out of seven tomato PPO genes showed two predicted α -helices in the C-terminal region which anchors the protein in the membrane suggesting presence of transmembrane domain in C terminal (Newman *et al.*, 1993).

2.5.3) Reaction mechanism:

Three different states of active site of PPO have been reported by Eicken *et al* (1999): the oxidized Cu (II) containing met form, the oxygenated oxy form and reduced Cu (I) containing deoxy form.

The whole reaction begins with the oxidized met form following the isolation of the enzyme. The enzyme accomplishes the oxidation of monophenols by oxygen, as it passes through its various enzymatic/ catalytic states(E_{deoxy} , E_{oxy} , $E_{\text{oxy-M}}$, and $E_{\text{met-D}}$); in cycle II, o-diphenols are oxidized as the enzyme passes through five enzyme states (E_{deoxy} , E_{oxy} , $E_{\text{oxy-D}}$, E_{met} and $E_{\text{met-D}}$). The two reaction led to the formation of o-quinones, which spontaneously react with each other to form oligomers (Sanchez *et al.*, 1995).

2.5.5 Genetics of PPO:

Lax *et al* (1984) compared the isozyme pattern of *Nicotiana* spp with their hybrids and found that inheritance of various PPO isoforms could be explained assuming that PPO is nuclear encoded. Another possibility is that the differences in PPO isozymes are due not to primary difference in structural genes but to other nuclear gene products that modify PPO. Thus, the idea that isoforms are the result of proteins which modify PPO secondarily is not unreasonable. Heating of the samples, raising the concentration of detergent or running the samples at room temperature tend to converge PPO forms in most plants. Treatments that result in the loss of chloroplast ribosomes or the mutants deficient in chloroplast ribosome were carried out to confirm that PPO is nuclear encoded. Vaughn *et al.*, 1981 found that a mutant of *Aegopodium podagraria* lacked 70s chloroplast ribosome but still retained PPO activity.

2.5.6) Multiplicity of PPO:

PPO displays a great disparity in its molecular weight (Robb *et al.*, 1965; Ganesha *et al.*, 1992). As PPO belongs to multigene family slight variations in molecular mass may result from differential expression of *PPO* gene family. Apart from genetic basis for the multiplicity it is also affected by other factors like import, targeting and processing of products of various gene members and secondary reactions between PPO enzyme and its products which result in the production of denatured, modified and cross- linked enzyme protein. Sometimes non-specific degradation of the protein results in the production of isolated PPO (Robinson *et al.*, 1992). Existence of PPO in latent state could also be one of the reasons of its multiple forms (Tolbert *et al.*, 1973; Mayer and Harel, 1979; Mayer, 1987).

2.6) Physiochemical properties of PPO:

2.6.1) Substrate specificity:

Phenolics are the primary substrates of PPO. Different types and concentration of natural phenolics vary widely for different plant sources e.g. catechin is a major phenolic compound found in grapes (Lee, 1987) and tea (Halder *et al.*, 1998), chlorogenic acid in apple (Murata *et al.*, 1995), potato (Sanchez-Ferrar *et al.*, 1993), and sunflower (Raymond *et al.*, 1993). Yang *et al* (2000) and Sojo *et al.*, 1998 reported dopamine as substrates of PPO in banana.

2.6.2) pH effects in PPO activity:

The pH optimum of PPO varies widely with plant source but is generally in the range of 4.0-8.0. The optimum pH is influenced by a number of factors as extraction methods, temperature, nature of phenolic substrate, buffer system used during determination (Whitkar, 1994).

2.6.3) Temperature effect on PPO activity:

The optimum temperature of PPO varies with different plant sources. PPOs from apple (Zhou *et al.*, 1993), banana (Yang *et al.*, 2000) and mango (Robinson *et al.*, 1993) have temperature optima of 30° C while PPO from cocoa bean (Lee *et al.*, 1991), tea (Halder *et al.*, 1998), sunflower (Raymond *et al.*, 1993) show 45 ° C optima. PPOs from strawberry (Serraadell *et al.*, 2000) have relatively high optima temperature of 50.

2.7) Physiological functions of Polyphenol oxidase:

2.7.1) Plant respiration:

Boswel and Whiting (1938) were first to study the role of tyrosinase in respiration in potato. They observed a sudden rise in rate of oxygen uptake upon addition of catechol. In 1979 Mayer and Harel showed that the cyanide insensitive respiration of some plants may be mediated by PPO.

2.7.2) Role in phenolics biosynthesis:

PPO is involved in the hydroxylation of many phenolic compounds in higher plants (Butt, 1985). As PPO enzymatically carry out many of these reactions *in vitro*, it has been

assumed that it is involved in synthesis of these compounds *in vivo* (Mayer and Harel, 1979). But the enzyme is normally bound to, or associated with, plastids (Vaughn and Duke, 1984) whereas phenolics are usually either vacuolar or sequestered in special cells (Mayer and Harel, 1979). Hence its role in phenolics biosynthesis could not be established.

2.7.3) Role in photosynthesis:

As polyphenol oxidase is located in the thylakoids of mature chloroplast and being a redox enzyme, it was suggested that it may have a role in the Mahler reaction (Vaughn and Duke, 1984; Vaughn *et al.*, 1988). PPOs involvement in Mahler reaction (pseudo cyclic photophosphorylation) was first of all suggested by Tolbert (1973). In 1976 Elstner *et al* proposed a diphenol–quinone cycle generating super oxide radicals powered by photosystem I and PPO activity in undamaged cells. Lax and Vaughn (1991) also suggested a possible structural and functional role in electron transport chain of photosystem.

2.7.4) Role in rooting of cuttings:

Hahlbrook and Grisebach (1979) reported that PPO indirectly regulates synthesis of phenolics and also play a role in organization and development of root primordial. Bassuki *et al*, 1981 found that PPO generated compounds from the flavanoid phloridzin enhanced rooting of cuttings of apple. According to Huystee and Cairns (1982) PPO also plays an important role in cell division, differentiation and primordium development.

Involvement of PPO activity in the beginning and length of walnut rooting phases confirmed its role in root development (Rios *et al.*, 1997)

2.7.5) Role in defense and disease resistance:

PPO activity has been associated with disease resistance and defense against insects and plant pathogens (Vaughn and Duke, 1988). The cellular damage results in the loss of PPO latency, decompartmentalization of the phenols and production of bactericidal and fungicidal hydroxyphenolics and quinones which may polymerize to seal off infected tissues. Constabel *et al* (2000) showed that PPO activity increased in poplar as a result of wounding and herbivory by leaf eating insects thus supporting the hypothesis that PPO is an important compound of the defense against leaf eating insects. Zinkernagel (1986) suggested that PPO might also be involved in oxidative detoxification of pathogen produced phytotoxins. Li and Steffens (2002) used transgenic tomato plants with modified PPO expression. These transgenic tomato plants under the control of Cauliflower mosaic Virus (CaMV) 35 S promoter constitutively over expressed a potato PPO. When these plants were exposed to bacterial pathogen *Pseudomonas syringae* pv. tomato exhibited increase in resistance towards the pathogen and there was less severity of disease as compared to control plants. Thus it proved the role of PPO in disease resistance.

2.8) Polyphenol oxidase: an industrial; / bioprospecting enzyme:

PPO is usually associated with browning reactions in fruits, vegetables and food products and hence its action causes considerable economic and nutritional loss

commercially. But PPO activity is very much desirable in tea, coffee, raisins and cocoa where it is the key factor involved in synthesis of organoleptic properties (Van Gelder *et al.*, 1997; Lee 1991). PPO plays an important role in tea processing where it catalysis conversion of catechins into theaflavin and thearubigins imparting a characteristic colour to black tea.

2.9) Polyphenol oxidase from Tea:

PPO has been purified and characterized from a large number of species such as potato (Marri *et al.*, 2003), apple (Janovitz-Klapp *et al.*, 1990), broadbean (Flurky, 1985; Robinson *et al.*, 1992), grape (Harel and Mayer, 1971), pear (Smith and Montgomery, 1985), spinach (Golbeck and Cammarata, 1981), carrot (Soderhall and Soderhall, 1989) mung bean (Shin *et al.*, 1997), banana (Yang *et al.*, 2001), tea (Gregory and Bendall, 1966; Halder *et al.*, 1998), coffee (Mazzafera and Robinson, 2000), sweet potato (Eicken *et al.*, 1998) and iceberg lettuce (Chazzara *et al.*, 2001). And most of these studies were carried out to study the kinetic properties of PPO. These studies reveal wide and significant variation in substrate specificities of PPO from various sources. No PPO except tea was found to show maximal affinity towards catechins and gallocatechins. These catechins are converted into pigments called theaflavin and thearubigins by PPO which are antioxidants with a very high commercial value apart from imparting characteristic colour, flavour and strength to black tea

Various workers as Sanderson, 1964; Gregory *et al.*, 1966; Takeo and Baker, 1972; Halder *et al.*, 1998 and Pruidze *et al.*, 2003 studied various kinetic properties of tea polyphenol oxidase. In various such studies molecular weight of PPO from tea was

measured by sedimentation and diffusion which was found to be 144000 ± 16000 , copper content was 0.32%, optimum pH was 5.7. Inhibition of activity by tropolone confirmed the presence of Cu^+ in the active site of the enzyme (Halder *et al.*, 1998).

Gregory *et al.*, 1966 showed that tea PPO differs from tyrosinases and laccases in its ability to oxidize monohydric phenols either by hydroxylation or by dehydrogenation. Thus PPO in tea readily oxidized o-diphenols as catechol and is highly specific towards catechin and epicatechins.

2.9.1) Potential medical applications of Tea:

It has been known since time immemorial that tea may exert a number of physiological effects on humans. Caffeine one of the noticeable components in tea, has been under a wide range of investigations and thought to be responsible for many of tea effects, beneficial or undesirable. In addition to caffeine, other components of tea, especially the polyphenol, have been proved by modern biomedical research to possess a variety of physiological pharmacological effects. The understanding on the biological effects of tea and its components especially polyphenol oxidase and related mechanism of action has been rapidly expanded, particularly over the last two decades.

2.9.1.1) Antioxidant and anticarcinogenic activity:

Free radicals produced in our body cause oxidative damage to sensitive biological structures, such as DNA and cell membranes and can lead to cancer, heart ailments and other autoimmune disease (Vendemiale, *et al.*, 1999; Kanter, 1998) although our body contains its own repair and antioxidant system but it still requires supplementation

by antioxidants like vitamin C, E, flavonoid etc. present in fruits vegetables and tea. Flavonoids in tea have been reported to show 20 times more powerful antioxidant activity than vitamin C (Vinson *et al.*, 1995). Green tea components were shown to be the most potential oxidants against various forms of tumors. Green tea catechins have been shown to inhibit tumor cell proliferation as well as promote the destruction of leukemia cells (Smith and Dou, 2001)

2.9.1.2) Antimicrobial activity of tea:

Medical books written as early as in the Song dynasty (960-1279 A.D.) in China mentioned that green tea in combination with ginger can effectively cure dysenteric disorder, including those so called red and white in appearance (Lin, 1992). Modern medical research has demonstrated that tea and tea products are active against a wide range of microorganisms, implying that tea may be potentially useful for the treatment of some infectious illness. A wide range of pathogenic bacteria like *Staphylococcus aureus*, *Shigella dysenteriae*, *Salmonella typhosa*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and others are inhibited by green and black tea.

2.9.1.3) Deodorising activity:

The anticaries activity of tea has drawn much attention. Tea product has shown inhibitory activity against mutants *Streptococci* and glucosyltransferase. Mutant *streptococci* that are known to synthesize glucans have been implicated as the primary causative agent of caries (Hamada *et al.*, 1984). Two types of glucans, water soluble and water insoluble are synthesized by two different groups of glucosyltransferases in

these bacteria. The water insoluble glucan is highly adhesive to tooth surface resulting in the formation of dental plaque. The bacteria grow in the dental plaque, metabolize various sugars there and produce organic acids, especially lactic acid, which retains in the plaque, decalcify the tooth enamel and develop dental caries (Koga *et al.*, 1986). Several catechins, the components from green tea, are active against carcinogenic bacteria. In addition tea polyphenols inhibit water insoluble glucan synthesis catalyzed by glucosyltransferase from *streptococci* mutant. Tea polyphenol especially EGCG, have been found to be active against a series of viruses under experimental conditions.

2.9.1.4) Preventing cardiovascular diseases (CVDs):

Polyphenols inhibit the oxidative modification of lipoprotein cholesterol which is known to be involved in the development of atherosclerosis. Thus tea helps in reducing the risk of cardiovascular diseases.

2.10) *Pichia pastoris* expression system:

Pichia pastoris is a highly successful system for the production of wide variety of recombinant proteins. As yeast, *P. pastoris* is unicellular microorganism that is easy to manipulate and culture. Being eukaryote it is capable of many post translational modifications performed by higher eukaryotic cells such as proteolytic processing, folding, disulphide bond formation and glycosylation. Thus many proteins that end up as inactive inclusion bodies in bacterial system are produced as biologically active molecules in *P. pastoris*. The *Pichia pastoris* system is also generally regarded as being faster, easier and less expensive to use than expression system derived from higher

eukaryotic systems as insects and mammalian tissue culture cell systems and usually give higher expression levels.

2.10.1) History of *Pichia pastoris* expression system:

The ability of certain yeasts to utilize methanol as a sole carbon source and energy was discovered less than 40 years ago by Koichi Ogata (Cregg *et al.*, 1993). Because methanol could be inexpensively synthesized from natural gas, there was immediate interest in exploiting these organisms for generating yeast biomass or single celled protein (SCP) to be marketed. During 1970s, Phillips Petroleum Company developed media and methods for growing *Pichia pastoris* on methanol in continuous culture at high cell densities. In early 1980s, Salk institute of Biotechnology, in La Jolla, California, developed *Pichia pastoris* gene expression system. Researchers at SIBIA isolated *AOX1* gene and developed vectors, strains and methods for molecular genetic manipulation of *Pichia pastoris*. The combination of strong regulated expression under control of *AOX1* promoter along with fermentation media and methods developed for SCP process resulted in strikingly high levels of foreign proteins in *Pichia pastoris*.

2.10.2) *Pichia pastoris* as a methylotropic yeast:

The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde, generating hydrogen peroxide in the process, by the enzyme alcohol oxidase. This first step in methanol metabolism takes place within a specialized organelle, called the peroxisome, which sequesters toxic hydrogen peroxide away from the rest of the cell.

2.10.3) Secretion of heterologous proteins:

A large number of heterologous proteins have been expressed in *Pichia pastoris* as shown in table 2.2:

Proteins	Expression levels grams/litre	Expression	Reference
Invertase	2.3	Secreted	Tschopp <i>et al.</i> , 1987
Hepatitis B surface antigen	0.55	Intracellular	Cregg <i>et al.</i> , 1987
Tetanus toxin	12.0	Intracellular	Clare <i>et al.</i> , 1991
Tumour necrosis factor	10.0	Intracellular	Sreekrishna <i>et al.</i> , 1989
Enterokinase	0.021	Secreted	Vozza <i>et al.</i> , 1996
Rabbit single chain antibody	> 0.1	Secreted	Ridder <i>et al.</i> , 1996
Alpha amylase	2.5	Secreted	Paifer <i>et al.</i> , 1994
Mouse serotonin receptor	0.001	Secreted	Weiss <i>et al.</i> , 1995
Pertussis Antigen P69	3.0	Intracellular	Romanos <i>et al.</i> , 1991
HIV-1 gp 120	1.25	Intracellular	Scorer <i>et al.</i> , 1993
Kunitz protease inhibitor	1.0	Secreted	Wagner <i>et al.</i> , 1992
Ghilanten	0.01	Secreted	Brankamp <i>et al.</i> , 1995

Table 2.2: Various proteins expressed in *Pichia pastoris*

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3. Materials:

3.1. Buffers and solution:

Details of buffers and solutions used are given in Appendix-1.

3.1.2) Molecular biology Kits:

Molecular biology kits that were used for DNA isolation, gel extraction, yeast cloning / expression and protein analysis are listed along with their protocols in Appendix-2.

3.1.3) Strain and plasmid:

Pichia pastoris strain GS115 and vector pPIC9 was purchased from Invitrogen Corporation (San Diego, CA, USA) for cloning and expression of gene.

3.1.4) Culture medium:

A) Maintenance media for *E. coli*:

Luria bertani for liquid media and Luria-agar (LB broth and 1.5% agar) was used for maintaining *E.coli* strain TOP10 F' in liquid and solid culture media.

B) Different media used for yeast:

B.a) Maintenance media for yeast (GS115 strain):

GS115 strain was streaked on YPD (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar) plates.

B.b) Media used for screening of yeast transformants:

MD (minimal dextrose) and MM (minimal methanol) containing 1.34% YNB; 4×10^{-5} % biotin; 2% dextrose for MD and 0.4% methanol for MM was used for screening of *Pichia pastoris* transformants.

B.c) Induction media for *Pichia pastoris*:

Protein induction was carried out in Buffered methanol glycerol complex (BMGY) and buffered methanol minimal complex (BMMY) having 1% yeast extract; 2% peptone; 100mM potassium phosphate buffer (pH 6.0); 1.3% YNB (without amino acid); 4×10^{-5} biotin; 1% glycerol for BMGY or methanol for BMMY.

3.2 Method:

3.2.1) Primer designing for cloning tea polyphenol oxidase (PPO) gene into expression vector:

Specific primers were designed on the basis of coding sequence of PPO gene of tea (*CS PPO*) containing restriction enzyme sites *Xho* I and *Not* I for the in-frame cloning of *PPO* gene in the expression vector pPIC9. In addition to that 6 x His tag sequence was also added to facilitate protein purification (Fig 3.1).

Contents	Quantity (μ l)
Autoclaved dH ₂ O	18.5
10 x PCR buffer	2.0
dNTPs (10 mM)	0.5
Forward primer (10 mM)	1.0
Reverse primer (10 mM)	1.0
Plasmid	1.0
Taq polymerase (5 U/ μ l)	0.25
Total volume	25.0

Thermocycler was programmed at 94° C, 3 min $\longrightarrow$ 94°C, 30 sec $\longrightarrow$ 45°C, 30 sec $\longrightarrow$ 72° C, 2 min $\longrightarrow$ 94° C, 30 sec $\longrightarrow$ 50° C, 30 sec $\longrightarrow$ 72° C, 2 min and PCR product was stored at 4°C. PCR product was run on 1% agarose gel in 1 x TAE buffer with an appropriate DNA size marker. The gel was viewed and captured on a gel documentation system (Alpha innotech corporation, USA).

3.2.3) Elution of the amplified products

The amplified product was excised along with agarose gel using a sterile blade and was extracted from agarose gel using GenEluteTM gel extraction kit (Sigma). The detailed procedure is given in Appendix-2.

3.2.4) Cloning of the eluted cDNA fragment in pGEM-T easy vector

Gel purified PCR product was cloned into a pGEM-T Easy Vector (Promega, USA) (Fig 3.2) which is a thymidine tailed vector. This vector exploits template-independent activity of thermostable polymerase, which preferentially adds a

single adenosine nucleotide to the 3' end of double stranded DNA, thus allowing cloning using A and T bonding.

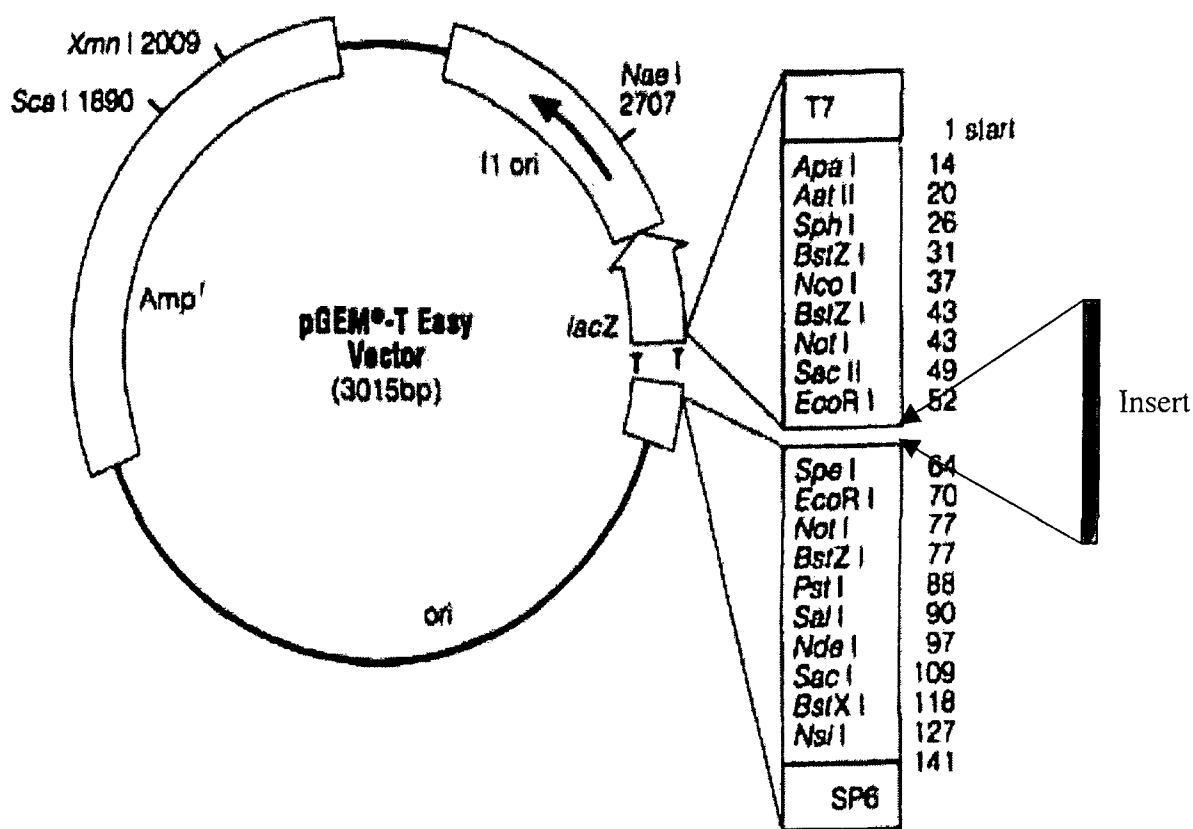


Fig. 3.2: Diagrammatic representation of pGEM-T Easy vector (Promega, USA), showing restriction sites in multiple cloning site and a unique 3' T overhang which is compatible to 5' A overhang in PCR products. Inserts in this vector can be sequenced by T7 and SP6 primers.

3.2.5) Ligation and transformation of PCR amplified product:

Amplified PCR product was ligated in pGEM-T Easy vector (Promega Inc, USA) and appropriate amount of vector and insert (usually equimolar) was mixed and ligation was set up. The components were given in table 3.1

Components	Amount
DNA(PPO insert)	3.0 μ l
Vector	1.0 μ l
2 x T4 DNA ligase buffer	5.0 μ l
T4 DNA ligase (3 Weiss U)	1.0 μ l
Total reaction volume	10 μ l

Table 3.1: various components of reaction mix for ligation

The reaction was incubated at 4°C for overnight.

3.2.6) Preparation of competent cells and their transformation:

- LB (250 ml) containing suitable antibiotic was inoculated with 1 ml of overnight culture of *E.coli* and grown at 37°C on a shaker till the O.D. 600 reached 0.5.
- Culture was cooled on ice immediately and cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C in SS-34 rotor.

- Supernatant was removed carefully and traces of supernatant were removed by inverting the centrifuge tube on paper towels.
- The tube was not removed from ice for long. The bacterial cells were resuspended in 50-70 ml of ice-cold 0.1M CaCl_2 (sterilized by filtration or autoclaving) and incubated on ice for 30 min.
- Cells were recovered by centrifugation at 6000 rpm and resuspended in 50-70 ml of 0.1M MgCl_2 and again incubated on ice for 30 min.
- The cells were finally recovered and suspended in 10 ml of 0.1 CaCl_2 containing 10% glycerol.
- Aliquots of 200 μl each were prepared and immediately stored at -70°C . For transformation, the cells were taken out from the deep freezer, thawed on ice for 10 min and the DNA to be transformed was added to it.

Transformation of *E. coli* (TOP10 F')

- Frozen competent cells were thawed on ice for 5-10 min.
- Ligated product (10 μl) was mixed gently with competent cells (200 μl) and the mixture was incubated on ice for 30 min.
- The cells were given heat shock at 42°C for 90 sec and immediately transferred to ice for at least 5 min.
- Luria broth (800 μl) was added and tube was incubated at 37°C for 1 h in a rotary shaker set at 200rpm.

- The cells were pelleted down and 800 µl LB was discarded. Pellet was resuspended in remaining broth and plated on luria agar plates supplemented with ampicillin and tetracycline.
- Plates were incubated at 37°C for 12-16 h till colonies are big enough for transfer.

3.2.7) Plasmid isolation by boiling lysis mini prep method (Holmes and Quigley, 1981):

Transformed colonies were picked and inoculated on LA-ampicillin-tetracycline plates with the help of sterile toothpick and same was used for the inoculation of 5ml LB-amp-tetra media. These plates and tubes were incubated overnight at 37°C.

- Overnight grown culture (1.5 ml) was pelleted down in a microfuge by centrifugation at 14,000 rpm for 30 sec.
- Supernatant was discarded and the pellet was resuspended in 110 µl of STET buffer.
- Freshly prepared 10.0 µl of lysozyme (10 mg / ml stock in 10 mM Tris (pH 8.0) was added to the resuspended cells and thoroughly mixed and incubated at room temperature for 5 min.
- Heat shock was given for 42 sec in boiling water followed by centrifugation at full speed for 20 min at room temperature.
- Pellet containing the cell debris was removed with the help of a sterile toothpick.

- DNA in the supernatant was precipitated by adding one volume of isopropanol (i.e. 120 μ l).
- Precipitated DNA was collected immediately by centrifuging for 20 minutes at 14,000 rpm.
- The supernatant was completely removed and the pellet was air dried to remove excess of isopropanol.
- DNA was resuspended in 50 μ l nuclease free water.

3.2.8) Restriction digestion of the plasmid for checking positive clones:

In order to find out positive clones among the transformed colonies plasmid were digested with *Eco* RI as follows:

Digestion mixture:

Components	Amount
Plasmid DNA (~2 μ g)	10.0 μ l
10 x Buffer H (Roche)	5.0 μ l
<i>Eco</i> RI (10 U/ μ l)	0.5 μ l
Water	34.5 μ l
Total	50.0 μ l

Procedure:

- The digestion mixture containing plasmid DNA, enzyme *Eco* RI and water was thoroughly mixed and centrifuged briefly.
- Mixture was incubated at 37°C for 3 h.
- Chilled ethanol (2.5 volumes) was added in each mixture and incubated at -20°C overnight for precipitation.
- Precipitated nucleic acid was centrifuged at 12,000 rpm for 20 min at 4° C.
- The supernatant was discarded pellet was dried and resuspended in RNase free water and 4 µl of dye and loaded on 1% agarose gel.

3.3) Directional cloning of amplified PPO gene in expression vector pPIC9:

PPO gene cloned in pGEM-T vector was then cloned in expression vector pPIC9 which is a shuttle vector of 8023bp designed for transformation into both *E. coli* and *P. pastoris*. For maintenance in *E. coli*, the vector contains an origin of replication and an ampicillin resistance gene. As a selectable marker for *P. pastoris*, vector contains the histidinol dehydrogenase (*HIS4*) gene. Unique feature of the vector is the presence of α -secretion signal sequence, which helps in secretion of the recombinant protein extracellularly. It also contains unique restriction site (*Sac* I, *Bgl* II and *Sal* I etc) that permits linearization of vector for efficient integration in *P. pastoris* genome.

Comments for pPIC9:
8023 nucleotides

5' *AOX1* promoter fragment: bases 1-948
 5' *AOX1* primer site: bases 855-875
 a-Factor secretion signal (S): bases 949-1218
 a-Factor primer site: bases 1152-1172
 Multiple Cloning Site Region: bases 1192-1241
 3' *AOX1* primer site: bases 1327-1347
 3' *AOX1* transcription
 termination (TT) fragment: bases 1253-1586
HIS4 ORF: bases 4514-1980
 3' *AOX1* fragment: bases 4870-5626
 ColE1 origin: bases 6708-6034
 Ampicillin resistance gene: bases 7713-6853

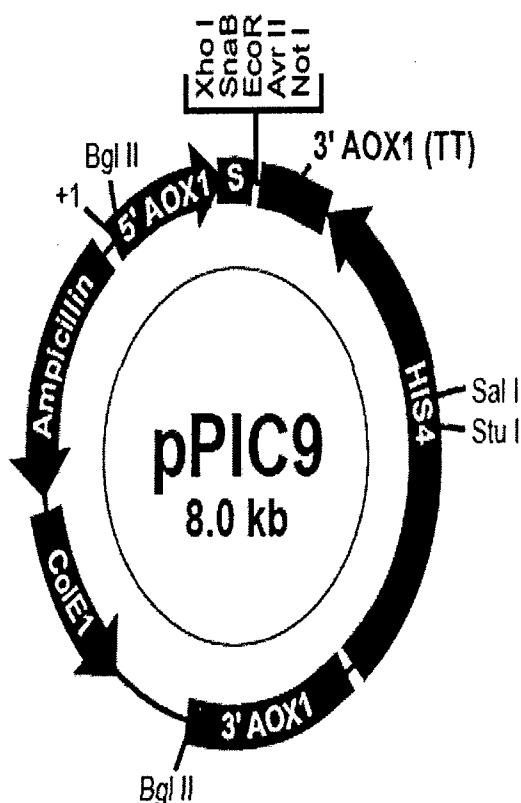


Fig 3.3: Expression vector pPIC9 vector map

Features of pPIC9:

- 5' AOX I promoter fragment: 1-948 bases.
- 5' AOX I primer site: 855-875 bases.
- Multiple cloning site: 1192-1241 bases.
- 3' AOX I primer site: 1327-1347.
- 3' AOX I transcription termination: 1253-1586 bases.

3.3.1) Double digestion of positive clones with *Xho* I and *Not* I restriction enzymes:

The positive plasmids having desired insert were again double digested with *Not* I and *Xho* I restriction enzymes along with parent expression vector for in-frame cloning of *PPO* gene in vector pPIC9.

Digestion mixture:

Components	Amount
Plasmid DNA (~2 µg)	10.0 µl
10 x Buffer H (Roche)	10.0 µl
<i>Xho</i> I (10 U/µl)	0.5 µl
<i>Not</i> I (10 U/µl)	0.5 µl
Water	29 µl
Total	50.0 µl

The procedure for restriction digestion was discussed earlier in section 3.2.8.

The digested bands of *PPO* and pPIC9 vector were eluted using GenElute™ gel extraction kit (Sigma, USA). All the centrifugation steps were performed at 13,000 rpm.

3.3.2) Ligation and transformation of *PPO* into pPIC9 vector:

The ligation reaction was set up as follows: -

Components	Amount
10 x ligation buffer	2.0 μ l
pPIC9 vector (2ng)	2.5 μ l
Eluted PPO fragment (6ng)	7.5 μ l
T4 DNA ligase (3 U/ μ l)	1 μ l
dH ₂ O	7 μ l
Total	20 μ l

✧ Mixture was incubated overnight at 16°C.

Transformation of the recombinant vector was carried out in *E.coli* as described in section 3.2.6.

3.3.3) Analysis of transformants through colony PCR:

Transformed colonies were randomly picked up using tooth pick. Single colonies were lysed in TE buffer by boiling in water bath and colony lysate were used as template for PCR using gene specific primers (PPO 1U & PPO 2D).

PCR reaction was set up as follows:

Contents	Quantity
Autoclaved dH ₂ O	14.3 μ l
10 x PCR buffer	2.0 μ l
dNTP (10 mM)	0.5 μ l
Forward primer (200 ng/ μ l)	0.5 μ l
Reverse primer (200 ng/ μ l)	0.5 μ l
Colony lysate	2.0 μ l
Taq DNA polymerase (3 U/ μ l)	0.20 μ l
Total volume	20.0 μ l

Cycling parameters for gene specific primers were described previously under section 3.2.2.

3.3.4) Large scale plasmid isolation:

Large scale pure plasmid was isolated using Genopure plasmid isolation kit (Appendix 2). The purified plasmid was consequently used in sequencing and transformation experiments in yeast.

3.3.5) Automated DNA Sequencing:

Sequencing of the amplified cloned PPO fragment was carried out using automated DNA sequencing system (Applied Biosystems model ABI Prism 310)

Big Dye terminator cycle sequencing ready reaction kit (Applied biosystems, USA) was used for sequencing reactions.

The sequencing PCR was performed as follows:

Contents	Quantity
Plasmid(200-500 ng/μl)	1μl
5 x sequencing buffer	1μl
Terminator ready reaction mix	1.0 μl
Primer(1.66 pmol)	0.5 μl
Autoclaved water	1.5 μl
Total	5 μl

The thin walled PCR tubes (0.2 ml) containing the reaction mix was placed in Gene Amp PCR systems 9700 (Applied biosystems) and thermal cycling for 25 reactions was carried out as follows:

Denaturation	94°C	10 sec
Annealing	50°C	40sec
Amplification	60°C	4min

After the completion of reaction the sample was precipitated with ethanol, washed with injection buffer for removing surplus dyes and taken for sequencing by ABI PRISM™, 310 Genetic analyzer. Sequence obtained was then matched with that of presequenced *PPO* and pPIC9 to ensure in-frame cloning of *PPO* gene.

3.4) Transformation of *Pichia pastoris*

Linear DNA generates stable integrants of *Pichia pastoris* via homologous recombination between the transforming DNA and regions of homology within the genome. Such integrants show extreme stability so transforming DNA was linearized and then used for transforming yeast through electroporation. The yeast strain GS115 was used for transformation.

3.4.1) Preparation of plasmid DNA:

Plasmid isolated from genopure plasmid isolation kit was used for linearization. For isolating His⁺ Mut⁺ transformants of GS115, plasmid was linearised with *Sa*/I and *Sac* I and for isolating His⁺ Mut^s transformants of GS115 plasmid was linearised with *Bgl* II. Both the recombinant construct as well as the parent vector without insert were digested. Parent vector was digested to be used as background control for protein expression.

Linearization reaction setup was as follows:

1) *Sac* I and *Sa*/I restriction digestion

Components	Amount
<i>Sac</i> I(10 U/μl)	1 μl
10 x Buffer A (Roche)	5.0 μl
Plasmid	10 μl
Water	34 μl
Total	50 μl

2) *Bgl* II restriction digestion:

Components	Amount
<i>Bgl</i> II (10 U/ μ l)	1 μ l
10 x Buffer M (Roche)	5.0 μ l
Plasmid	10 μ l
Water	34 μ l
Total	50 μ l

Digestion steps were performed as described in section 3.2.8.

3.4.2. Electroporation

Transformation of *Pichia pastoris* with recombinant vector was carried out by electroporation method for the integration of the linearized vector into the yeast genome. The electrocompetent cells of yeast strain, GS115 were prepared and transformed as described in manufacturers manual (Invitrogen, USA). Following steps were carried out:

Preparation of electrocompetent cells:

1. *Pichia pastoris* strain GS115 (5ml) was grown in YPD in a 50 ml conical at 30°C overnight.
2. Inoculated 500ml of fresh YPD medium in a liter flask with 0.1-0.5 ml of the overnight culture and again grown overnight to an $A_{600} = 1.3-1.5$.

3. Cells were centrifuged at 1500 x g for 5 minutes at 4°C and the pellet was resuspended in 500 ml of ice-cold, sterile water.
4. Cells were again centrifuged at 1500 x g for 5 min at 4°C and then resuspended the pellet in 250 ml of ice-cold, sterile water.
5. Cells were centrifuged once again at 1500 x g for 5 min at 4°C and resuspended the pellet in 20 ml of ice-cold 1 M sorbitol.
6. Again the cells were centrifuged at 1500 x g for 5 min at 4°C and resuspended the pellet in 1 ml of ice-cold 1 M sorbitol for a final volume of approximately 1.5 ml.

Electrocompetent cells so prepared were kept in 80 µl aliquots and were used fresh at that time as the transformation frequencies of these electrocompetent cells decrease significantly with time

3.4.3) Transformation

- Competent cells (80 µl) so prepared were mixed with 5-20 µg of linearized DNA (in 5-10 µl TE Buffer) and then transferred to an ice-cold 0.2 cm electroporation cuvette.
- The cuvettes were incubated on ice for 10 minutes.
- Cells were pulsed accordingly:

Mode	HV
Voltage	1200-1500 V
Capacitance	25 µF
Resistance	200 ohms

- Immediately 1 ml of ice cold 1M sorbitol was added to the cuvette and contents were transferred to a sterile micro centrifuge tube.
- Aliquot (200-600 μ l) was spread on Minimal Dextrose (MD) plates and plates were incubated at 30°C 36-48 h.

3.4.4) Verifying the Mut⁺ (Methanol utilization) phenotype:

Transformed GS115 colonies were streaked on both Minimal Dextrose (MD) and minimal methanol (MM) plates. After 2 days incubation at 30°C, plates were analyzed for their methanol utilization phenotype by comparing their characteristics relative to control strains. Mut⁺ control strain, GS115/pPICZC//acZ shows normal growth on both plates, whereas control strain GS115 albumin is Mut^S and shows little or no growth on MM plate.

3.5) PCR analysis of *Pichia* integrants

Transformed colonies were then analyzed by using gene specific primers. For this genomic DNA of recombinant *P. pastoris* was first isolated as described by Ausubel *et al.* (1994) and PCR was carried out as follows:

3.5.1) Isolation of genomic DNA

- Liquid culture (10 ml) of yeast grown for 20 - 24 h at 30°C in YPD yeast was transferred into a micro centrifuge tube. Cells were pelleted by centrifugation at 1200 $\times$ g for 5 min.

- Cells were resuspended in 200 µl of breaking- buffer. After adding 0.3 g glass beads and 200 µl phenol/chloroform/isoamyl alcohol (25:24:1) cells were vortexed for 3 min.
- Added 200 µl of TE buffer to it and then vortexed for 2 min followed by centrifugation at 12000rpm for 5 min.
- Supernatant was transferred to a new tube and 1ml 100% EtOH was added and mixed by inversion.
- Centrifuged for 3min at 12000 rpm supernatant was discarded and the pellet was suspended in 0.4 ml TE buffer(pH 8) containing 3 µg /ml RNase
- After 5 min incubation at 37°C, 10µl of 4M NH₄OAc was added along with 1ml 100% EtOH and stored at -20°C for few hours.
- Precipitated DNA was isolated by centrifuging for 10 min at 13,000rpm and pellet was resuspended in 50 µl in TE buffer.

3.5.2) Genomic DNA PCR:

Genomic DNA was used as template for PCR reaction using gene specific primers. PCR conditions and cyclic parameters used for genomic DNA PCR have been described in section 3.2.2.

3.6) Expression of PPO in *Pichia pastoris*:

The PCR verified GS115 integrants were induced as described in manufacturer's manual (Invitrogen, USA). Cells were grown in Buffered minimal glycerol (BMGY)

medium until the culture reaches an A_{600} of approximately 2-6. Harvested cells were resuspended in Buffered minimal methanol (BMMY) medium and 0.5% methanol was added every 24 hours to maintain induction. Baffled flasks were used for expression, and cells were grown at 28-30°C at 250 rpm. In order to analyze the background extracellular expression, strain transformed with the parent vector pPIC9 without *PPO* gene insert was also induced. At defined time points samples were taken from the cultures for absorption measurements. In order to analyze extracellularly expressed proteins samples were pelleted and supernatants containing secreted soluble proteins were stored for further analysis.

3.6.1) Analysis by SDS-PAGE

Molecular weight of the expressed PPO was determined by SDS-PAGE.

Preparation of polyacrylamide gel (PAGE)

Composition of stacking gel and resolving gel are given below:

(i) Resolving gel/Separating Gel /lower gel Composition:

Components	7.5%	10%	12%	15%
Distilled Water	19.4 ml	16.05ml	13.4 ml	9.4 ml
10% SDS (w/v) stock	400 μ l	400 μ l	400 μ l	400 μ l
Tris-HCl (1.5 M; pH 8.8)	10 ml	10 ml	10 ml	10 ml
Acrylamide/Bis-acrylamide(30% stock)	10 ml	13.33ml	16 ml	20 ml
10% Ammonium persulphate	200 μ l	200 μ l	200 μ l	200 μ l
TEMED	20 μ l	20 μ l	20 μ l	20 μ l
Total	40 ml	40 ml	40 ml	40 ml

(ii) Stacking gel (5%), upper gel:

Distilled Water	6.1 ml
10% SDS (w/v) stock	2.5 ml
Tris-HCl (1.5 M; pH 8.8)	100 μ l
Acrylamide/Bis-acrylamide(30% stock)	1.3 ml
10% Ammonium persulphate	50 μ l
TEMED	10 μ l
Total	10.0 ml

Ammonium persulphate (APS) was prepared fresh (10%) and added to the gel solution just before pouring.

10%-12% resolving gel and 4-5% stacking gel was used to standardize the best separation. Above mentioned amount of components were mixed for both resolving and stacking gel (excluding APS and TEMED) solutions separately in a properly covered flask and the solutions were degassed for 20 min in a vacuum desicator before pouring to the gel assembly. After degassing, APS and TEMED were mixed properly to the resolving gel solution and immediately poured to the assembly, such that $2/3^{\text{rd}}$ of the assembly should be filled, the solution was over layered with 500 μ l of water saturated butanol to check aeration. It was then kept for polymerization at room temperature (20-30 min). After polymerization butanol was removed completely, APS and TEMED were mixed to the stacking gel solution and poured above the resolving gel. A comb of 10-13 wells was fitted in the assembly to make the wells so that 20-50 μ l of protein preparation can be

loaded in the wells. The assembly was kept for 1-2 hour at room temperature for proper polymerization. After the polymerization the gel assembly was kept in the running tank filled with 1x gel running buffer in both top and bottom chambers. Thereafter the comb was removed from the stacking gel carefully and washed with the running buffer properly.

Buffer composition for SDS PAGE has been described in Appendix 1

Sample preparation:

Supernatant was mixed with denaturing buffer (5 x loading dye). The samples were denatured in boiling water for 10 minutes and immediately chilled on ice for 5 min, centrifuged for 30 seconds to allow debris to settle down and then loaded onto gel. In case of pellet sample was prepared as follows:

- Cell pellets were thawed quickly and kept on ice.
- Pellets were resuspended in 100 μ l of breaking buffer.
- Acid washed glass beads (0.5 mm) were added to the pellet and vortexed for 30 sec.
- Sample was incubated on ice for 30 sec and the whole process repeated for 8 cycles.
- Finally sample was centrifuged at 14,000 rpm for 10 min at 4°C.
- Clear lysate was mixed with 50 μ l of 5 x SDS PAGE loading dye, boiled for 10 min and loaded onto gel after centrifuging for 30 sec at 12, 000 rpm.

Electrophoresis:

The wells were rinsed thoroughly before loading the samples and pre-run was carried out for 15 min at 25mA. Denatured protein samples were loaded into the wells. Electrophoresis was carried out in 1x electrode buffer at 15 mA in stacking gel and 25 mA in resolving gel until the bromophenol blue dye reached the bottom of the gel.

Silver staining of PAGE:

After electrophoresis gel was soaked in 50% methanol for 1 h in a glass dish. Staining solution was prepared by adding solution A dropwise to solution B with constant stirring and then making volume to 100 ml with deionised water (this silver stain should be prepared immediately before use). The gel was stained in this solution for 15 min with gentle agitation on a gel rocker in dark. Gel was washed with deionized water for 5 min with gentle agitation. The gel was then soaked in developing solution until band appears. After proper band development the gel was washed with deionised water and placed in 50% methanol to stop further reaction.

Solution A: 0.8 gm silver nitrate in 4 ml distilled water.

Solution B: Mix 21 ml of 0.36% NaOH with 1.4 ml of freshly prepared 14.8 ammonium hydroxide.

Developing solution: Mix 2.5 ml of 1% citric acid with 0.25 ml of 37% formaldehyde and make the volume to 500 ml (freshly prepared).

3.7. Estimation of PPO activity:

3.7.1) PPO enzyme assay:

- PPO activity analysis was carried out according to Halder *et al* (1998).
- Catechol (50 µl of 0.5 M), 445 µl of 0.5 M citrate buffer and 5 µl of induced sample (supernatant) having enzyme were placed in cuvette at room temperature.
- Increase in absorbance at 410 nm was recorded for 5-10 minutes and linear portion of curve was plotted for enzyme activity calculations.

One unit of enzyme activity was defined as the amount of enzyme required to cause a change in absorbance of 0.001 at 410 nm.

3.7.2) In gel activity analysis of PPO:

- For the in gel activity of PPO native PAGE without SDS was run. A small portion of the gel was first soaked in sodium citrate buffer (pH 7.0) for 5 minutes at room temperature.
- Buffer was poured off and methyl catechol was added for 2-3 minutes. Orange brown spots were observed corresponding to catechol oxidase.

3.7) Protein estimation:

Proteins from the supernatant were estimated following the procedure of Bradford method (Bradford, 1976) using BSA as standard.

3.8) Purification of PPO using Ni-NTA columns:

Recombinant *P. pastoris* were induced with methanol as described previously under section 3.6. 6 x His tagged protein was purified by immobilized metal affinity chromatography (IMAC) using a prepacked column of nickel-chelated agarose. PPO secreted into medium contain 6 × His sequence which binds to nickel chelated agarose. After a thorough washing to remove unbound proteins, the His-tagged attached protein were eluted and recovered from the column with an imidazole buffer (Pierce, Belgium).

Purification was set up as follows:

- Nickel Chelated Column was uncapped and the Sodium azide storage solution was allowed to drain from the gel bed. The column stopped flowing when the liquid level reached the top disc.
- Gel bed was prepared by adding 10ml (2 × 5ml) of B-PER reagent (Contains 20mM Tris buffer, pH7.5)
- Applied upto 10 ml of sample (2 × 5ml) to the column and allowed it to flow through the gel bed.
- Column was washed by adding 6ml (2(3ml) of wash buffer1 (Contains 35mM Tris, 150mM NaCl, 10mM imidazole, 5% glycerol, pH 7.2 and 0.5 x concentration of proprietary B-PER reagents additives) and allowed it to flow through the gel bed.
- Column was washed by adding 3 x 3ml of wash buffer2 (Contains 50mM Tris, 300 mM NaCl, 25mM imidazole, 10% glycerol, pH 6.8).

- Eluted the 6 x His-tagged protein by adding 2ml of elution buffer (Contain 50mM Tris, 300 mM NaCl, 200mM imidazole, 10% glycerol, pH6.8)
- Eluted protein was then analyzed by SDS PAGE and in gel PPO assay as described under section 3.6.1 and 3.7.

RESULTS

4. RESULT

4.1 Amplification and cloning of Polyphenol oxidase (PPO) gene in *Pichia pastoris*

For cloning of CS *PPO* gene in *P. pastoris*, it was strategized to adopt a two step process. First step was to amplify the gene and clone into pGem-T easy vector. In second step the gene was excised, subcloned into vector pPIC9 (the *Pichia* expression vector). The recombinant plasmid was selected in *E.coli* and after confirmation the purified DNA was used for transformation of *P. pastoris*.

PPO was amplified from the original construct containing the tea (*Camellia sinensis*) *PPO* (CS *PPO*) gene using the primers designed with restriction enzyme sites *Xho* I, *Not* I and His tag and an amplified product of 1.8 kb fragment corresponding to *PPO* was obtained by PCR (Fig 4.1).

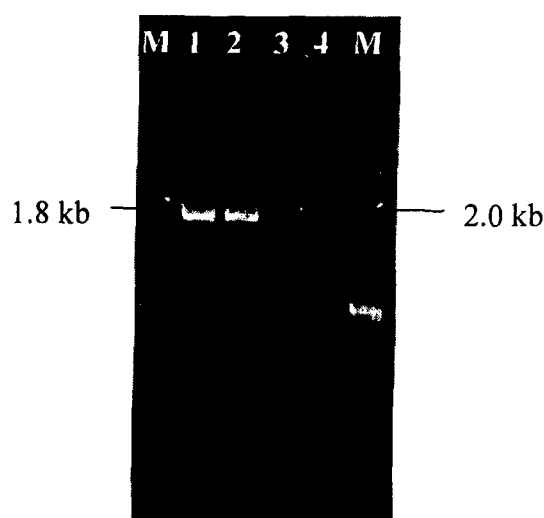


Fig 4.1: Gel photograph showing amplified PPO. Lane M: Low range 3 kb DNA ladder; lane 1, 2 3, 4: amplified PPO ~1.8 kb

The fragment was cloned into pGEM-T easy vector (Promega, USA) and transformed into TOP10 F' strain of *E. coli*. Plasmids were isolated from transformants and checked by restriction digestion with *Eco* RI. Insert size of ~ 1.8 kb was obtained upon restriction digestion with *Eco* RI (Fig 4.2) which confirmed the presence of insert of the desired size into recombinant *E.coli*.

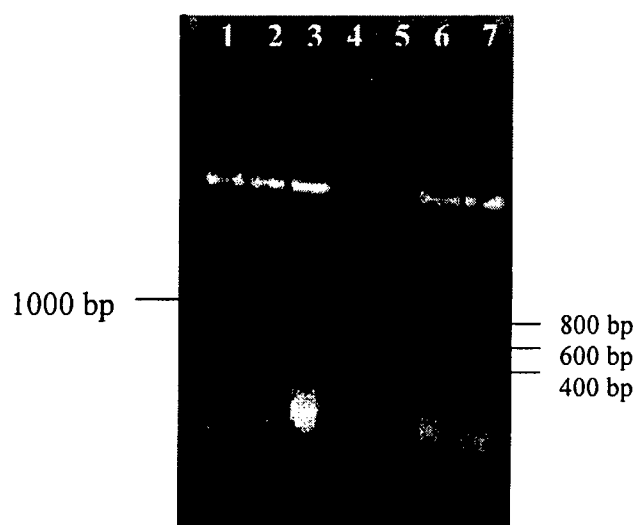


Fig 4.2: Gel photograph showing digestions of recombinant pGEM- T easy vector having PPO insert. Lane 4: 100 bp marker; lane 3, 6: positive clones showing total insert size of ~1.8 kb corresponding to PPO

4.2) Cloning and transformation of recombinant expression vector into *E. coli*:

PPO gene was isolated from recombinant pGEM-T easy vector by digestion with *Xho* I and *Not* I restriction enzymes. Gel showed the digested product of PPO as well as parent vector pPIC9 in pGEM-T vector (Fig 4.3)

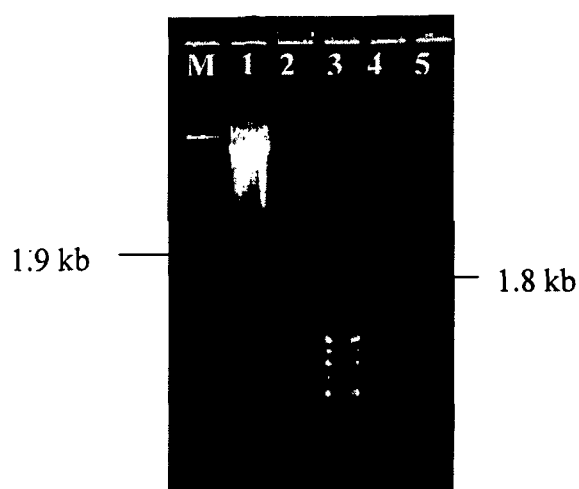


Fig 4.3: Gel photograph showing digestions of recombinant pGEM- T easy and pPIC9 vector with *Xho* I and *Not* I having PPO insert. Lane M: Lambda DNA marker *Eco* RI / *Hind* III digested; lane 2, 4: positive clones showing 1.8 kb PPO insert; lane 1: digested expression vector pPIC9.

The digested PPO fragment and pPIC9 expression vector were excised from the gel, eluted and transformed in TOP10 F' strain of *E. coli* selected on ampicillin and tetracycline Luria agar plates Transformants were also screened by colony PCR using gene specific primers. Results showed the presence of an insert of ~1.8 kb corresponding to PPO gene as expected in the expression vector (Fig 4.4).

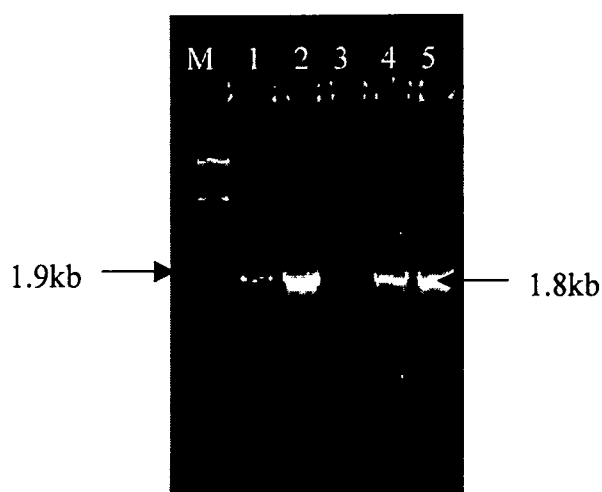


Fig 4. 4: Colony PCR showing PPO insert in *Pichia* expression vector pPIC9. Lane M: Lambda DNA Eco RI / Hind III digested marker. Lane1, 2, 4, 5: positive clones having PPO insert (~1.8kb)

Further confirmation of positive clones was carried out by restriction digestion of plasmid isolated from the positive clones. Digestion with appropriate enzymes yielded an insert of 1.8 kb from above clones (Fig 4.5).

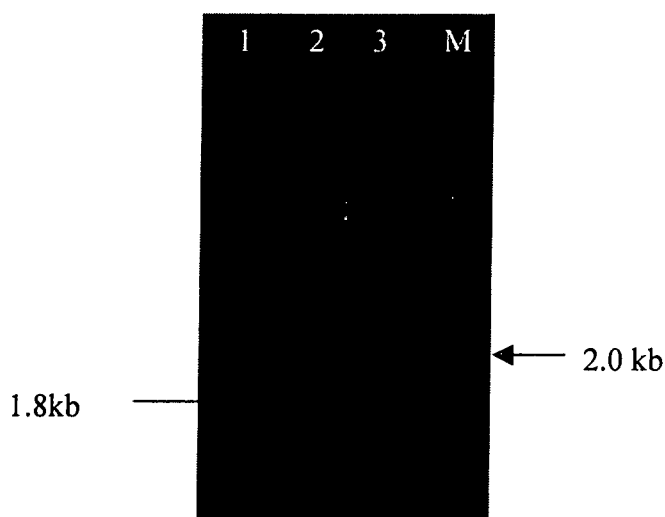


Fig 4.5: Agarose gel electrophoresis of restriction digested plasmid DNA from recombinant clones. Lanes 1, 2, 3: 1.8 kb insert corresponding to PPO gene in positive clones of pPIC9. Lane M: Fast ruler marker (Fermentas, Lithuania)

4.3) Sequencing:

The sequencing of recombinant vector was performed using sequencing primers (5'AOX I' and 3'AOX I') to confirm in- frame cloning of PPO in expression vector. Sequencing of the above clone using AOX primers revealed the in-frame cloning of PPO gene in expression vector (Fig 4.6).

```

TTCCATTTACGACACTTGAGAAGTCAAAAAACAATAATTATTCGAAGGATCCAAACG
ATGAGATTTAAACAATTTTTACTGCAGTTTTATTTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAG
AAGATGAAACGGCACAATTCGGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTGATGTTGCTGTTT
TGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAG
GGGTATCTCTCGAGAAAAGAATGGCTTCCATTCTCCCTCCAACCACCACCAAGACTACCACCACCTCCTCCACCCCTCT
ATTCTTATCCCATTTTCAAGAACACCTCTAAAATTCCCACAATTAGAAAGCATAACCATTCCTTCAATGTGTCATGCA
GCAAAGCCAAAGATAGTGACCCAAACCTTACTCCCCCTCCCAAAATACACAAACCTCCCTAGGGAAGTTTGATAGGA
GAAACATGCTCATTGGCTTAGGAGGCCTCTACGGAGCAGCCGGCCTCACCGACACTGACCCACCAGCCTTGGCCGCTC
CGGTCACCGCCCCGGATTTATCCAAATGCGGGGCGGCGGACTTACCCGCGGATGCAAACCGACCAACTGTTGTCCCTC
CAAAAACCAACAAAATCATCGAATTCAGCTCCCTCCTCCCTCCAACATTTTACGAGCCGACCCGCGGCCCATTTAGC
CGACGAAAAATACATAGCCAAGTTTTCTAAAGCCCTCCAACCTCATGAAATCGCTCCCGATGACGACCCACGAAGCTT
CAAGCAACAATCCAATATTTCACTGCGCTTATTGCGAAGGCGCTTATCACCAAGTCGGTTTTCCAAGCACAGAACTCC
CAAGTTCACAACTCATGGCTCTTCTTTCCCTTTCCATAGATTCTATCTCTACTTTCTTCGAAAAAGATTTTGGGAATG
CTGCTCGATGATCCAGCGTTGCAATTCCTTTTTGGAATTGGGATTCTCCGGGCGGGCATGAAAATACCCGCCATTGTA
TTGCGGACATAAATTCGCCACTCTATAACGTCTCTGACGCACACAGCACGGACATAATTTGATCTTGACTACTATTTT
TGACCTGTGTATCTCCGA

```

Fig 4.6: Nucleotide sequence of PPO cDNA cloned in expression vector pPIC9. Sequence highlighted in pink is vector pPIC9 sequence while those highlighted with orange is *Camellia* PPO sequence and sequence highlighted with blue is alpha factor primer site.

4.4) Expression of PPO in *Pichia pastoris*:

4.4.1) Linearization of plasmid DNA:

For transformation of *Pichia pastoris*, linearized recombinant expression vector (having PPO insert) was required for recombination into yeast genome. For this purpose, sequence verified construct was isolated from *E.coli* TOP 10 F' cells using plasmid isolation kit (Sigma, USA). The isolated plasmid was digested with *Sac* I, *Sal* I and *Bgl* II separately for 3 hours. The parent vector without any insert was also linearized with these restriction enzymes as control. Results of restriction digestion with these enzymes are shown in Fig 4.7.

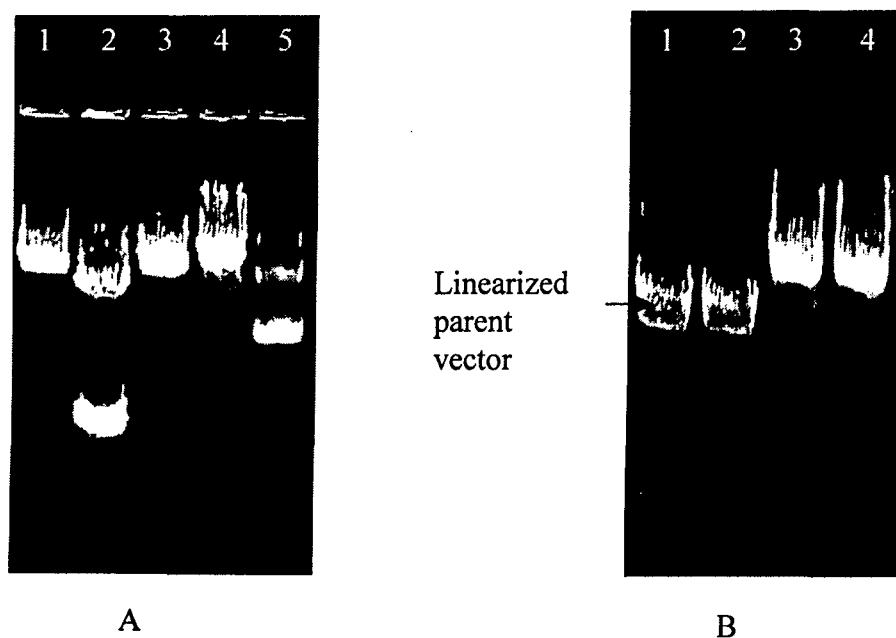


Fig 4.7: Gel photograph showing linearization of recombinant expression plasmid pPIC9 with *Sal* I, *Sac* I and *Bgl* II.

- (A):** Lane 1: *Sac* linearized r pPIC9- -PPO plasmid. Lane 2: *Bgl* II linearized pPIC9 plasmid. Lane 3, 4: *Sal* I linearized r- pPIC9 -PPO plasmid. Lane 5: unlinearized pPIC9 expression vector as control
- (B):** Lane 1: *Sal* I linearized parent vector for background control. Lane 2: *Sal* I linearized parent vector pPIC9 without insert. Lane 3, 4: *Sal* I linearized r- pPIC9 -PPO plasmid.

4.4.2) Cloning of PPO in *P. pastoris*:

The linearized plasmids were excised from the gel and eluted for yeast transformation (Fig 4.8)

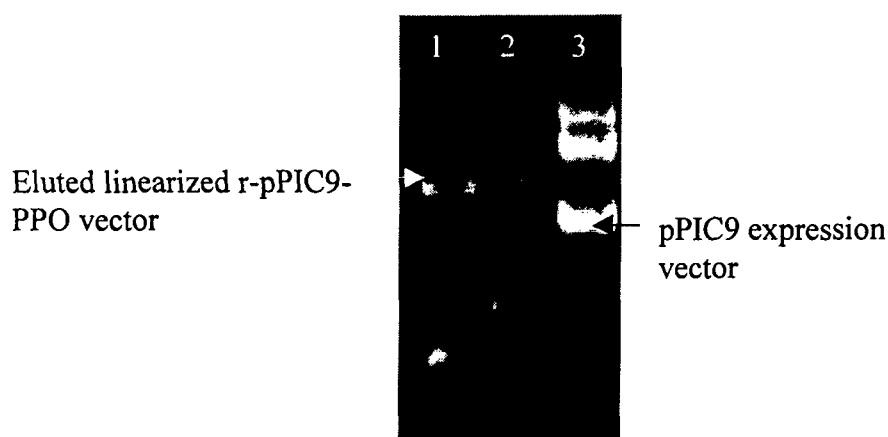


Fig 4.8: Gel photograph showing linearized recombinant expression plasmid (pPIC9-PPO) eluted from agarose gel. Lane 1, 2: eluted linearized plasmid. Lane 3: undigested parent plasmid (pPIC9).

4.4.3) Transformation of yeast *Pichia pastoris*

Electroporation method was used for the transformation of *Pichia pastoris*. After transformation plates were incubated at 30°C for 48 hours and colonies were obtained in all the cases (*Sac* I, *Sal* I and *Bgl* II digested vectors). The transformants were selected on MD (minimal dextrose) media without histidine. Strain GS115 of *Pichia pastoris* is histidine deficient but the expression vector pPIC9 is His⁺ and thus allows selection of transformants on His⁻ media after electroporation. Fig 4.9 shows the *Pichia*

recombination at the AOX / locus. Due to the disruption of AOX / Mut^s transformants are not able to produce alcohol oxidase and thus cannot efficiently metabolize methanol as their sole carbon source and so grow poorly on minimal methanol(MM) medium and therefore such transformants called as Mut^s and vice versa.

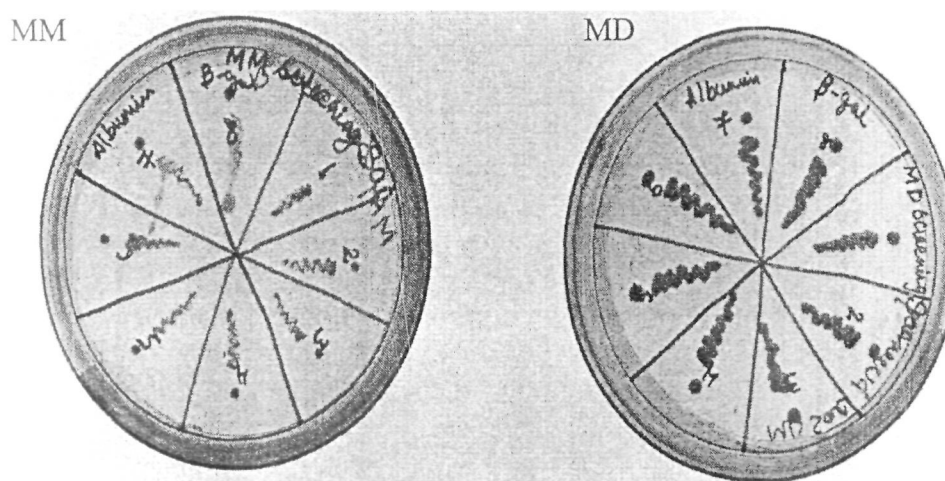


Fig: 4.10: Screening of *P. pastoris* transformants for methanol utilization (Mut) phenotype. Growth of colonies was compared to those of the control strains. *Pichia* transformants were found to be Mut⁺ they exhibited similar growth pattern to Mut⁺ GS115/pPICZC//lacZ control strain on MM media.

Lane 7: Mut⁺ GS115/pPICZC//lacZ control strain.

Lane 8: Mut^s GS115 Albumin control strain.

3,4,5 and 6: Mut⁺ *Pichia* transformants

4.4.5) Confirmation of *Pichia* transformants by genomic DNA PCR:

Total DNA was isolated from the transformants and was taken for amplification of the PPO gene using gene specific primers which were used for initial amplification of the gene.

4.4.5.1) PCR analysis of *Pichia* integrants:

To identify positive integrants, amplification of the gene of PPO was performed by gene specific primers. These primers amplified ~1.8 kb band corresponding to PPO gene insert in the total DNA from yeast (Fig 4.11)

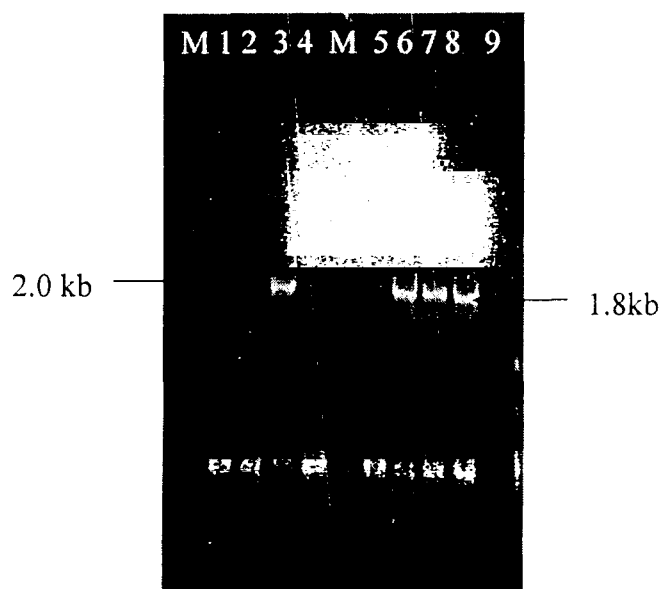


Fig 4.11: Gel electrophoresis showing PCR analysis of *Pichia* integrants using genomic DNA of recombinant transformants. Lanes M: fast ruler DNA marker (Fermentas). Lanes 3, 6, 7, 8: positive transformants having the desired PPO fragment ~ 1.8 kb (as shown by arrow). Lane 9: negative control (*Pich* transformed with parent vector without PPO gene.)

4.4.6) Protein induction and analysis by SDS PAGE:

Protein induction was carried out by addition of methanol to a final concentration of 0.5% every 24 hours to maintain the induction in BMMY media (Buffered Methanol-complex Medium) for 6 days at 30°C .1 ml sample was taken out at every 12 hours of induction to

analyze the expression levels by SDS PAGE post induction. Sample from *Pichia* transformed with parent vector without the PPO insert was also induced and taken as control. As has been described in construct preparation, due to the presence of secretion signal it is expected that the protein will come in the supernatant. So crude supernatant was analyzed for secreted expression by SDS PAGE. The optimum induction of protein was found at 60-72 hours post induction (Fig 4.12.1 and 4.12.2).

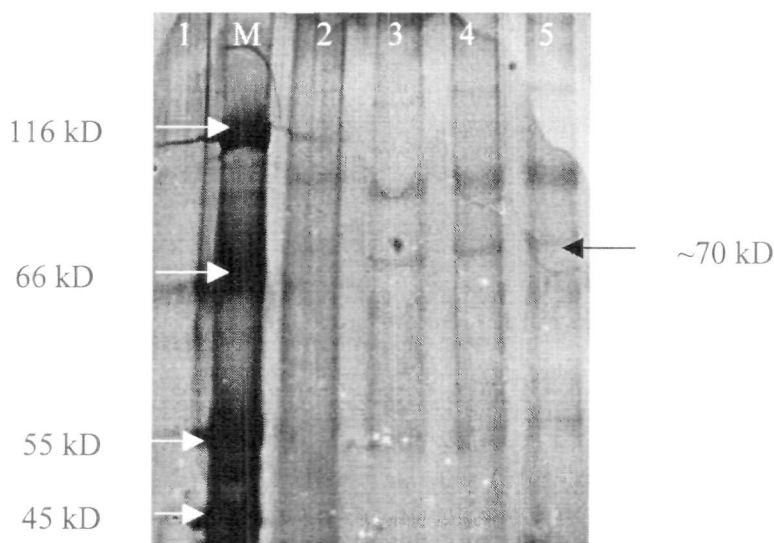


Fig 4.12.1: SDS PAGE analysis of supernatant of positive *Pichia* transformants of various time intervals. Lane 1: supernatant form zero hour induction. Lane 2-5 supernatant from 12, 36, 48, 60 hours induction respectively. Lane M: protein molecular

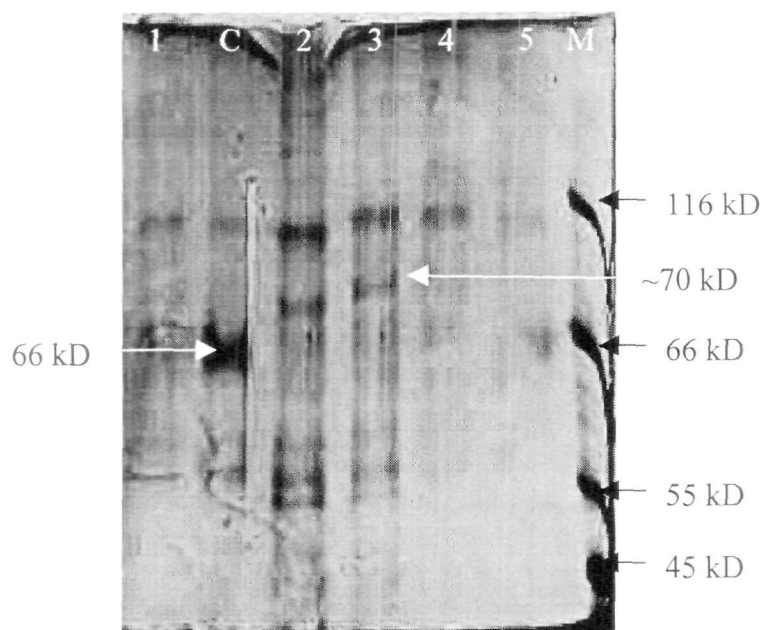


Fig 4.12.2: SDS PAGE analysis of supernatants from positive *Pichia* transformants taken at various time intervals. Lanes 1-5 show protein as expressed in the supernatant from samples taken at 12, 72, 84, negative control (vector without any insert) and zero hour respectively. Lane C: control albumin 66 kD protein as provided in the kit. Lane M: protein molecular weight marker (Fermentas, Lithuania).

4.4.7) Protein analysis from pellet:

Though due to presence of secretion signal, secreted expression is expected but in order to rule out any kind of possibilities of inclusion body formation, pellets of the induced samples were also sequentially checked. For the analysis of expressed protein, if any, in the pellets, they were lysed using breaking buffer and agitated using acid washed glass beads following incubation on ice for 30 seconds. The whole process was repeated for 8 times and finally the supernatant called as cell lysate obtained after centrifugation was mixed with gel loading dye and analyzed for PPO protein. Pellets from different time

induction were analyzed and it was found that there was no protein corresponding to PPO in the pellet (Fig 4.13).

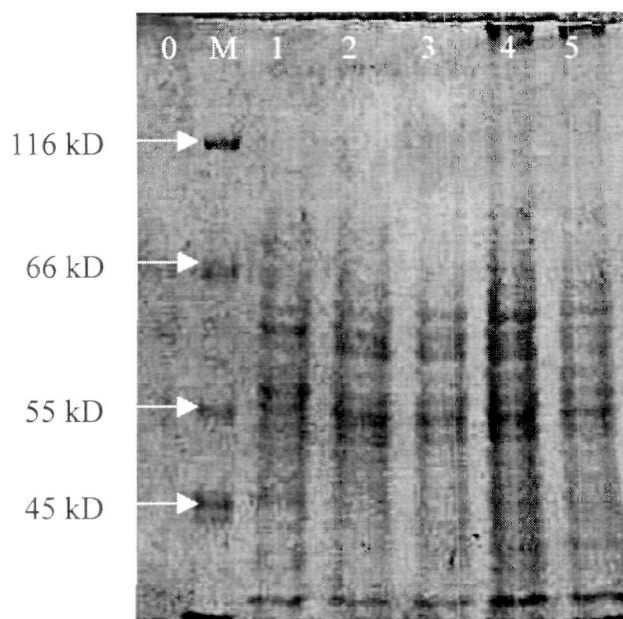


Fig 4.13: SDS PAGE analysis of pellets from positive *Pichia* transformants taken at various time intervals. Lanes 1-5 show protein as expressed in the pellet from samples taken at 24, 36, 48, 72 post induction and negative control (vector without any insert). Lane 0: pellet from zero hour induction. Lane M: protein molecular weight marker (Fermentas,Lithunia).

4.5) Ammonium sulfate precipitation of protein for concentration and further analysis

Total protein was subjected to ammonium sulfate precipitation and concentrated from crude supernatant taken at 24, 36, 48 and 72 hour intervals which showed induction of a distinct band in comparison to control. Ammonium sulfate upto 80% concentration was used and precipitated protein was analyzed on SDS PAGE (Fig 4.14). Though the protein concentration increased but due to the presence of salts the band was not clearly

visible and there was a thick blackened patch of protein in the ~70 kD size range. Hence it was decided to purify the induced protein for further analysis.

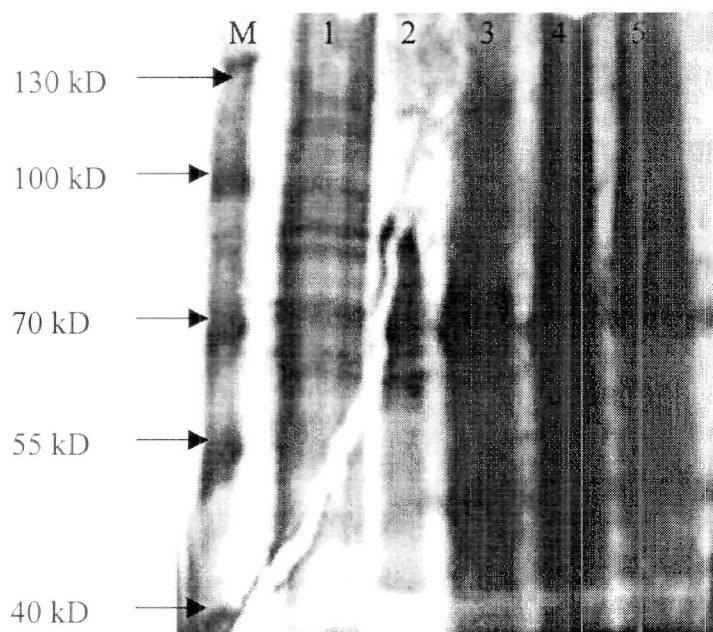


Fig 4.14: SDS PAGE analysis of ammonium sulfate precipitated total protein from supernatant of transformants. Lane M: protein marker (Page Ruler, Fermentas). Lane 1-5: ammonium sulfate precipitated protein from samples taken at 24, 36, 48, 72 and 84 hours post

4.6) PPO enzyme assay:

Assay was carried out to check the activity of PPO enzyme secreted in the supernatant of *Pichia* transformants. Supernatant from *Sal I* sample from 72 hours induction showed highest increase in the absorbance followed by 96 hours post induction (Fig 4.15.a)

whereas the negative control i.e. transformants from parent vector without insert showed negative absorbance indicating the absence of PPO protein in the sample.

One unit of enzyme activity was defined as the amount of enzyme required to cause a change in absorbance of 0.001 per minute at 410 nm.

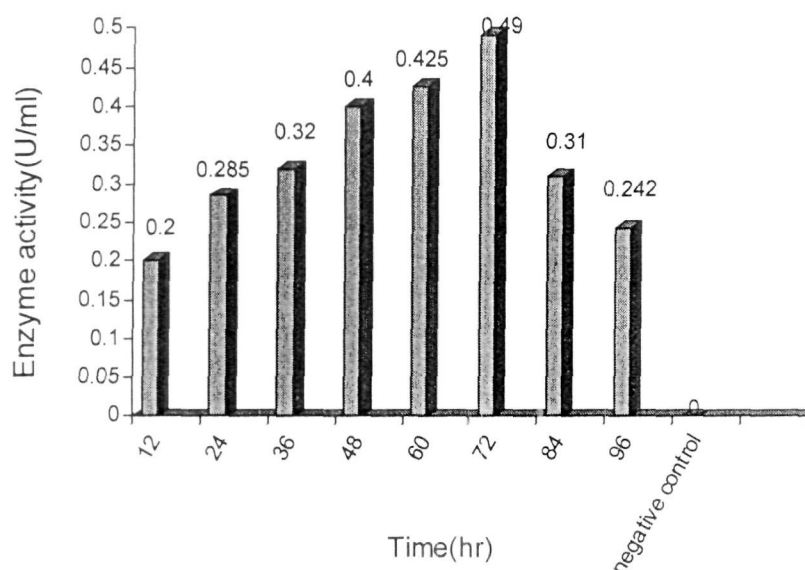


Fig 4.15(a): Diagrammatic representation of PPO enzyme assay showing the total absorbance units of crude supernatant from *Sal* linearized *Pichia* colonies as well as from negative control i.e. *Pichia* transformed with parent vector without insert and purified PPO from *Sal* supernatant after 72 hours induction.

Similarly PPO activity was checked for *Sac* I induced samples and highest activity was found after 60 hours of induction as shown in fig 4.15(b). Therefore PPO was purified

from *Sac* I samples induced for 60 hours and its maximum activity was found to be 0.48 total absorbance units / ml

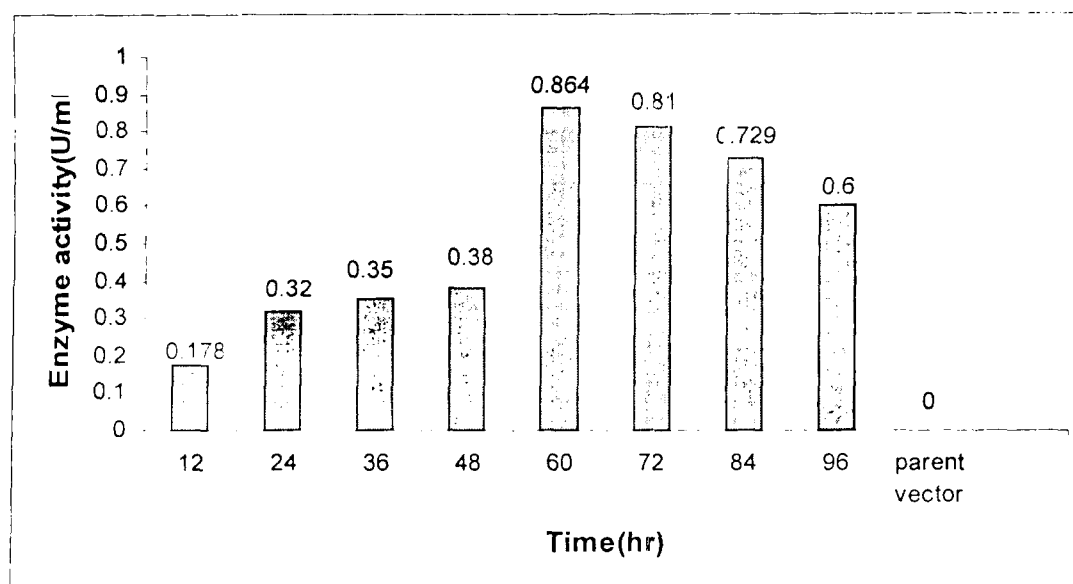


Fig 4.15(b):Diagrammatic representation of PPO enzyme assay showing total absorbance units of crude supernatant from *Sac* linearized *Pichia* colonies at different time intervals as well as from parent vector (*Pichia* transformed with pPIC9 without insert) and purified sample (*Sac* 60).

4.7) Purification of PPO:

PPO was purified from *Pichia* transformants. Owing to the presence of His tag in the protein, Nickel chelated columns (B-Per 6 X His Fusion Protein Purification Kit, Pierce, Belgium) were used for its purification. As the earlier results showed that optimum amount of protein was induced after 60 and 72 hours of induction. Therefore samples from *Sac* I (60 hour post induction) and *Sal* I corresponding to 72 hours induction was

taken for the purification of PPO protein. A single major band of purified PPO was obtained after purification which was approximately 70 kD as shown in Fig 4.16.

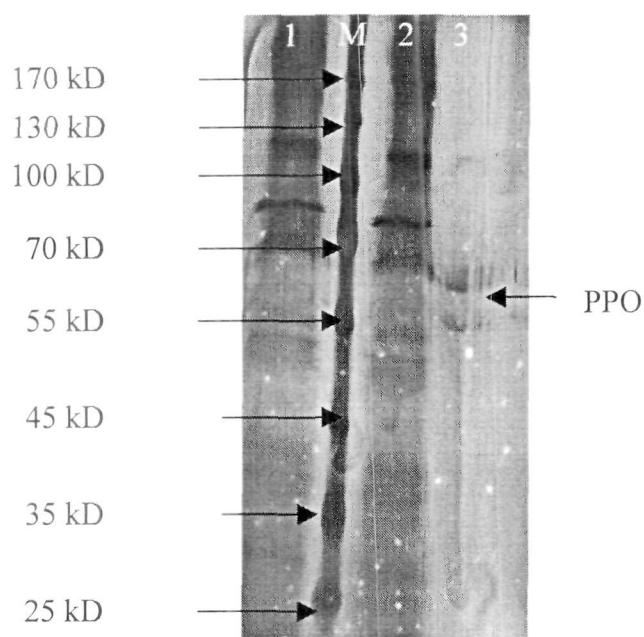


Figure 4.16: SDS PAGE showing His tag purified PPO. Lane 1: crude supernatant from 96 hrs induction. Lane 2: supernatant from Sac 60 hrs induction. Lane 3: purified PPO from Sac I 60 hours induction. Lane M: protein molecular weight marker (Fermentas, Lithuania)

4.8) Protein estimation and specific activity of PPO:

Proteins from the crude supernatant were estimated following the procedure of Bradford (1976) using BSA as standard. Total protein was found to increase with the increase in time of induction in crude supernatant but was quite less in case of purified samples implying the presence of just the purified protein and not other proteins in them.

4.9) Specific activity estimation of PPO:

Specific activity of crude as well as of purified PPO protein was calculated. Purified PPO from *Sac* and *Sal Pichia* clones showed higher specific activity about 10.66 units / ml and 4.66 U / ml (Fig 4.17)

Specific activity from *Sac* supernatant

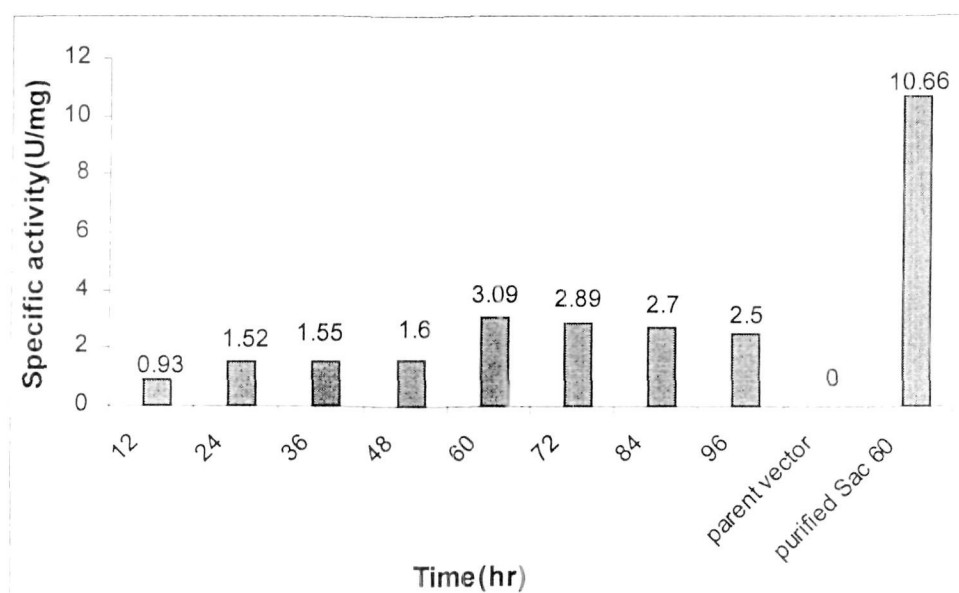


Fig 4.17.(a): Bar diagram showing comparison between the specific activity of crude *Sac* supernatant, parent vector (*Pichia* transformed with pPIC9 without insert) as well as from purified sample (*Sac* 60 h)

Specific activity from *Sal* supernatant:

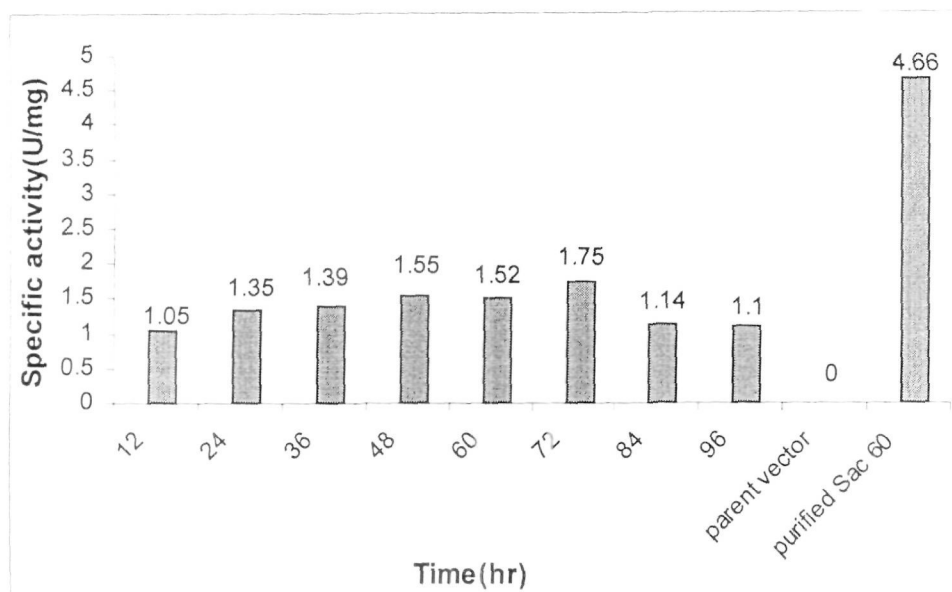


Fig 4.17.(b): Bar diagram showing comparison between the specific activity of crude *Sal* supernatant, parent vector (*Pichia* transformed with pPIC9 without insert) as well as from purified sample (*Sal* 72 h)

4.10) Analysis of the purified PPO by in-gel assay:

Native protein gel was run and soaked in sodium citrate buffer (2M) which was then dipped in methyl catechol (0.5M). Presence of orange brown band indicated the presence of catecholase activity of PPO in the supernatant samples as shown in Fig 4.18.

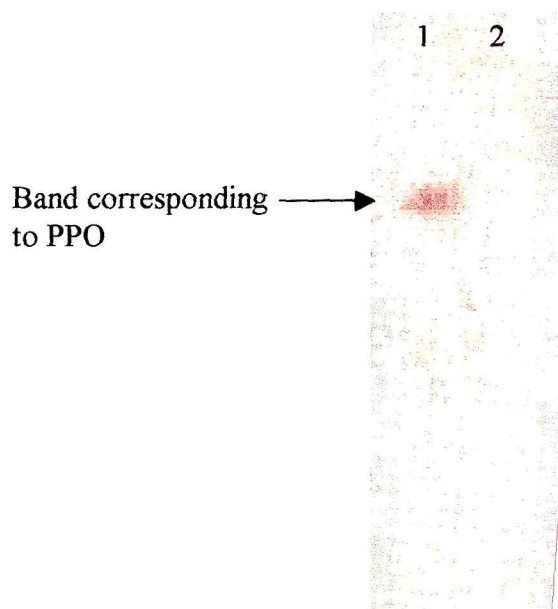


Fig 4.18: Native in gel activity analysis of purified PPO showing a dark orange band corresponding to PPO activity. Lane1: showing positive transformant and lane 2 showing negative control respectively.

DISCUSSION

5. DISCUSSION

Polyphenol oxidases (PPO) are the copper containing oxidoreductases catalyzing the hydroxylation of monophenols to o-diphenols (monophenol monooxygenases) and further oxidation of o-diphenols to o-quinones by utilizing molecular oxygen (Mayer, 1987). The o-quinones thus generated polymerize to form colored compounds usually black or brown pigments causing characteristic enzymatic browning of fruits and vegetables (Walker and Ferrar, 1975) and thus are responsible for losses in nutrient quality.

Though enzymatic browning due to PPO is undesirable in fruits and vegetables but it imparts organoleptic properties to certain foods like flavour and color to tea, coffee and cocoa. Polyphenol oxidase imparts golden brown color in dried fruits like figs, prunus, dates etc (Marshall *et al.*, 2000). Polyphenol oxidase is also involved in phenolics biosynthesis, defense against insect attack and disease resistance (Li and Steffens, 2000).

PPO has been isolated and cloned from various sources but PPO from tea show greatest affinity towards catechins which are oxidized into antioxidants of high commercial value, theaflavins and thearubigins, tea PPO (CS PPO) was cloned, characterized and was sequenced at IHBT (Raizada J, 2004). Owing to the involvement of tea PPO in the production of high value compounds i.e. theaflavin and thearubigins which possess antioxidant, antibacterial, deodorizer and anticarcinogenic properties; there is immense industrial need of the enzyme. To alleviate the pressure on tea bushes for the extraction of PPO and to get PPO

year around an effort was made to develop heterologous expression of tea PPO in yeast, *Pichia pastoris*.

Pichia pastoris:

Pichia pastoris is unicellular eukaryotic methanoltrophic yeast which has been developed as an outstanding host for the production of a variety of recombinant proteins. It is easy to manipulate, culture and being eukaryotic in nature it is capable of various post translational modifications performed by higher eukaryotic cells such as proteolytic processing, protein folding, disulphide bond formation and glycosylation. Thus, many proteins that end up as inactive inclusion bodies in bacterial systems are produced as biologically active molecules in *Pichia pastoris*. Several other factors have also contributed to it's rapid acceptance are (1) a promoter derived from the alcohol oxidase I gene of *Pichia pastoris* that is uniquely suited for the controlled expression of foreign genes, (2) strong preference of *Pichia pastoris* for respiratory growth which facilitates its culturing at high cell densities relative to fermentive yeasts, (3) it does not pose endotoxin problem associated with bacterial or viral contamination of proteins produced in animal cell culture, (5) its prolific growth rate which makes it easy to culture in shake flask and by switching over to fermenter culture protein production in *Pichia* could be increased by over 140% (Chen *et al.*, 1996).

5.1) Selection of *Pichia pastoris* expression strain:

Numerous strains of *Pichia pastoris* with wide range of genotypes like GS115, KM71, and SMD1168 are available but strain GS115 was used for the expression of polyphenol oxidase in yeast. Use of GS115 allowed transformants to be selected based on their ability to grow in non-histidine containing media. It contains a functional copy of *AOX1* gene responsible for approximately 85% of utilization of methanol by alcohol oxidase enzyme and thus has wild type methanol utilization phenotype.

5.2) Selection of expression vector:

Various expression vectors for the heterologous protein expression in *Pichia pastoris* are available like pHIL-s1, pHIL-d2, pPIC3.5, pPIC9. Among these, expression vector pPIC9 was selected for PPO gene expression. It is composed of DNA sequences of *AOX1* promoter, followed by unique restriction sites *Xho* I and *Not* I (same restriction sites included in the primers for the amplification of PPO gene) for in frame cloning of PPO gene followed by transcriptional terminator sequence from *Pichia pastoris* *AOX* I which directs efficient 3' processing and polyadenylation of mRNAs. It also contains functional histidine dehydrogenase gene (*HIS4*) which acts as selectable marker following transformation into *his4* (histidine dehydrogenase deficient GS115 strain). Presence of alpha factor secretion signal in pPIC9 allows the protein to be secreted out in media and hence helps in easy purification of the PPO from total crude protein. Various workers have used pPIC9 as well as other expression

vectors for foreign gene expression studies. Vector pPIC9 have been used by Alan *et al.*, 1998 for the expression of soybean root nodule phosphatase, Tomoyoshi *et al.* (2000) for the expression of pea seedling copper amine oxidase whereas pPIC3 vector has been used for expression of pepper endoglucanases by Ferrarese *et al.* (1998), expression of hexose oxidase by Wolf *et al* (2001), pHILD-2 vector has been used for the expression of spinach phosphoribulokinase by Brandes *et al.*, 1996.

Large size of pPIC9 (~8kb) makes *in vitro* cloning steps more difficult and integration of such large vectors typically results in transformants that are genetically less stable.

5.3) Amplification, cloning and transformation of cloned fragment in *E.coli*:

Primers were designed from the already sequenced *PPO* gene (Raizada. J, 2004) that could be ligated in frame within the expression vector. For in- frame cloning, the restriction enzyme sites *Xho* I and *Not* I were included in the upstream and downstream primers, respectively. Apart from these sites, 6 x His tag sequence was also included for facilitating protein purification. The PPO gene thus amplified (Fig 4.1) was successfully cloned in *Xho* I / *Not* I digested pPIC9 (Fig 4.3). Similarly, other workers have used various restriction sites for in-frame cloning of their desired gene as Sugrue *et al.*, 1997; Zhu *et al.*, 1995; Brandes *et al.*, 1996; Alan *et al.*, 1998; Wen-ChinHuang *et al.*, 2003 in yeast expression vectors.

5.4) Transformation into yeast:

Expression vector pPIC9 is a shuttle vector but lacks yeast origin of replication so *Pichia pastoris* was transformed by integration of expression cassette into yeast genome in order to yield genetically stable transformants. Moreover, chromosomal integration is more desirable than episomal plasmid expression systems as episomal plasmids tend to have low copy number which can affect the amount of protein expressed. Large sized episomal plasmids also tend to be lost during repeated generations as they are mitotically unstable. On the other hand shuttle vector pPIC9 which integrates into the genome have selectable markers (*HIS4*) that enables detection of transformants. For integration of pPIC9 it was linearized using *Sal* I / *Sac* I or *Bgl* II restriction enzymes (Fig 4.7).

Though the introduction of expression cassette into the yeast genome can be achieved by various ways like spheroplasting, PEG transformation, electroporation, LiCl transformation but electroporation was carried out as its efficiency is quite high compared to other methods such as LiCl and PEG transformation. Spheroplasting have similar efficiencies to electroporation but spheroplasting requires several steps with the risk of high contamination during transformation especially in case of pPIC9 vector as it does not have antibiotic selection. Moreover in spheroplasting over digestion with cell lysing enzyme, zymolase can reduce cell viability (Daly *et al.*, 2004). Therefore electroporation was found having an edge over other methods in all respects and carried out successfully. Similarly electroporation was carried by other workers in expression studies (Penheiter *et al.*, 1998; Wolf *et al.*, 2000; Jia da Li *et al.*, 2001).

5.5) Screening for Mut⁺ and Mut^{slow} transformants:

Screening of transformants for Mut⁺/ Mut^s phenotype decides the type of integration / recombination between the recombinant linearized plasmid constructs and yeast genome. Such screening also decides on the culture conditions to be used for protein induction. Transformation of GS115 strain of *Pichia pastoris* with *Sal* I and *Sac* I linearized constructs favour recombination at *HIS* 4 locus and therefore all the transformants were found to be Mut⁺ (methanol utilization plus, Fig 4.10), which refers to wild type ability of the strain to metabolize methanol as the sole carbon source. Transformants with *Bgl* II linearized constructs had Mut^{slow} phenotype due to loss of alcohol oxidase activity encoded by the *AOX* I gene.

5.6) PCR analysis of *Pichia* integrants:

PCR analysis of HIS⁺ Mut⁺ *Pichia* integrants revealed the presence of gene of interest (*PPO*) in the transformants. Using gene specific primers an amplified product of ~1.8 kb (Fig 4.11) was obtained in *Sac* I and *Sal* I linearized constructs, whereas no positive clone was obtained in *Bgl* II transformants DNA and fresh.

5.7) Evaluating expression of tea PPO in *Pichia pastoris*:

Protein expression from positive *Pichia* transformants was analyzed by SDS PAGE. Transformants from *Sal* I linearized constructs showed maximum PPO expression level at 72 hours induction (Fig 4.12), whereas transformants from *Sac* I linearized constructs have optimum protein production after 60 hours

induction. Although there are no previous reports on tea PPO production in *Pichia pastoris*. Similarly, optimum induction time for other proteins in *Pichia pastoris* was found at 60-72 hours by Brandes *et al.*, 1996; Geol *et al.*, 2000; Li *et al.*, 2001 and Muraki (2006) whereas optimum induction at 96 hours was reported by like Sugrue *et al.*, 1997 and even after 12 hours by Ferrarese *et al.*, 1998.

Expression of PPO was expected in supernatant due to the presence of secretion signal. Induced analysis of pellet and supernatant showed PPO expression only in the supernatant and not in the pellet (Fig 4.13) suggesting synthesis of soluble protein and not of inclusion bodies.

Usually 0.5-1.0% methanol concentration is used for protein induction in *Pichia pastoris*. In certain studies increasing methanol concentration from 0.5 to 2% v/v could increase the expression levels (Katakura *et al.*, 1998). However in the present study, increasing the concentration did not effect protein production. Certain studies infact revealed that higher methanol had negative effects on the cell growth and lead to decreased expression of proteins (Sarramegma *et al.*, 2002).

The type of culture flask used in smaller scale expression studies was also found important. Use of baffled flask was found to have an edge over simple conical flasks in protein production, as a result of improved aeration enhancing protein production (Villatte *et al.*, 2001).

For preventing the proteolysis of the recombinant protein, peptone was added in the induction media. Ammonium sulfate present in YNB was also responsible for preventing the proteolysis to a great extent (Tsujioka *et al.*, 1996).

5.8) Activity analysis:

The crude supernatant was analyzed for PPO activity. Highest PPO activity in the crude supernatant was found at 60 and 72 hours post induction respectively in *Sac I* and *Sal I* *Pichia* constructs. Control transformants of *Pichia* without PPO insert did not exhibit any PPO activity. Thus indicated the presence of tea PPO protein only in the recombinant *Pichia* transformants. Purified PPO from *Sac I* linearized constructs showed a specific activity of 10.6 U/ml (Fig 4.17).

5.9) Purification of PPO:

Recombinant PPO was purified from the culture supernatant by affinity chromatography (Ni-chelated His tag column). SDS PAGE analysis of the purified protein predicted a molecular weight of PPO to be 70 kD. Though the molecular weight of tea PPO was calculated to be 65 kD but an increase in the molecular weight of PPO expressed in *Pichia* could be due to the post translational modifications.

Conclusion and future prospective:

This study is the first report of cloning tea PPO in yeast expression vector. The sequence verified construct was integrated properly into *Pichia pastoris* genome.

The recombinant protein expression in Mut⁺ *Pichia pastoris* strain was induced by methanol. Expressed protein was purified using Ni-NTA purification column.. The present study demonstrates the capacity of *Pichia pastoris* to synthesize PPO. Although the *Pichia pastoris* systems are efficient and easy to use with well defined process, some degree of optimization is required to achieve maximum production of the heterologous PPO protein in *Pichia pastoris* to achieve maximum activity of the protein.

Leads obtained in the present thesis could be used to optimize high PPO production, even in fermentors. Also, expression vector may be modified to enhance yield of recombinant PPO.

SUMMARY

6. SUMMARY

Polyphenol oxidases are the plastid associated metalloproteins catalyzing the hydroxylation of monophenols to o-diphenols and oxidation of o-diphenols to o-quinones (Mayer, 1987). PPO protein has been purified from several higher plant species as tomato (Yu *et al.*, 1992), wild potato (Kowalski *et al.*, 1992), grapevine (Dry and Robinson, 1994) and tea (Halder *et al.*, 1998). Polyphenol oxidases isolated from several sources have different substrate affinity but PPO from tea has maximal affinity for catechins and gallocatechins leading to formation of theaflavins and thearubigins (Halder *et al.*, 1998). These pigments are commercially valuable antioxidants having beneficial implications in human health. Gene of tea PPO was cloned by Raizada (2004) thus providing possibilities to exploit the *PPO* gene. In view of its importance, an effort was made to develop heterologous expression of tea PPO in an alternate host *Pichia pastoris*.

cDNA encoding tea *PPO* was initially amplified using gene specific primers containing *Xho* I / *Not* I unique restriction sites for the directional cloning of PPO in the expression vector pPIC9. 6 x His tag sequence was also included in the primers to facilitate protein recovery from the supernatant. The amplified product of 1.8 kb corresponding to *PPO* gene was cloned in pGEM-T easy vector followed by in expression vector pPIC9. In-frame cloning of PPO was checked through sequencing and recombinant plasmid was linearized with restriction enzymes *Sal* I, *Sac* I and *Bgl* II and transformed into *Pichia pastoris* through

electroporation. Subsequent screening of the transformants for determining their growth phenotype on MD and MM media showed that all transformants obtained by *Sa*I and *Sac*I linearized constructs were Mut⁺. Genomic DNA PCR using gene specific primers confirmed the integration of *PPO* gene in *Pichia* genome. The positive clones so obtained were induced by methanol for protein expression in BMMY media. Protein analysis through SDS PAGE as well as enzymatic assay confirmed the induced PPO protein (~70 kD) in supernatant. Protein was purified from supernatant using Ni-chelated NTA columns. Specific activity analysis as well as in gel activity assays were carried out which showed successful expression of PPO in *Pichia pastoris*.

REFERENCES

REFERENCES

- Alan, R., Klucas, R.V. and Sarath, G. 1998. Purification and characterization of a soybean root nodule phosphatase expressed in *Pichia pastoris*. *Protein Expression Purif.* 14: 125-130.
- Baker, D. and Nelson, J. M. 1943. Tyrosinase and plant respiration. *J. Gen. Physiol.* 26: 269-276.
- Bassuki, N. L., Hunter, L. D. and Howard, B. H. 1981. The apparent involvement of polyphenol oxidase and phloridzin in production of apple rooting. *J. Hort. Sci.* 56: 313-322.
- Bertrand, M. G. 1895. Sur la recherche et la présence de la laccase dans les végétaux. *C. R. Acad. Sci.* 121: 166-168.
- Bertrand, M. G. 1896. Sur la présence simultanée de la tyrosine dans le suc de quelques champignons. *C. R. Acad. Sci.* 122: 1215-1219.
- Bertrand, M. G. and Mutttermilch, W. 1907. Sur l'existence d'une tyrosinase dans le son de froment (on the presence of a tyrosinase in the wheat bran). *Compt. Rend. Hebdomadaire des Séances Acad. Sci.* 144: 1285.
- Bonner, Jr., W. D. 1957. Soluble oxidases and their functions. *Ann. Rev. Plant Physiol.* 8: 427-452.
- Boswell, J. G. and Whiting, G. C. 1938. A study of polyphenol oxidase system in potato tubers. *Ann. Bot.* 11: 847-852.

- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Brandes, H. K., Hartman, F. C., Lus, Tse-Yuan and Lrimers, F. W. 1996. Efficient expression of the gene for spinach phosphoribulokinase in *Pichia pastoris* and utilization of the recombinant enzyme to explore the role of regulatory cysteinyl residues by site directed mutagenesis. *J. Biol. Chem.* 271(11): 6490-6496.
- Brankamp, R. G., Sreekrishna, K., Smith, P.L., Blankenship, D.T. and Cardin, A.D. 1995. Expression of a synthetic gene encoding the anticoagulant-antimetastatic protein ghilanten by the methylotropic yeast *Pichia pastoris*. *Protein Expression Purif.* 6: 813-820.
- Bruhn, J. N., Johnson, T. E., Karr, A. L. and Leininger, T. D. 1907. Identification of *Armillaria* field isolates using isoenzymes and mycelial growth characteristics. *Mycopathologia.* 142: 89-96.
- Bucheli, C. S., Dry, I. B. and Robinson, S. P. 1996. Isolation of full length cDNA encoding polyphenol oxidase from sugarcane, a C4 grass. *Plant Mol Biol.* 31: 1233-1238.
- Butt, V.S. 1985. Oxygenation and oxidation in the metabolism of aromatic compounds. *Annual proceedings of the Pytochemical society of Europe.* 25: 349-369.
- Cary, J. M., Lax, A. R. and Flurky, W. H. 1992. Cloning and characterization of cDNAs coding for *Vicia faba* polyphenol oxidase. *Plant Mol. Biol.* 20. 245-253.

- Chazarra, S., Garcia-Carmona, F. and Cabanes, J. 2001. Evidence for a tetrameric form of Iceberg Lettuce (*Lactuca sativa* L.) polyphenol oxidase: Purification and characterization. *J. Agric Food Chem.* 49: 4870-4875.
- Chevalier, T., de rigal, D., Mbeguie-A-Mbeguie, D., Gauillard, F. and Fils-Lycaon, B.R. 1999: Molecular cloning and characterization of apricot fruti polyphenol xoidase. *Plant Pysiol.* 119: 1261-1269.
- Chen, Y., Krol, J., Cino, J., Freedman, D., White, C., and Komives, E. 1996. Continous production of Thrombo-modulin from a *Pichia pastoris* fermentation . *J. Chem. Tech. Biotechnol.* 67: 143-148.
- Clare, J. J., Rayment, F. B., Ballantine, S.P., Sreekrishna, K and Romanos, M. A. 1991. High level expression of tetanus toxin frgment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Bio/TECHNOLOGY.* 9: 455-460.
- Constabel, C. P., Bergey, D. R. and Ryan, C. A. 1995. Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proc. Natl. Acad. Sci. USA.* 92: 407-411.
- Constabel, C. P., Yip, L., Patton, J. J. and Christopher, M. E. 2000. Polyphenol oxidase from Hybrid Poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.* 124: 285-295.
- Cregg, J. M., Tschopp, J.F. Stillman, C, Stilman, R. Siegel, M, Akong, W. S. Craig, Buckholz, R.G., Madden, L. R., Kellaris, P. A., Davis, G. R., Smiley, B.L., Cruje, J. and Thill, G. P. 1987. High-level expression and efficient assembly of hepatitis B surface antigen in the methylotropic yeast, *Pichia pastoris*. *Bio/Technology.* 5: 479-485.

- Daly, R. and Hearn, Milton, T. W. 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *Mol. Recognit.* 18: 119-138.
- de-Boer, A. D. and Weisbeck, P. J. 1991. Chloroplast protein topogenesis: import, sorting and assembly. *Biochem. Biophys. Acta.* 1071: 221-253.
- Dry, I. B. and Robinson, S. P. 1994. Molecular cloning and characterization of grape berry polyphenol oxidase. *Plant Mol. Biol.* 26: 495-502.
- Eicken, C., Krebs, B. and Sacchettini, J. C. 1999. Catechol oxidase- structure and activity. *Curr. Opi. Struc. Biol.* 9: 677-683.
- Eicken, C., Zippel, F., Buldt-Karentzopoulos, K. and Krebs, B. 1998. Biochemical and spectroscopic characterization of catechol oxidase from sweet potatoes (*Ipomoea batatas*) containing a type-3 dicopper center. *FEBS Lett.* 436: 293-299.
- Elstner, E. F., Konze, J. R., Selman, B. R. and Stoffer, C. 1976. Ethylene formation in sugar beet leaves. Evidence for the involvement of 3-hydroxytyramine and phenoloxidase after wounding. *Plant Physiol.* 58: 163-168.
- Ferrarese, L., Trainotti, L., Gattolin, S. and Casadora, G. 1998. Secretion, purification and activity of two recombinant pepper endo- β -1, 4 glucanases expressed in yeast *Pichia pastoris*. *FEBS Lett.* 422(1): 23-26.
- Flurkey, W. H. 1985. *In vitro* biosynthesis of *Vicia faba* polyphenol oxidase. *Plant Physiol.* 79: 564-567.

- Ganesha, C., Fox, M. T. and Flurkey, W. H. 1992. Microheterogeneity in purified broad bean polyphenol oxidase. *Plant Physiol.* 98: 472-479.
- Golbeck, J. H. and Cammarata, K. V. 1981. Spinach thylakoid polyphenol oxidase. Isolation, activation and properties of the native chloroplast enzyme. *Plant Physiol.* 67: 977-984.
- Goel, A., Beresford, G. W., Colcher, D., Pavlinkova, G., Booth, J. M., Baranaowska, J. and Batra, S. K. 2000. Divalent forms of cc49 single chain antibody constructs in *Pichia pastoris*: Expression, Purification and Characterization. *J. Biochem.* 127: 829-836.
- Goldman, M. H. S., Seurinck, J., Marius, M., Goldman, G. H. and Mariani, C. 1998. A tobacco flower- specific gene encodes a PPO. *Plant Mol. Biol.* 36: 479-485.
- Gooding, P. S., Bird, C. and Robinson, S. P. 2001. Molecular cloning and characterization of banana fruit polyphenol oxidase. *Planta.* 213: 748-757.
- Gregory, R. P. F. and Bendall, D. S. 1966. The purification and some properties of the polyphenol oxidase from tea (*Camellia sinensis* L.). *Biochem. J.* 101: 569-581.
- Hahlbrock, K. and Grisebach, H. 1979. Enzymatic controls in the biosynthesis of lignin and flavanoids. *Ann. Rev. Plant Physiol.* 30: 105-136.
- Halder, J., Tamuli, P. and Bhaduri, A. N. 1998. Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camellia sinensis*). *Nutr. Biochem.* 9: 75-80.

- Hamada, S., Koga, T., Ooshima, T. 1984. Virulence factors of *Streptococcus mutans* and caries prevention. *J. Dent. Res.*, 63, 407-411.
- Harel, E. and Mayer, A. M. 1971. Partial purification and properties of catechol oxidases in grapes. *Pytochemistry*. 10: 17-22.
- Haruta, M., Pederson, J. A. and Constabel, C. P. 2001. Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression and potential substates. *Physiol. Plant*. 112: 552-558.
- Hind, G., Marshak, D. and Coughlan, S. 1995. Spinach thylakoid polyphenol oxidase: cloning, characterization and relation to a putative protein kinase. *Biochemistry*. 34: 8157-164.
- Hunt, M. D., Eanetta, N. T., Yu, H., Newman, S. M. and Steffens, J. C. 1993. cDNA cloning and expression of potato polyphenol oxidase . *Plant Mol. Biol.* 21: 59-68.
- Huystee, R. B. and Cairns, W. L. 1982. Progreno and prospects in the use of peroxidase to study cell development. *Phytochemistry*. 21: 1843-1847.
- Jackman, M. P., Hajnal, A. and Lerch, K. 1991. Albino mutants of *Streptomyces glaucescens* tyrosinase. *Biochem. J.* 274: 707-713.
- Janovitz-Klapp, A. H., Richard, F. C., Goupy, P. M. and Nicolas, J. J. 1990. Kinetic studies on apple polyphenol oxidase. *J. Agric. Food Chem.* 38: 1437-1441.
- Jia, daLi, Qiang, Xu and Wang, Ke-yi. 2001. Functional expression of galactose-binding c-type lectin in *Pichia pastoris*. *Protein and Peptide Letters*. 8(3): 217-224.

- Joy, N. R. W., Sugiyama, M., Fukuda, H. and Kananine, A. 1995. Cloning and characterization of polyphenol oxidase cDNAs of *Phytolacca americana*. Plant Mol. Biol. 107: 1083-1089.
- Kanter, M. 1998. Free radicals, exercise and antioxidant supplementation. Proc. Nutr. Soc. 9.
- Katakura, Y., Zhang, W. H., Zhuang, G. Q., Omasa, T., and Suga, K. I. 1998. Effect of methanol concentration on the production of human glycoprotein by a recombinant *Pichia pastoris*. J. Ferment. Bioengng. 86: 482-487.
- Keilin, D. and Mann, T. 1938. Proc. Roy. Soc.(London). 125: 187-204.
- Kieselbach, T., Hagman, A., Anderson, B. and Schroder, W. P. 1998. The thylakoid lumen of chloroplasts. Isolation and characterization. J. Biol. Chem. 273: 6710-6716.
- Koga, T., Okahashi, N., Asakava, H., Hamada, S. 1986. Adherence of streptococci mutans to tooth surfaces. Molecular microbiology and immunology of Stretococcus mutans, 111-120.
- Kowalski, S. P., Eanetta, N. T., Hirzel, A. T. and Steffens, J. C. 1992. Purification and characterization of polyphenol oxidase from glandular trichomes of *Solanum berthaulti*. Plant Phsiol. 100: 677-684.
- Kubowitz, F. 1937. Biochem Zeitschrift. 292: 221-229.
- Kubowitz, T. 1938. Spaltung and resynthese der polyphenol oxydase und des hamocyanins. Biochem Zeitsschrift. 299: 32-57.

- Lax, A. R. and Vaughn, K. C. 1991. Colocalization of polyphenol oxidase and photosystem II proteins. *Plant Physiol.* 96: 26-31.
- Lax, A. R., Vaughn, K. C. and Templeton, G. E. 1984. Nuclear inheritance of polyphenol oxidase in *Nicotiana*. *J. Hered.* 75: 285-287.
- Lee, P. M. 1991. Biochemical studies of cocoa bean polyphenol oxidase. *J. Sci. Food Agric.* 55: 251-260.
- Li, L. and Steffens, J. C. 2002. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta.* 215: 239-247.
- Li, Z. J., Xiong, F., Lin, Q. S. and Yang, D. S. C. 2001. Low temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. *Protein Expression Purif.* 21: 438-445.
- Lin, Q. L. 1992. Pharmacological properties of tea. *China Tea Book*, 91-101.
- Malkin, R. and Malmstrom, B. G. 1970. The state and function of copper in biological systems. *Adv. Enzymol.* 32: 177-244.
- Marri, C., Frazzoli, A., Hochkoeppler, A. and Poggi, V. 2003. Purification of polyphenol oxidase isoforms from potato (*Solanum tuberosum*) tubers. *Phytochemistry.* 63: 745-752.
- Marshall, M. R., Kim, J and Wei, C-I. 2000. Enzymatic browning in fruits, vegetables and seafoods. *Food Agric. Assoc.*

- Mason, H. S. and Peterson, E. W. 1965. Melanoprotein I. Reactions between enzyme generated quinines and amino acids. *Biochem. Biophys. Acta.* 3: 134-146.
- Mayer, A. M. 1987. Polyphenol oxidases in plants-Recent progress. *Phytochemistry.* 26: 11-20.
- Mayer, A. M. and Harel, E. 1979. Polyphenol oxidases in Plants. *Phytochemistry.* 18: 193-215.
- Mazzafera, P. and Robinson, S. P. 2000. Characterization of polyphenol oxidase in coffee. *Phytochemistry.* 55: 285-296.
- Mueller, W. C. and Beckman, C. H. 1978. Ultrastructural localization of polyphenol oxidase and peroxidase in roots and hypocotyls of cotton seedlings. *Can J. Bot.* 56: 1579-1587.
- Muraki, M. 2006. Secretory expression of synthetic human Fas ligand extracellular domain gene in *Pichia pastoris*: Influences of tag addition and N- glycosylation site deletion, and development of a purification method. *Protein Expression Purif.* doi: 10.1016/j.pep. 2006.08.006.
- Murata M, Tsurutani M, Tomita M, Homma S, Kaneko K. 1995. Relationship between napple ripening and browning: Changes in polyphenol content and polyphenol oxidase. *J. Agric. Food Chem.* 43: 1115-1121.
- Nakayama, T., Yonekura-Sakakibara, K., Sato, T., Kikuchi, S., Fukui, Y., Fukuchi-Mizutani, M., UEDA, T., Nakao, M., Tanaka, Y., Kusumi, T. and Nishino, T. 2000. Aureusidin synthase: A polyphenol oxidase homolog responsible for flower coloration. *Science.* 290: 1163-1166.

- Newman, S. M., Eanetta, N. T., Yu, H. and Prince, J. P. 1993. Organization of tomato polyphenol oxidase family. *Plant Mol. Biol.* 21: 1035-1051.
- Nicolas, J., Richard-Forget, F. C., Goupy, P. M., Amiot, M. J. and Aubert, S. Y. 1994. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci. Nutr.* 34: 109-157.
- Paifer, E., Margolles, E., Cremata, J. Herrera, L. and Delgado, J. M. 1994. Efficient expresión and secretion of recombinant Alpha amylase in *Pichia pastoris* using two different signal sequences. *Yeast.* 10: 1415-1419.
- Penheiter, A. R., Klucas, R. V. and Sarath, G. 1998. Purification and characterization of a root nodule phosphatase expressed in *Pichia pastoris*. *Protein Expression Purif.* 14: 125-130.
- Pruidze, G. N., Mchedlishvili, N. I., Omiadze, N. T., Gulua, L. K. and Pruidze, N. G. 2003. Multiple forms of phenol oxidase from Kolkhida tea leaves (*Camellia sinensis* L.) and *Mycelia Sterilia* IBR 35219/2 and their role in tea production. *Food Res Int.* 36: 587-595.
- Raizada, J. 2004. Cloning and characterization of Polyphenol oxidase gene from *Camellia sinensis* (L.). O. Kuntze. Ph.D. Thesis. GNDU, Amritsar, Punjab, India.
- Rathjen, A. H. and Robinson, S. P. 1992. Characterization of variegated grapevine mutant showing reduced polyphenol oxidase activity. *Aust. J. Plant. Physiol.* 19: 43-54.
- Raymond, J., Rakariyatham, N. and Azanza, J. L. 1993. Purification and some properties of polyphenol oxidase from sunflower seeds. *Phytochemistry.* 34: 927-931.

- Russell, I. M. and Burton, S. G. 1999. Development and demonstration of an immobilized polyphenol oxidase bioprobe for the detection of phenolic pollutants in water. *Anal. Chem. Acta.* 389: 161-170.
- Sanchez-Ferrer A, Laveda F, Garcia-Carmona F. 1993. Partial purification of soluble potato polyphenol oxidase by partitioning in aqueous two-phase system. *J. Agric. Food Chem.* 41: 1219-1224.
- Sanchez-Ferraz, A., Roriguez-Lopez, J. N., Garcia-Canovas, F. and Garcia Carmona, F. 1995. Tyrosinase: a comprehensive review of its mechanism. *Biochem. Biophys. Acta.* 1247: 1-11.
- Sanderson, G. W. 1964. Extraction of soluble catechol oxidase from tea shoot tips. *Biochem. Biophys. Acta.* 92: 622-624.
- Sarramegna, V., Demagne, P., Milon, A. and Talmont, F. 2002. Optimizing functional versus total expression of the human mu-opioid receptor in *Pichia pastoris*. *Protein Expression Purif.* 24: 212-220.
- Scorer, C.A., Buckholz, R.G., Clare, J.J. and Romanos, M.A. 1993. The intracellular production and secretion of HIV-1 Envelope Protein in methylotropic yeast *Pichia pastoris*. *Gene.* 136: 111-119.
- Seo, S-Y., Sharma, V. K. and Sharma, N. 2003. mushroom tyrosinase: recent prospects. *J. Agric. Food Chem.* 51: 2837-2853.
- Serradell MA, Rozenfeld PA, Martinez GA, Civello PM, Chaves AR, Anon MC. 2000. Polyphenol oxidase activity from strawberry fruit (*Fragaria x*

- ananassa, Duch, cv Selva): characterization and partial purification. J. Sci. Food Agric. 80: 1421-1427.
- Shahar, T., Henning, N., Gutfinger, T. and Haeven, D. 1992. The tomato 63 kD polyphenol oxidase gene: molecular identification and developmental expression. Plant Cell. 4: 135-147.
- Sherman, T. D., Vaughn, K. C. and Duke, S. O. 1991. A limited survey of the phylogenetic distribution of polyphenol oxidase. Phytochemistry. 30: 2499-2506.
- Shin, R., Froderman, T. and Flurkey, W. H. 1997. Isolation and characterization of a mung bean leaf polyphenol oxidase. Phytochemistry. 45: 15-21.
- Smith, D. M. and Montgomery, M. W. 1985. Improved methods for the extraction of polyphenol oxidase from d'Anjou pears. Phytochemistry. 24: 901-904.
- Smith, D. M. and Dou, Q. P. 2001. Green tea polyphenol epigallocatechin inhibits DNA replication and consequently induces leukemia cells apoptosis. Int. J. Mol. Med. 7: 645-662.
- Soderhall, I and Soderhall, K. 1989. Purification of polyphenol oxidase from *Daucus carota* cell cultures. Phytochemistry. 28: 1805-1808.
- Sojo M. M, Nunez-Delicado E, Garcia-Carmona F, Sanchez-Ferrer A. 1998a. Partial purification of a banana polyphenol oxidase using triton X-114 and PEG 8000 for removal of polyphenols. J. Agric. Food Chem. 46: 4924-4930.

- transgenic alfalfa. A. Hopkins, Z. Y. Wang, R. Mian and R. Barker (eds.), Molecular breeding of forage and turf, 189-195.
- Takeo, T. and Baker, J. E. 1972. Changes in multiple forms of polyphenol oxidase during maturation of tea leaves. *Pytochemistry*. 12: 21-24.
- Thipyapong, P., Hunt, M. D. and Steffens, J. 1995. Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry*. 40: 673-676.
- Tolbert, N. E. 1973. Activation of polyphenol oxidase of chloroplasts. *Plant Physiol*. 51: 234-244.
- Tomoyoshi, K., Matsumura, K., Shunichi, K. and Tanzania, K. 2000. Molecular cloning and heterologous expression of pea seedling copper amine oxidase. *Biosci. Biotechnol. Biochem*. 64(4): 717-722.
- Trebst, A and Depka, B. 1995. Polyphenol oxidase and photosynthesis research. *Photosynth Res*. 46: 41-44.
- Tschopp, J.F., Sverlow, G., Kosson, R., Craig, W. and Grinna, L. 1987. High level secretion of glycosylated invertase in the methylotropic yeast *Pichia pastoris*. *Bio/Technology*. 5: 1305-1308.
- Tsujikawa, M., Okabayashi, K., Morita, M. and Tanabe, T. 1996. Secretion of a variant of human single-chain urokinase-type plasminogen activator without glycosylation site in the methylotropic yeast, *Pichia pastoris* and characterization of the secreted product. *Yeast*. 12: 541-553.
- Van Gelder, C. W. G., Flurkey, W. H. and Wichers, H. J. 1997. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry*. 45: 1309-1323.

- Vaughn, K. C. and Duke, S. O. 1981. Tentoxin-induced loss of plastidic polyphenol oxidase. *Physiol. Plant.* 53: 421-428.
- Vaughn, K. C. and Duke, S. O. 1981. Tissue localization of polyphenol oxidase in *Sorghum*. *Protoplasma.* 108: 319-327.
- Vaughn, K. C. and Duke, S. O. 1984. Tentoxin stops the processing of polyphenol oxidase into an active enzyme. *Plant Physiology.* 60: 106-112.
- Vaughn, K. C., Lax, A. R. and Duke, S. O. 1988. Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiol Plant.* 72: 659-665.
- Vendemiale, G., Grattaglino, E. and Altomare, E. 1999. An update on the role of free radicals and antioxidant defense in human disease. *Int. J. Lab. Res.* 29: 49-52.
- Villatte, F., Hussain, A. S., Bachmann, T. T. and Schmid, R. D. 2001. Expression level of heterologous proteins in *Pichia pastoris* is influenced by flask design. *Appl. Microbial. Biotechnol.* 55: 463-465.
- Vinson, J. A., Dabbagh, Y., Serry, M. and Jang, J. 1995. Plant flavanoids, especially tea flavonols are powerful antioxidants using an *in vitro* oxidation model for heart disease. *J. Agric. Food Chem.* 43: 2800-2811.
- Vozza, L. A., Wittwer, L., Higgins, D. R., Purcell, T. J. and Hoeffler, J.P. 1996. Production of a recombinant bovine enterokinase catalytic subunit in the methylotropic yeast *Pichia pastoris*. *Bio/Technology.* 14: 77-81.

- Wagner, S. L., Siegel, R. S., Vedvick, T. S., Raschke, W. C. and VanNostrand, W. E. 1992. High level expression, purification and characterization of the Kunitz-type protease inhibitor domain. *Biochem. Biophys. Res. Commun.* 186: 1138-1145.
- Walker, J. R. L. 1975. Enzymic browning in foods; a review. *Enz.Tech. Digest.* 4: 80-100.
- Walker, J. R. L. and Ferrar, P. H. 1998. Diphenol oxidases, Enzyme- catalysed browning and plant disease resistance. *Biotech. Genet. Engnr. Rev.* 15: 457-490.
- Weiss, H. M., Michel, H., Hasse, W and Reilander, H. 1995. Expression of functional mouse serotonin receptor in the methylotropic yeast *Pichia pastoris*: Pharmacological characterization and localization. *FEBS.* 377: 451-456.
- Wen-Chin Huang, Wang, Ai-Yu., Wang, Li-Ting and Sung Li-Hsien. 2003. Expression and characterization of sweet potato invertase in *Pichia pastoris*. *J. Agric. Food Chem.* 51: 1494-1499.
- Whitaker JR. 1994. Polyphenol oxidase. In: *Princeples of Enzymology for the Food Sciences. 2nd ed.* Marcel Dekker, Inc., New York. pp 543-556.
- Wolf, A. M., Hansen, O. C., Madrid, S. and Stougaard, P. 2001. Optimization of the production of *Chondrus crispus* hexose oxidase in *Pichia pastoris*. *Protein Expression Puif.* 22: 189-199.
- Yang, C. S. and Landau, J. M. 2000. Effects of tea consumption on nutrition and health. *J. Nutr.* 130: 2409-2412.

- Yang, C. P., Fujita, S., Cono, K., Kusubayashi, A., Ashrafuzzaman, MD and Yací, N. 2001. Partial purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) peel. J. Agric. Food Chem. 49: 1446-1449.
- Yu, H., Kowalski, S. P. and Steffens, J. C. 1992. Comparison of polyphenol oxidase expression in glandular trichomes of *Solanum* and *Lycopersicon* species. Plant Physiol. 100: 1885-1890.
- Zawistowski, J., Biliaderis, C. G. and Eskin, N. A. M. 1991. Polyphenol oxidases. In. Oxidative enzymes in foods. Eds. Robinson, D. S. and Eskin, N. A. M. Elsevier Science Publishers, London. pp 217-273.
- Zinkernagel, V. 1986. Peroxydase-peroxydatische Kalatase- and Polyphenol oxydasee- Aktivitäten. J. Phytopathol. 115: 257-266.
- Zhou P, Smith NL, Lee CY. 1993. Potential purification and some properties of Monroe apple peel polyphenol oxidase. J. Agric. Food Chem. 41: 532-536.
- Zhu, A., Monahan, C., Zhang, Z., Hurst, R., Leng, L. and Goldstein, J. 1995. High level expression and purification of coffee bean alpha- galactosidase produced in yeast *Pichia pastois*. Arch. Biochem. Biophys. 324: 65-70.

APPENDICES

APPENDIX I

Buffers and Solutions

Reagents used in agarose gel electrophoresis:

Sample loading dye (6X)

Ficoll 400	15%
Bromophenol blue	0.25%
Xylene cyanol	0.25%

TAE buffer (50X, per lit):

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8)	100 ml

Reagent used in plasmid isolation using boiling prep method:

STET buffer: 8% sucrose, 0.5% Triton X-100, 50 mM Tris-HCl (pH 8.0), and 50 mM EDTA (pH 8.0). Add Triton X-100 after autoclaving rest of the components in solution.

Ethidium bromide (10 mg/ ml):

Prepare 10 mg/ml stock of ethidium bromide in autoclaved dH₂O. Store it in dark colour bottle at 4°C for longer use.

Taq DNA polymerase buffer: 10 mM TAPS (pH 8.8); 15 mM MgCl₂; 50 mM KCl and 0.01% gelatin.

dNTPs:

Prepare mixture of 2.5 mM of dATP, dCTP, dGTP and dTTP. Mix well and store at 20°C for longer use.

Ampicillin (100mg/ml):

Dissolve 100 mg of Ampicillin in 1 ml of autoclave dH₂O. Filter sterilize and dispense in aliquots. Store at -20°C for longer use.

Tetracycline:

Prepared 50 mg/ml stock of tetracycline in absolute ethanol (100%). Filter sterilized and dispensed in aliquots. Stored at -20°C for longer use.

Breaking buffer composition for genomic DNA isolation:

2% v/v Triton X-100,

1% v/v SDS,

100 mM NaCl,

10 mM TrisCl pH8,

1 mM EDTA pH8.

1 M potassium phosphate buffer, pH 6.0:

Combine 132 ml of 1M K₂HPO₄, 868 ml of 1M KH₂PO₄ and confirm that the pH=6.0 (if pH needs to be adjusted, use phosphoric acid or KOH). Autoclave and store at room temperature.

Phenol:Chloroform:Isoamyl alcohol (25:24:1):

Equilibrate phenol with 0.1 M Tris-HCl (pH 7.8). 25 ml of phenol mixed with 24 ml of chloroform and 1 ml of isoamyl alcohol. Mix well and store the solution at 4°C in dark bottle.

TE buffer:

Tris (pH 7.0) 10 mM

EDTA (pH 7.0) 1 mM

Stock solutions used for *P. pastoris* medium:

- i. **YNB (13.4% Yeast Nitrogen Base with Ammonium sulfate without amino acids):** Added 34 g of YNB without ammonium sulfate and amino acids and 100 g of ammonium sulfate in litre distilled water and filter sterilized. Store at room temperature
- ii. **Biotin (0.02%):** Dissolved 20 mg biotin in 100 ml of water and filter sterilized. Store at +4°C.
- iii. **Histidine (0.4%):** Dissolved 400 mg of L-histidine in 100 ml of water. Heated the solution, if necessary, to no greater than 50°C in order to dissolve. Filter sterilized and stored at +4°C.

- iv. **Dextrose (20%):** Dissolved 20 g of D-glucose in 100 ml of water and filter sterilized.
- v. **Methanol (5%):** Mixed 5 ml of methanol with 95 ml of water. Filter sterilized and stored at +4°C.
- vi. **Glycerol (10%):** Mixed 10 ml of glycerol with 90 ml of water and filter sterilized. Stored at room temperature.

Buffer composition for SDS PAGE:

- i. **Acrylamide/Bisacrylamide stock:** Added 29.2 g Acrylamide and 0.8 g Bis-Acrylamide in 50 ml sterile water and stirred to dissolve completely. Made the final volume to 100 ml with water and kept at 4°C in an amber colored bottle.
- ii. **Resolving gel buffer:** 1.5 M Tris-HCl, pH 8.8 (Dissolved 18.15 gm Tris-base in 50 ml sterile water and adjusted the pH to 8.8 with 1 N HCl and made final volume to 100 ml) and kept at 4°C.
- iii. **Stacking gel buffer:** 0.5 M Tris-HCl, pH 6.8 (Dissolved 6.0 gm Tris-base in 50 ml sterile water and adjusted the pH to 6.8 with 1 N HCl and made final volume to 100 ml) and kept at 4°C.
- iv. **10 % SDS Stock solution:** Dissolved 10 g Sodium dodecyl sulphate in water with gentle stirring and made the final volume to 100 ml and kept at room temperature.

- v. **10% Ammonium persulphate (APS):** Dissolved 100 mg APS in 1 ml sterile water (Prepared fresh each time and added to the gel solution just before pouring)
- vi. **5x Electrode buffer (Gel running buffer):** Dissolved 15.0 g Tris-base, 72 g glycine and 5 g SDS in 800 ml sterile water and adjusted the pH to 8.3 and made the final volume to 1000 ml and kept the solution at room temperature.
- vii. **Denaturing buffer (5x loading dye):** 250 mM Tris HCl (pH 6.5); 10% SDS, 1% β -mercaptoethanol, 0.625% bromophenol blue and 50% glycerol. 1% β -mercaptoethanol was added every time just before denaturing protein suspension.

Stock solutions for PPO assay:

A) 4-methyl catechol (MC) Solution:

1.24 g 4-methyl catechol was dissolved in 100 ml of distilled water.

B) Sodium phosphate Buffer, pH 7:

Stock Solutions;

B.1): 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml of distilled water)

B.2): 0.2 M solution of dibasic sodium phosphate (53.65 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or 71.7 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml of distilled water)

Solutions A about 39.0 ml and 61.0 ml of B are mixed and diluted to a total volume of 200 ml with distilled water.

APPENDIX II

1. Elution of the DNA band from the gel using GenElute™ gel extraction kit (Sigma, USA)

The procedure used was as follows:

- Weighed empty eppendorf tubes.
- Sliced the electrophoresed products of required length bands using sharp sterilized blades under UV illuminator.
- The sliced bands were put in the empty eppendorf tubes and weighed again.
- Gel solubilization solution was added thrice the volume of the gel (weight we got after subtraction the weight of eppendorf with gel and the empty eppendorf) into the gel slice and kept on water bath at 60°C for 10 minutes.
- In the mean time column was prepared. For preparation of binding column the GenElute Binding Column was placed into one of the provided 2 ml collection tubes. 500µl of the Column preparation solution was added to each binding column. Centrifuged for 1 min at 12,000 rpm. Flow through liquid was discarded.
- Isopropanol equal to weight of the gel was added.
- Solubilized gel solution (700 µl) mixture was added to the prepared column.
- Centrifuged for 1 minute at 12,000 rpm after loading and flow-through liquid was discarded.

- Wash solution (700 µl) was added to the binding column. Centrifuged for 1 minute at 12,000 rpm, the flow through liquid was discarded.
- The binding column was transferred to a fresh collection tube. 30-50 µl of elution solution was added to the center of the membrane and centrifuged for 1 minute. The binding column was discarded and the DNA was collected in collection tube.

2. Plasmid isolation using Genelute plasmid miniprep kit (Sigma, USA)

The procedure used was as follows:

- Overnight culture (3 ml) was pelleted down by centrifugation at 12,000 rpm for 1 min. Supernatant was discarded. The cells were resuspended in 200 µl resuspension solution. Pipette up and down or vortex. 200 µl of lysis solution was added and inverted gently to mix. It was allowed to clear for 5 minutes.
- Neutralization solution (350 µl) was added and inverted 4-5 times to mix. Centrifuged to pellet debris for 10 min at 12,000 rpm.
- Column preparation solution (500 µl) was added to binding column in a collection tube and spun at 12,000 rpm for 1 min. The flow through was discarded
- Transferred cleared lysate into binding column.
- Spun for 1 min and flow through was discarded.
- Wash solution (750 µl) was added to the column. Spun for 1 min at 12,000 rpm and flow through was discarded.
- Spun for 1 min at 12,000 rpm to dry the column.
- Column was transferred to new collection tube.

- Elution solution (50 μ l) was added and spun for 1 min.
- Plasmid DNA was collected and suspended in distilled water.

3) Plasmid isolation by Genopure isolation kit:

The procedure used was as follows:

- Inoculated single recombinant colony in 2-10 ml LB supplemented with appropriate antibiotic and incubated at 37°C on a shaker at 220-250 rpm for 16 hours.
- Inoculated 100-500 ml culture with 1-3 ml of overnight starter culture and again incubated at 37° C on a rotary shaker at 220-250 rpm for 12-16 hours.
- Pelleted the cells for 5-10 min at 3000-5000 rpm at 2°C- 8°C.
- Supernatant was discarded and pellet resuspended in suspension buffer (available with kit) and mixed properly.
- Added 12 ml of lysis buffer and the solution was gently inverted 6-8 times.the solution was incubated at room temperature for 2-3 min.
- Chilled neutralization buffer was added to the suspension, mixed well by inverting for 6-8 times and incubated for 5 min on ice.
- Suspension was centrifuged for 45 min at 12, 000 rpm at 4°C.
- The cleared lysate was put onto the equilibrated column.
- Allowed the column to empty by gravity. The flow through so obtained was discarded.
- Wash buffer about 16 ml was added onto the column and allowed to empty by gravity.

- Flow through was again discarded and wash buffer was added to the column and allowed to empty.
- The column was re-inserted into a new collection tube.
- Plasmid was eluted with 15 ml of prewarmed elution buffer and precipitated using 11 ml of isopropanol.
- Plasmid was washed with 4 ml of chilled 70% ethanol, centrifuged for 10 min at 15,000 rpm at 4°C.
- Supernatant was discarded; plasmid pellet was air dried and redissolved in 100-500 µl of autoclaved millipore water.