Bioprospecting endophytic fungi for production of acid stable laccases and tyrosinase inhibitors for their development into user friendly skin bleaching formulations

A

Thesis submitted

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Submitted By

Jenia Garg

(301501005)

Under the supervision of

Dr.Sanjai Saxena

Professor

DEPARTMENT OF BIOTECHNOLOGY

Thapar University, Patiala, Punjab

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Certificate

This is to certified that the thesis **entitled "Bioprospecting endophytic fungi for production of acid stable laccases and tyrosinase inhibitors for their development into user friendly skin bleaching formulations"** being submitted by **Ms. Jenia Garg** (Roll No-301501005) in the partial fulfilment of the requirements for the award of the degree of master of science in Biotechnology , Thapar university, Patiala, is bonafide work carried out under the esteemed supervision and conception of **Dr. Sanjai Saxena** and the no part of this thesis has been submitted for the any other award of degree.

14/7/17

Dr. Sanjai Saxena
Professor/Supervisor
DBT,Thapar university

Candidate's Declaration

I hereby declared that the work being presented in thesis entitled "**Bioprospecting endophytic fungi for production of acid stable laccases and tyrosinase inhibitors for their development into user friendly skin bleaching formulations**" in the partial fulfilment of the requirements for the award of degree of Master of Science, Department of Biotechnology, Thapar Uuniversity , Patiala is my own laboratory work during the period of January 2017 to July 2017 under the esteemed supervision and conception of **Dr. Sanjai Saxena** , Professor, Department of Biotechnology , Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Patiala

Date: 14/7/17

Jenia Garg

Roll No. 301501005

This is to certify that the above statement made by candidate is correct and true to the best of our knowledge.

14/7/17

Dr. Sanjai Saxena

Professor/Supervisor

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Matre devo bhava, Pitr devo bhava, Guru devo bhava!

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Jenia garg

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Abbreviations

S.NO.	abbreviation	full form
1.	SLA's	Skin Lightening agents
2.	DQ	Dopaquinone
3.	DHI	5,6-Dihydroxy indole
4.	SLA's	Skin Lightening agents
5.	L-DOPA	Levo-3,4- dihydroxyphenyalanine
6.	DCT	Dopachrome tautomerase
7.	TYRP1	Tyrosinase protein 1
8.	TYRP2	Tyrosinase protein 2
9.	PDA	Potato Dextrose agar
10.	PDB	Potato Dextrose Broth
11.	MEA	Malt Extract Agar
12.	MHA	Mueller hinton agar
13.	WA	Water agar
14.	PA	Pine agar
15.	SNA	Synthetic Nutrient agar
16.	TLC	Thin Layer Chromatography
17.	µg	Micro gram
18.	mg	Milligram
19.	ml	Milliliter
20.	DCM	Dichloromethane
21.	E.A.	Ethyl Acetate
22.	DMSO	Dimethyl sulphoxide
23.	Conc.	Concentration
24.	Abs.	Absorbance
25.	µl	Microlitre

Executive Summary

Skin lightening agents (SLA's) are the agents which are incorporated into creams and lotions for development of cosmeceutical products for over the counter and therapeutical use. The market of SLA's expected to reach US$ 31.2 billion by 2024. SLA's inhibits the synthesis of melanin by acting upon the tyrosinase or they bleach the melanin which is already present in the cells. However, due to some side effects of the existing SLA's, there is utmost need to explore alternative natural source.

Endophytic fungi are endosymbionts which colonize the healthy living tissue of host plant. They are considered as reservoir of bioactive compounds which exhibits anti-cancer, anti-microbial and anti-fungal activities. The present study was based upon the screening and identification of tyrosinase inhibitor and laccase producing endophytic fungi. Initially, culture filtrates of 91 cultures isolated from *Aegle marmelos , Cinnamomum sp. , Vitis vinifera* were screened for Tyrosinase inhibition as well as laccase production by agar well diffusion method. Out of 91 cultures, #20CMBANEY and #20(b)VVLPM showed the potential tyrosinase inhibitory and laccase activity respectively. Further, Crude Dichloromethane extract of #20CMBANEY was resolved into 6 bands using Hexane: Toluene solvent system. In vitro anti microbial assay was carried out by agar well diffusion method in which methanol fraction of #20CMBANEY exhibited the maximum anti microbial activity against *E.coli* and ethyl acetate fraction against *B.subtilis.* In vitro anti-oxidant assay was carried out by using spectrophotometer, hexane fraction exhibits the maximum 63.2% of free radical scavenging activity.The potential endophytic fungus #20CMBANEY was identified as *Nigrospora* sp. by morphotaxonomy. ITS region of approximately 500bp was amplified and further speciation of fungi will be deduced after analyzing sequencing data of ITS region.

Chapter – 1

INTRODUCTION

INTRODUCTION

Fairness is considered as symbol of beauty, grace and high social status in most of the communities. The skin colour of mammals is due to the expression level of heat shock protein 70-1A (Murase et al., 2016). This protein is correlated with "tyrosinase" which is rate limited enzyme of melanin synthesis pathway. Melanin is a pigment which is synthesized by the melanosomes in the cells in presence of "tyrosinase'. Tyrosinase carries out the conversion of an amino acid L-tyrosine into L-DOPA which gets convert into dopachrome by the same enzyme. Dopachrome formation leads to the synthesis of Eumelanin (Black/brown pigment) whereas in presence of cysteine, Dopachrome gets convert into Pheomelanin (Red/yellow pigment) *(*Ferrer et al., 1995*).* Difference in melanin content is due to several factors viz:

a. Rate of synthesis and activity of Tyrosinase
b. Size of melanosomes
c. Transfer rate of melanocytes to keratinocyte*s (*Iozumi et al., 1993*).*

Any change in above factors, results in occurrence of skin disorders such as Melasma and post-inflammatory hyper- pigmentation (Pandya et al., 2000). Apart from the clinical applications of SLA's they are also incorporated in cosmeceutical formulations viz: creams and lotions. The mode of action of SLA's is either through tyrosinase inhibition or melanin degradation in keratinocytes.

Various SLA's which are tyrosinase inhibitors are isolated from the botanical region such as polyphenols from strawberry and grapes, flavonoids from citrus fruits and licorice roots , α-arbutin from blueberry where as from the microbial origin kojic acid from *Aspergillus niger* and Azelaic acid from *Pityrosporum ovale* has been reported for the treatment of hyper-pigmentation. The problem associated with botanical agents is, they cause some allergic and photo-toxic reactions where as microbial origin sla's causes the erythema and irritation in most of the cases, due to which they are not safer to use (Fisk et al., 2014)

SLA's which bleach the melanin such as hydrogen peroxide and mercury mainly incorporated into cosmetic products (Gimeno et al., 2015*).* Mercury is also incorporated in the bleaching creams. Long exposure of the creams containing mercury leads to health problems such as nausea, tremors, affects vision and hearing and leads to loss of appetite. Prominently, it is considered as neurotoxin and nephrotoxic *(*Agrawal et al., 2015*).*

Introduction

15% of the world population invest in the skin lightening agents. The global market of SLA's is expected to increase by US$31.2 billion till 2024 *(*global skin lighteners market (MCP/ 6140*)*. Currently, skin whitening agents which are in market having some side effects associated with them. So, there is need to explore new skin lightening agents which is presumed to be safe and may have cosmeceutical application. In the present study, the endophytic fungi have been exploited as a novel source of tyrosinase inhibitors and bleaching agent.

Endophytic fungi live in the tissue of plants and symbiotic relationship with their host. Their relationship affects the quality and quantity of metabolic products of plants that can be used as drugs. Endophytes produce secondary metabolites such as alkaloids, flavonoids, to increase the resistance to biotic and abiotic stress of their host plant (Jia *et al.,* 2016) So it is hypothesized during abiotic stress , may be there is increase in transfer of fluids across the plant which mainly happens due to break down of melanin which provides energy to plant for transportation. To protect itself from ex osmosis, endophytic fungi may produce tyrosinase inhibitors where as laccase help in morphology and sporulation of fungi. Hence, in the present work we have explored the endophytic fungi of *Vitis sp., Cinnamoum sp., Aegle sp*. to get potential producer of tyrosinase inhibitor and laccase in free fermenting medium.

Chapter – 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1. MELANOGENISIS

Melanin protects the skin from harmful solar radiations. It helps in maintaining the haemostasis of the body but long exposure to UV rays, results in photosensitization. It leads to generation of super oxides which lethal to cell tissue. Exposure of melanin to toxic compounds leads to deposition of melanin in the cells. (Slominski et al., 2004) Melanin synthesis occur in melanosome which is a membrane bound organelle same as lysosome. Some structural proteins like gp100 (Pmel17) and MART1 required for the maturation of melanosomes. Melanin production requires the key enzyme i.e. TYR (tyrosinase) and others are TYRP1, TYRP2, DCT. Mutation in any of the enzyme leads to affect the quality and quantity of melanin. (Yamaguchi et al., 2007) . Basically, two types of melanin synthesis occur in mammals' i.e.

1. Eumelanin
2. Pheomelanin (Thody et al., 1991).

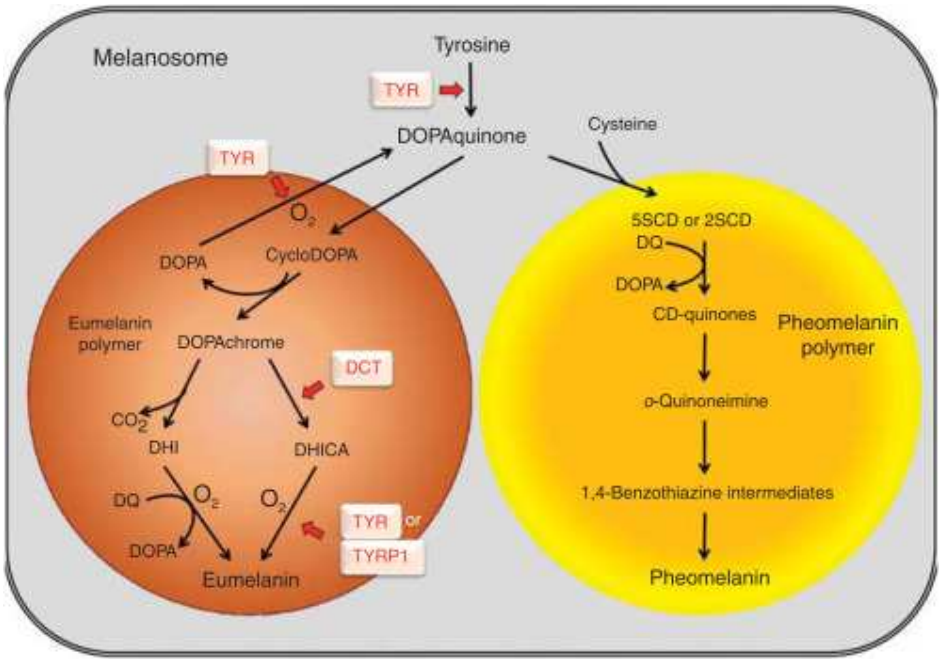

Figure 2.1: Melanin synthesis (Hearing 2011)

2.2 Pigment disorders

Pigment disorders mainly occur by influence of internal or external factors. External factor includes the exposure of skin to UV rays, results in "Tanning". It leads to erythema and patches on skin. (Lin et al., 2007). Melasma, drugs induced hyper pigmentation, post inflammatory hyper pigmentation mainly occurs due to external factors (Pandya et al., 2000). Internal factors such as mutations in the genes lead to hypo pigmentation. It results in oculocutaneous albinism, Hermansky-Pudlak syndrome (Dessinioti et al., 2009). To overcome the pigment disorders mainly which occurs by external factors, people use Skin lightening agents.

2.3. Skin Lightening Agents

To get fairer skin and to cure skin disorders, people mainly use skin lightening agents (SLA's). SLA mainly works by inhibiting the tyrosinase activity. As it is easy to target and considered as key enzyme. So, tyrosinase inhibitors are in great demand in cosmeceutical. The number of the inhibitors has been found till date from different sources (Momtaz et al., 2008*)*.

2.3.1 Therapeutic mechanism of action of skin lightening agents

Hyper-pigmentation occurs due to the deposition of melanin in the cells which results in unusual dispersion of pigment. Inhibition of melanin synthesis may affect the clinical pigmentation and skin lightening agents inhibits the pigmentation by different mechanisms viz:

1. By inhibition of the tyrosinase activity
2. By degrading the melanin which is already present in the cells
3. By anti-oxidant effect
4. By inhibiting DNA and RNA synthesis of melanocytes (Fisk et al., 2014)

   Mainly, skin lightening agents which are in market, are tyrosinase inhibitors. Tyrosinase is being targeted because it is key enzyme in melanin synthesis. There are different tyrosinase inhibitors isolated from plants and microbes listed below.

2.3.2 Tyrosinase inhibitors from Plants

Botanical extracts has been reported for exhibiting the property of tyrosinase inhibition. Hydroquinone considered as gold standard for treatment of hyper-pigmentation. However, it is associated with side effects such as irritation, permanent degradation of melanocytes. There are

herbs which are used in traditional Chinese medicine for the treatment of hyper-pigmentation. So the concept of exploiting botanicals for tyrosinase inhibiton has come to existence (Zhu et al., 2008). Large number of plants has been explored for the preparations of cosmetic products. Tyrosinase inhibitors which are isolated from plants as in table 1, mainly belongs to the families *Asteraceae, Fabaceae* (Dorni et al., 2016).

Table 1: Tyrosinase inhibitors from plants

plant	**compound**	**mechanism**	**reference**
Bauhinia rufescens	alpha amyrin acetate	Competitive inhibition	Muhammad et al., (2013)
Sophora flavescens	Kuraridin, kurarirone.	Not known	Kim et al., (2003)
Ginseng leaves	P-coumaric acid	Not known	Lim et al.,(1999)
Arctostaphylos uva-uris	Arbutin	Competitive	Pietraszek et al., (2016)
Aloe vera	9-dihydroxyl-2'-O-cinnamoyl-7-meyhoxy-aloesin	Competitive inhibitor	Kim et al., (2017)
Morus alba	Oxyresveratrol	Non competitive	YM et al.,(2002)
Citrus fruit peel	Nobiletin	Competitive	Sasaki et al., (2002)
Licorice roots	Glabrene and Isoliquiritigenin	Not known	Nerya et al., (2003)
Pomegranate	Ellagic acid	Competitive	Yoshimura et al., (2005)
Kaempferia pandurata	Isopanduratin A , 4-hydroxypanduratin A	Not known	JH et al.,(2007)
Euphorbia lathyris	Esculetin	Competitive inhibition	Masamoto et al., (2002)

2.3.3 Tyrosinase inhibitors from Micro organisms

Micro organisms such as bacteria and fungi have been explored for producing the tyrosinase inhibitors. Fungi produce diverse bioactive compounds, including, enzymes, enzyme inhibitors, growth promoters and antibiotics exploited in the agriculture and pharmaceutical industries. Fungi from different genera have been found to show anti-tyrosinase activity. One of the genera, Aspergillus found to produce several compounds having tyrosinase inhibitory activity (Fernandes et al., 2017). Kojic acid, a good tyrosinase inhibitor from *Aspergillus niger* has been reported (Vasantha et al., 2014).Other tyrosinase inhibitors from microbes are discussed in (table 2).

Table 2: Tyrosinase inhibitors from microbes

source	inhibitor	mechanism	reference
Bacteria			
Streptomyces.sp	OH-3984 K1 and K2	Not known	Takamastu et al*.,* (1993)
Actinomycete(KP-3052)	Amphistin	Post translational modification of enzyme	Arai et al.,(1997)
Enterobacter	Byelyankacin	Isocyanide binds to active copper site of enzyme	Takahashi et al., (2007)
Streptomyces roseolilacinus NBRC 12815	12815A and B	Not known	Nakashima et al., (2009)
Pseudomonas	Methylene chloride	Inhibits expression of enzyme	Corinaldesi et al .,(2017)
Thalassotalic sp. (PP2-459)	Thalassotalamides A and B	Not known	RW et al., (2016)
Fungi			
A.decumbens	Decumbenone A	Not known	Fujji et al., *(*2002)
Alternaria sp	Phomaligol A		Li et al., (2003)
Dictyophora indusiata	5-Hydroxymethyl-2-furfural	Non competitive inhibition	Sharma et al., (2004)

Daedalea dickinsii	Daedalin A	Competitive inhibitor	Morimura et al.,(2007)
Trichoderma viridae	Homothallin	Competitive inhibition	Tsuchiya et al., (2008)
Paecilomyces gunni	Paecilomycones A , B , C	Competes for active sitte	Lu et al., (2014)
A.oryzae BCRC 32288	6,7,4-trihydroxyisoflavone, Daidzein, glycitein	Not known	Fernandes et al., (2017)
Trichoderma harzianum	MR566A, MR566B	Isocyano group in compound helps in inanition	Fernandes et al., (2017)
Pityrosporum ovale	Azelaic acid	Competes for α-carboxylate bonding site of L-tyrosine	Fernandes et al., (2017)
Polyporus confluens	Neogrifolin	Not known	Fernandes et al., (2017)

2.3.4 Tyrosinase inhibitor from algae

Various marine algae such as *Endarachne binghamiae, Schizymenia dubyi , Ecklonia cava (EC) Sargassum silquastrum (SS)* has been reported for showing the tyrosinase inhibitory activity. Amongst the four, only two EC and SS showed best activity and could be used in cosmetics. (SH et al*.,* 2011)

Four different tyrosinase inhibitors such as (1) eckstolonol (2) eckol (3) Phlorofucifuroeckol (4) dieckol has been isolated from edible brown algae *Ecklonia stolonifera*. Eckstolonol and eckol act as competitive inhibitor of L-tyrosine where as Phlorofucofuroeckol A , dieckol act as non competitive inhibitor. (HS et al., 2004).

Red algae *Grateloupia lancifolia* has been reported for showing anti tyrosinase activity. Diethyl ether extract showed the maximum inhibitory activity. It is considered safe so it can be used in food additives also (Nguyen et al., 2012).

2.4. Laccase

In 1883, Yoshida was first who isolated laccase from exudates of Japanese lacquer tree *Rhus vernicifera* (Yoshida 1883). Laccase belongs to polyphenol oxidases. They have copper atom in their catalytic activity site. Laccase acts on the broad range of substrates including monophenols and diphenols. The different origin of laccase imparts the different reduced product. They are widely distributed in plants, fungi and bacteria. Laccase involve in lignin bio synthesis in plants and lignin degradation in bacteria and fungi where as they also help in in sporulation and morphogenesis of the fungi (Baldrian 2005). They polymerize the natural phenols which help in development of new cosmetics.

2.5 Sources of laccases

2.5.1 Bacterial laccases

In 1993, the first bacterial laccase was reported from *Azospirillum lipoferum* from rice rhizosphere (Givaudan et al., 1993). *Azospirillum lipoferum* found in soil and in rhizosphere of plants due to which it improves the growth of plant. Other bacteria such as *cyanobacterium, Anabaena azollae, Streptomyces lavendulae, Streptomyces cyaneus, Marinomonas mediterranea* also reported as source of laccases. Apart from these, *Bacillus subtilis* reported for producing more stable laccase which protects the bacteria spore coat from UV and hydrogen peroxide (Dwivedi et al., 2011).

2.5.2 Fungal laccases

In fungi, laccases are mainly found in ascomycetes, basidiomycetes and duteromycetes. Due to the hyphal organization, fungal laccases has better penetrating power due to which they efficiently degrade the lignin (Shraddha et al., 2011*). Myceliophthora thermophila* , a thermophillic fungi reported to produce laccase(Berk et al., 1997). *Chaetomium thermophilium a cellulolytic fungus*, isolated from municipal waste, exhibited the laccase activity (chefetz et al., 1998). *Cerrena maxima, Coriolopsis fulvocenerea,* and *Coriolus hirsutus* reported for producing laccase as it degrades atrazine (Koroleva et al., 2002). Some other fungi such as *Pycnoporus*

cinnabarinus, Coniophora puteana, Neurospora crassa reported for producing the laccase (Shraddha et al., 2011)

2.5.3 Plant laccases

Plant laccases are divergent in nature. Laccase are encoded by multigene family of plants. Five different type of laccases were reported from *Populus trichocarpa (Ranocha et al.,2002).* Other plants such as lacquer, mung bean, mango, peach, prune, pine, Arabidopsis, rice, *Liriodendron tulipifera* and *Zinnia elegans* were reported for producing the laccase(Fisk et al., 2014)

2.6. Applications of fungal Laccase

Laccase is industrially important enzyme as it act on the broad spectrum of substrate. Two main areas are included for using the laccase i.e. Delignification and pulp bleaching, bioremediation (Kunamneni et al., 2007)

2.6.1 Delignification and pulp bleaching

In pulp industry, chlorides were used to remove the pulp from wood and it becomes the costly process. In 2006, for delignification laccase was introduced in industry, which is eco friendly and reduce the cost also. (Camarero et al., 2007)

2.6.2 Bio remediation

10% of the toxic compounds are disposed properly in the environment where as other hazardous compounds such as poly aromatic hydrocarbons are not degradable in the environment. An alternative was needed to degrade the hazardous compounds, so laccase comes into existence. Laccase can degrade the complex compounds into simpler ones. Laccase from the strain *Coriolopsis gallica* has been reported to degrade carbozole,N-ethylcarbozole, fluorine, and dibenzothiophene which mainly comes under the xenobiotics (Viswanath et al., 2014)

2.7 Marketing of SLA's

Fairer skin is considered as sign of beauty in most of countries, so there in increment in use of SLA's every year. 15% of world population invest in the SLA's. Market size is expected to US$31.2 billion by 2024 (Global industry analysts, Inc.)

2.8. Why should we look for endophytic fungi as source of sla's ?

Endophytic fungi inhabit plants and highly categorized as diverse group of Ascomycetous fungi. It built the symbiotic relationship with their host plant due to which it affects the formation of metabolic products in plants. It leads to affect the quality as well as the quantity of the secondary metabolites which can be used as drugs of medicinal plants. *(*Jia et al*.,* 2016*).* Endophytic fungi help in defence mechanism of host plant. Chiefly, endophytes which present in grasses, are considered as plant mutualist, because endophytes release alkaloids which protect grass from herbivores (Faeth et al., 2002*).* Endophytes considered as tremendous source of bio active compounds which can be used as drugs. For e.g. Taxol, anti cancer agent has been isolated from endophyte *Taxomyces andreanae* which lives in *Taxus brevifolia* host plant. Except, taxol other agents such as Vinblastine, vincristine, Camptothecin and podophyllotoxin has been reported from endophytes only (Chandra et al., 2012). Same as melanin pigment is present in the plants. Melanin provides the energy to plants for transportation, after breaking down into water and energy carry molecules (Herrera 2015). As endophytes are in symbiotic relationship with plants, so there is possibility endophytic fungi may secrete tyrosinase inhibitors to prevent the ex-osmosis of it. Laccase may be produced by endophytes as laccases plays role in sporulation and morphogenesis of fungi. This is new approach to explore endophytes for tyrosinase inhibitors and laccase.

Chapter – 3

AIM OF THE STUDY

AIM OF STUDY

1. Screening of endophytic fungal isolates for tyrosinase inhibition and laccase production.
2. Identification of tyrosinase inhibitor and laccase producing endophytic isolates.

Chapter – 4

MATERIALS AND METHODS

MATERIAL AND METHODS

4.1 Procurement and maintenance of endophytic cultures

Out of 91 isolates, 30 isolates of *Cinnamomum,* 31 isolates of *Vitis vinifera* and 30 isolates of *Aegle marmelos* were procured from the repository of Dr. Sanjai Saxena Lab, Thapar University, Patiala. A mycelial plug of 5mm was prepared from the mother culture plate and placed on the freshly prepared PDA (Potato Dextrose Agar) which was subsequently incubated at 27±2˚ C for 5-6 days. These were then used for preparation of glycerol stocks as well as inoculums for the liquid culture.

4.2 Production of culture filtrates (Rodrigues et al. , 2000)

All 91 fungal isolates were inoculated in PDB (Potato Dextrose Broth) for induction of secondary metabolites. Briefly, 5mm mycelial plug of 3-4 days old culture was inoculated in 50 ml pre-sterilized PDB (pH 5.1, Himedia) followed by incubation at 27±2˚ C for 8 days at 120rpm. Subsequently, mycelial mass was collected by filtration by using Whatmann filter paper no. 4 (GE Health Care , U.S.A.).The supernatant so obtained centrifuged (Hitachi, Japan) at 8000 rpm for 10 minutes. The supernatant obtained was then stored in sterile vials at -20˚ C till further use.

4.3 Screening of laccase and tyrosinase inhibitory potential of endophytic fungi

The cell free culture filtrates of endophytic fungi was thawed and tested for laccase production and tyrosinase inhibitors by in vitro well diffusion method.

4.3.1 Preliminary and quantitative screening for tyrosinase inhibition (Grund et al., 1986)

Preliminary screening was carried out with slight modifications in Darryl et al., (1986) method. The method comprised of preparation of tyrosine agar plates having 2.5% agar, 7.5mg/ml tyrosine. 5mm wells were prepared aseptically by using sterile cork borer. Subsequently, 24µl of reaction mixture containing 12µl of each culture filtrate and 23.8 U of Tyrosinase (Source: Mushroom Tyrosinase, Sigma Aldrich) was dispensed in each well followed by incubation at 37˚

C for 4-5 h. The control well consisted of uninoculated broth and 23.8 U of tyrosinase. Appearance of a brown colour halo indicated the tyrosinase activity in control well while reduction in the diameter of halo in comparison to control indicated tyrosinase inhibition. The percentage of inhibition of all 91 isolates was calculated as follows:

$$\% \text{ of inhibition} = \text{control} - \text{sample} / \text{control} * 100$$

All the tests were carried out in triplicates. The halo diameter was recorded and all the data was represented as Mean ± SD values.

4.3.2 Preliminary screening of laccase (Kumar et al., 2011)

In vitro screening for laccase activity was carried out by the method of Kumar et al., (2011) with minor modifications. The method comprised of preparation of guaiacol plates using 2% agar and 0.2 g of guaiacol. Subsequently, 5mm wells were prepared aseptically by using sterile cork borer. 25µl of culture filtrate of each isolate was dispensed in the wells followed by overnight incubation at 37° C. Appearance of red halo indicated the laccase activity.

4.4 Liquid-Liquid extraction for isolation of tyrosinase inhibitor (Yamauchi et al., 2011)

Liquid -liquid extraction with slight modifications in method of Yamauchi et al., (2011) was adopted to partially purify the tyrosinase inhibitor. The cell free culture filtrates of selected cultures were extracted with the different solvents in order of increasing polarity from hexane, Dichloromethane, chloroform, Ethyl acetate, methanol to water (figure 4.1). Extraction of filtrates was carried out three times with each solvent and solvent layers were pooled. The organic layer containing the compounds of interest was collected and the remaining solvent was evaporated by rota evaporator. After removal of solvent, extract was reconstituted with DMSO and stored at -20° C till use.

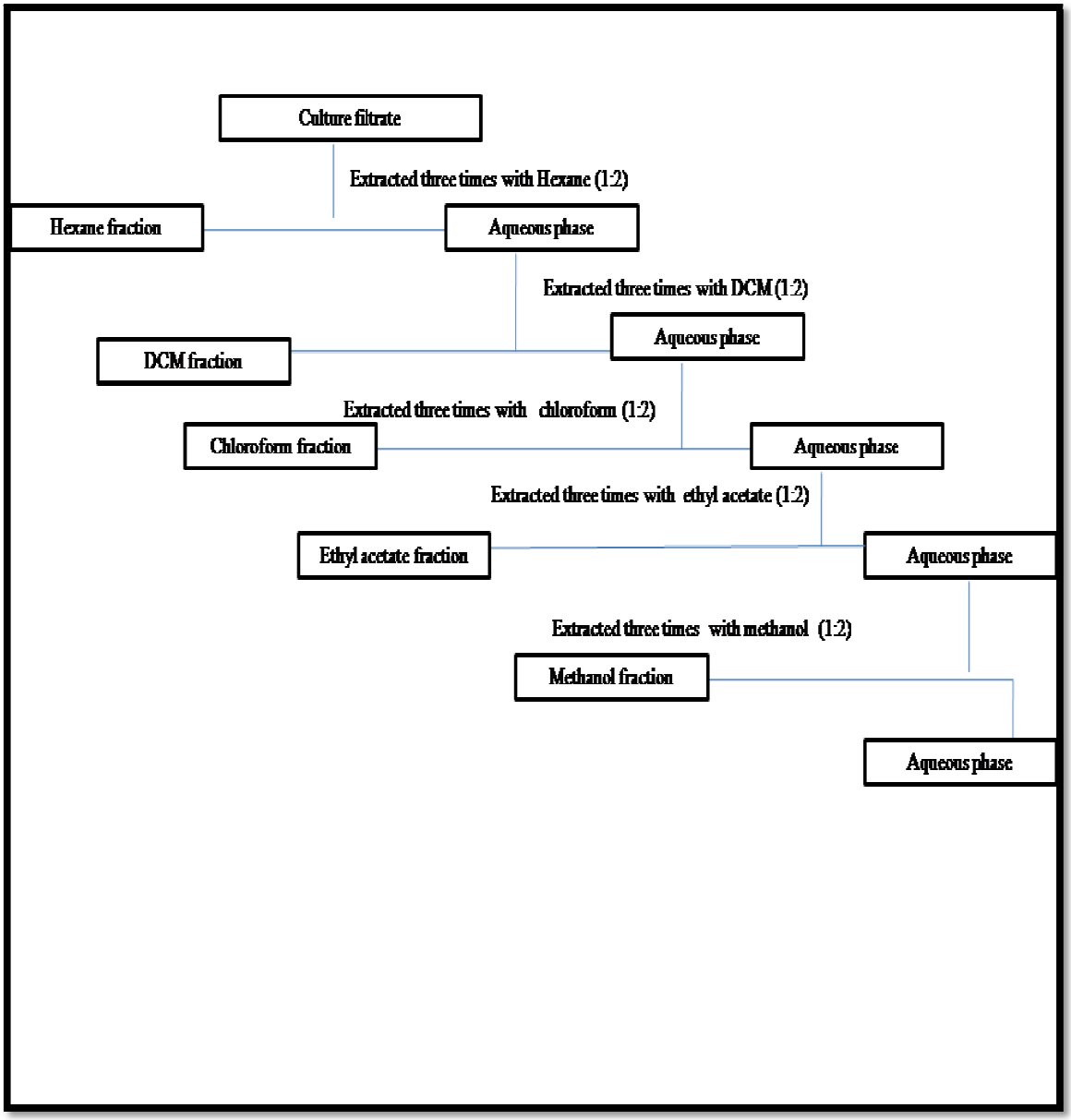

Figure 4.1 Schematic representation of solvent extraction (Yamauchi et al., 2011)

4.5 Kinetics of tyrosinase (Valipour et al., 2015)

Optimization of the enzyme activity is pre-requisite to establish the effect of inhibitor. All the tests were performed in triplicates and data represented as Mean± S.D.

4.5.1 Optimization of substrate concentration

A 5mM stock of the L-tyrosine, substrate of the enzyme tyrosinase was prepared by using the double distilled water. Dilutions were prepared from the stock in the concentration ranges from 0.1mM to 0.9mM. Total reaction mixture of each concentration was prepared of 200µl which contain different concentration of substrate , phosphate buffer(pH-6.5) , tyrosinase enzyme (5µl for each concentration) and 3M HCL reaction stopping agent (80µl for each concentration) as in Table 3.The reaction mixture was incubated for 2h and stopping reagent added to stop the reaction. Absorbance of formation of product was measured at 475nm.

Table 3: Reaction mixture for substrate optimization

Concentration(mm) of L-tyrosine	Vol. of stock (µl)	Phosphate buffer(µl)	Tyrosinase (µl)	3M HCL(µl)
0.1	4	111	5	80
0.2	8	107	5	80
0.3	12	103	5	80
0.4	16	99	5	80
0.5	20	95	5	80
0.6	24	91	5	80
0.7	28	87	5	80
0.8	32	83	5	80
0.9	36	79	5	80

4.5.2 Optimization of time of Tyrosinase activity

Assay time of Tyrosinase activity was optimized. The reaction mixture contains the substrate of 0.5mM (20 µl) concentration, phosphate buffer (pH-6.5) 95µl and tyrosinase enzyme (0.4 mg/ml) 5µl in it. The reaction mixture was incubated for 3h. Subsequently, the reaction was stopped by adding 3M HCL (80µl) at an interval of every 5min. Then absorbance noted at 475nm.

4.5.3 Optimization of enzyme concentration

Different concentrations of tyrosinase enzyme ranges from 0.1mg/ml to 1mg/ml and substrate concentration of 0.5mM (20µl) were taken (Table 4)and incubated at optimized time i.e. for 90

minutes at 37° C. The reaction was stopped by adding 3M HCL (80µl) and absorbance noted at 475nm.

Table 4: Reaction mixture for enzyme optimization

Tyrosinase conc.(mg/ml)	**substrate vol.(µl)**	**enzyme volume (µl)**	**phophate buffer(ph-6.5) (µl)**	**3M HCL (µl)**
0.1	20	5	95	80
0.2	20	5	95	80
0.3	20	5	95	80
0.4	20	5	95	80
0.5	20	5	95	80
0.6	20	5	95	80
0.7	20	5	95	80
0.8	20	5	95	80
0.9	20	5	95	80
1	20	5	95	80

4.6 Quantitative assay for Tyrosinase inhibitor (Vasantha et al ., 2014)

Briefly the assay comprised of L-tyrosine (20µl, 5mM), tyrosinase 19.8U/ml, fractions of selected culture (40µl each), phosphate buffer (pH-6.5, 55µl) where as control contain DMSO instead of inhibitor fraction. Firstly, tyrosinase and fractions were incubated for 15 minutes and then the whole reaction was completed. Assay was carried out in 96 well plate. Reaction mixture was incubated for 90 minutes at 37° C and the absorbance measured at 475 nm. All test were carried out in triplicates and data represented as Mean±S.D. The percentage of inhibition was calculated as

$$\% \text{ of inhibition} = O.D._{control} - O.D._{sample} / O.D._{control} * 100$$

4.7 Identification of Endophytic fungi

The selected isolate showing tyrosinase inhibitor production was identified by classical and molecular taxonomy.

4.7.1 Morphotaxonomy (Barnett et al., 1998)

The isolate which exhibited tyrosinase inhibition was microscopically examined and its plate characteristics were recorded.. The culture was grown on different media viz Potato dextrose agar (PDA) , Water agar (WA), Synthetic nutrient deficient agar (SNA), Malt Extract Agar (MEA), Pine agar(P.A) for 25 days at 28±2° C under 12 hr dark/light . For microscopic features, a drop of water was put on clean glass slide, upon which the mycelia mass was placed and teased properly with help of needle. It was then stained with Lacto phenol cotton blue (Himedia) and slide was covered with cover slip. The slide was observed at 10X, 40X and 100X using microscope.

4.7.2 Molecular identification of Endophytic fungi

4.7.2.1 DNA isolation

The fungal genomic DNA was isolated from 5-7 day old culture grown on PDA plate using Genomic DNA purification kit (Promega, USA). 2-3 mycelial plug of 5 mm diameter was crushed into very fine powder in sterile pestle and mortar by using liquid nitrogen. 660-750µl of nuclei lysis buffer was added and it was crushed. The contents were transferred to a 1.5 ml micro centrifuge tube and vortexed followed by incubation at 65° C in water bath for 15 min. After the incubation, the micro centrifuge tubes were centrifuged at 12,000 rpm for 5 min to remove cell debris. Further 5 µl of RNase was added to each tube and incubated at 37° C for 15 min followed by addition of 200µl of protein precipitation solution. After this, the micro centrifuge tubes were centrifuged at 12,000 rpm for 3 min to remove contaminating proteins. The aqueous phase containing DNA was transferred to iso-propanol containing micro centrifuge tube and centrifuged at 13,000 rpm for 3 min. The DNA pellet was rinsed with 70% ethanol followed by centrifugation at 13,000 rpm for 1 min. Then pellet was air dried and dissolved in 50µl of DNA dehydration buffer (Tris EDTA buffer (pH 8)). The qualitative estimation of DNA isolated was done by Agrose gel electrophoresis.

4.7.2.2 Agarose gel electrophoresis

0.8% Agarose gel containing 0.5µg/ml of ethidium bromide was prepared in 1X TAE (Tris Acetate EDTA) buffer and gel was casted in electrophoretic apparatus. The gel was allowed to solidify and the comb was carefully removed. The running buffer (1X TAE) was poured into electrophoretic tank . The DNA sample was mixed with 6X loading dye and subsequently loaded into wells and allowed to run 50V for 1h. The DNA fragments were visualized under UV transilluminator. Gel imaging was performed under UV light in Bio-Rad gel documentation system using quantity 1-D anaylsis software.

Quantitative estimation of genomic DNA was done by Nanodrop. The absorbance of sample was taken at 260nm and 280nm to determine concentration and purity of sample. 1 OD is equivalent to 50µg/ml DNA sample. The concentration of DNA sample was calculated using following Formula.

$$\text{Concentration (µg/ml)} = \text{O.D.}_{260nm} \times 50\text{µg/ml} \times \text{Dilution factor}$$

The purity of DNA sample was determined by taking the ratio of absorbance at 260nm and at 280nm. If the ratio is less than 1.6 then there is RNA contamination, if ratio lies between 1.6-1.8 then DNA sample is pure. If the ratio is more than 1.8, DNA is contaminated with protein.

4.7.2.3 PCR amplification (White *et al.,* 1990)

ITS1-5.8S-ITS2 rDNA sequence was amplified using universal primer pair i.e. ITS1 and ITS 4, synthesized by integrated DNA technologies (IDT), USA, in a thermocycler (My cycler, Bio-Rad Laboratories, Inc.). Amplification reaction was carried out by using primer ITS 1(5'TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT TGA TAT GC 3'). Amplification was performed in 25µl reaction mixture containing 25ng of extracted fungal DNA, 0.8µM of each primers , 2.5mM of dNTP , 1.5mM MgCl2, 1.5 U of Taq DNA polymerase in 10 X Taq buffer. The thermal cycling conditions consisted of initial denaturation at 96° C for 5 min followed by 39 cycles of 95° C for 1 min , 58° C for 1.30 min , 72° C for 1 min followed by final extension at 72° C for 5 min. The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio-Rad documentation system.

4.8 In vitro anti microbial assay (Hady et al., 2014)

The culture filtrate of the selected isolate screened for its anti microbial activity against bacteria viz: *Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus*. Cultures were revived by inoculating them on Mueller Hinton Broth (MHB) one day before performing the test. The test micro organisms were diluted in 0.9% w/v saline and visually adjusted with 0.5 Mc^{+} Farland solution to achieve 10^6 cfu/ml. Subsequently, 5mm wells were scooped and on pre-made MHA plates, 25µl of solvent fractions of selected culture were dispensed in each well. Streptomycin (1mg/ml) used as control. Plates were incubated at 37° C for 24 h. The zone of clearance was measured to know the antimicrobial activity. Readings were taken in triplicates and data stored as mean ± SD values.

4.9 In vitro anti oxidant assay (Hady et al., 2014)

The anti oxidant potential of the solvent fractions of the selected isolate was carried out by using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as be the method of Kitts et al., (2000) with minor modifications. Briefly, 230 µl of DPPH, 20µl of test sample (1mg/ml) was added and mixed well. The reaction mixture then incubated in dark at room temperature for 30 minutes. After incubation, the absorbance was measured at 517 nm. Quercetin (10-50µg/ml) used as positive control in assay. The DPPH radical scavenging capacity was expressed as µg of quercetin equivalents per milligram of extract. Free radical scavenging activity calculated as:

$$\% \text{ free radical scavenging activity} = (O.D._{control} - O.D._{test}) / O.D._{control} * 100$$

4.10 TLC analysis (Zhou et al., 2016)

The crude DCM fraction of culture exhibiting tyrosinase inhibition was fractionated by subjecting to preparative thin layer chromatography (TLC). The TLC plate of 0.5 mm thickness was prepared by coating silica gel on a 20 x 15 x 5mm clean glass plates and was activated by incubating at 80° C for 2 h prior to use. The sample was spotted on the activated TLC plate just 1 cm above the edge of plate with help of capillary. Simultaneously, the TLC chamber was saturated with different solvent system consisting of mixture of solvents of different polarties and ratios for 40 min. The loaded TLC plate was then placed in the `TLC chamber in such that the

sample spot is above the solvent level. When the solvent reaches to desired level, plate was taken out and viewed under UV light at 254nm. The retention factor (R_f) value was calculated as follows:

Retention factor= distance covered by solute/ distance covered by solvent

Chapter – 5

RESULTS

RESULTS

5.1 Procurement and maintenance of endophytic fungi

All the 91 cultures isolated from medicinal plants namely *Aegle marmelos , Cinnamomum malabaricum and Vitis vinifera* were sub cultured on PDA plates and further preserved as glycerol stocks (Table 5). Research on endophyte fungi as source of bio active compounds have strengthened in last two decades where in they have been screened for Immunosuppressant, anti cancer and anti microbial properties. However exploration of endophyte fungi for inhibitor of tyrosinase and as producers of laccase is a new concept.

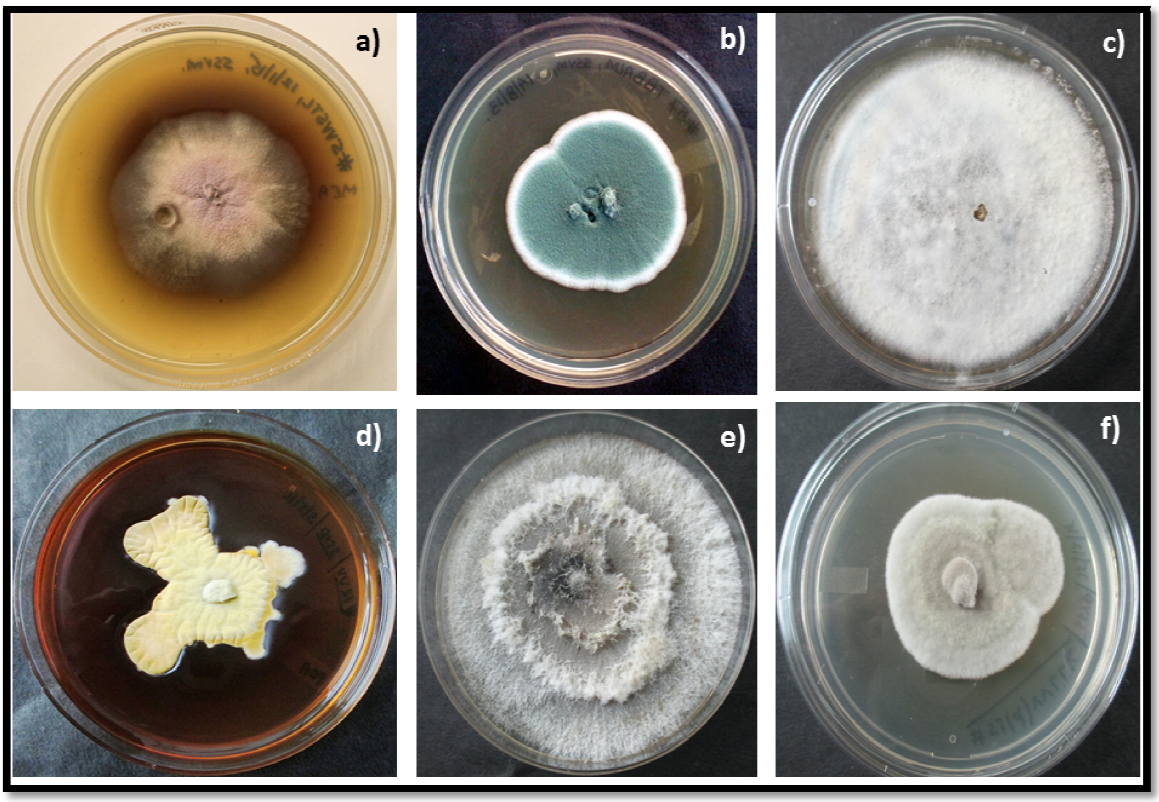

Figure 5.1: Endophytic fungi procured from lab a) *Quambuleria* sp., b) *Aspergillus* sp., c) *Fusarium* sp., .d) Cheatomium sp., e) and f) Unidentified

Results

s.no.	culture code	host plant	part of plant	location	tyrosinae inhibitor	laccase activity
1	# 2CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-	-
2.	# 4CMBABRT	*Cinnamomum malabaricum*	Bark	BRT Wildlife Sanctuary, Karnataka	+	-
3.	# 12CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-	-
4.	# 12CMBABRT	*Cinnamomum malabaricum*	Bark	BRT Wildlife Sanctuary, Karnataka	-	-
5.	# 18CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-	-
6.	# 18CMBABRT	*Cinnamomum malabaricum*	Bark	BRT Wildlife Sanctuary, Karnataka	++	-
7.	# 20CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	+++	-
8.	# 21CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-	-
9.	# 22CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-	-
10.	# 1CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala	-	-
11.	# 2CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala	-	-
12.	# 4CMLBRT	*Cinnamomum malabaricum*	Leaves	BRT Wildlife Sanctuary, Karnataka	-	-
13.	# 11CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
14.	# 17CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala	-	-
15.	# 18CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala	-	-
16.	# 23CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala	-	-
17.	# 27CMLBRT	*Cinnamomum malabaricum*	Leaves	BRT Wildlife Sanctuary, Karnataka	- -	- -
18.	# 29CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala		
19.	# 37CMLNEY	*Cinnamomum malabaricum*	Leaves	Neyyar, Kerala	-	-
20.	# 40CMLBRT	*Cinnamomum malabaricum*	Leaves	BRT Wildlife Sanctuary, Karnataka	-	-
21.	# # 1CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
22.	# 13CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
23.	# 26CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-

24.	# 35CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
25.	# 36CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
26.	# 43CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	++	-
27.	# 44CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
28.	# 45CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
29.	# 49CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
30.	# 55CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
31.	#23(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
32.	#51(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
33.	#129(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
34.	#14(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
35.	#57(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
36.	#140(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
37.	#117(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
38	#11VSMP	*Vitis vinifera*	Stem	Pune, Maharashtra	-	-
39.	#43(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
40	#9VVGSTL	*Vitis vinifera*	Stem	Rajendra nager, Lucknow	-	-
41	#131(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
42	#133(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
43	#113(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
44	#5VV	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
45	#12VV	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
46	#20VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
47	#19(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
48	#22(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
49	#13(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
50	#15(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
51	#41(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-

52	#33(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
53	#71(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
54	#55(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
55	#30(b)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
56	#103(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
57	#101(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
58	#111VVLSWN	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
59	# 20(b)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	+++
60	#11AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	-	-
61	#6AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	-	-
62	#61AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	-	-
63	#53AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-	-
64	#1AMSTYEL	*Aegle marmelos*	Stem	Yelundur , Karnataka	-	-
65	#3AMSTYEL	*Aegle marmelos*	Stem	Yelundur , Karnataka	-	-
66	#5AMSTYEL	*Aegle marmelos*	Stem	Yelundur , Karnataka	-	-
67	#7AMSTYEL	*Aegle marmelos*	Stem	Yelundur , Karnataka	-	-
68	#8AMSTYEL	*Aegle marmelos*	Stem	Yelundur , Karnataka	-	-
69	#9AMSTYEL	*Aegle marmelos*	Stem	Yelundur , Karnataka	-	-
70.	#1032AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur , Karnataka	++	-
71	#1048AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur , Karnataka	++	-
72	#1070AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur , Karnataka	-	-
73	#1118AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur , Karnataka	-	-
74	#1003MSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur , Karnataka	-	-

75	#28AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-	-
76	#57AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-	-
77	#47AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-	-
78	#53AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-	-
79	#59AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-	-
80	#9(b)AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
81	#11AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
82	#17AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
83	#22 (b) AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
84	#23AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
85	#4AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
86	#9AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife , sanctuary	-	-
87	#31VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
88	#1007AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife , sanctuary	-	-
89	#1016AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife , sanctuary	-	-
90	#10AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife , sanctuary	-	-
91.	#2158CZSTITG	*Cinnamamum zeylanicum*	Stem internal tissue	Guwahati	+	-

Note : (+) and (-) indicates the severity of tyrosinase inhibitor and laccase activity. Where (+++) indicates very good activity, (++) indicates good activity and (+) indicates fair activity.

Table 5: Preliminary screening of endophytic fungal isolates under study for tyrosinase inhibitor and Laccase producer.

5.2 Production of Culture filtrates of endophytes

All 91 cultures were subjected to secondary metabolite production. The growth rate of the fungal cultures can be determined through biomass production. pH of the filtrates were measured and it's mainly acidic in nature (Table 6)

Table6: Culture filtrates

s.no.	culture code	Biomass (gm)	pH
1.	# 18CMBABRT	0.27	2.8
2.	# 43CMSTNEY	7.95	5.98
3.	# 1032AMSTITYEL	0.16	3.83
4.	# 1048AMSTITYEL	0.29	2.7
5.	# 2158CZSTIT	0.14	5.98
6.	# 4CMBABART	0.29	6.55
7.	# 20CMBANEY	0.07	5.8
8.	# 2CMBANEY	0.21	4.5
9.	#61AMLWLS	0.34	4.2
10.	# 12CMBANEY	0.25	3.4
11.	# 12CMBABRT	0.28	2.5
12.	# 18CMBANEY	0.31	1.2
13.	#6AMLWLS	3.1	3.5
14.	# 21CMBANEY	2.4	4.1
15.	# 22CMBANEY	1.2	2.8
16.	# 1CMLNEY	0.58	3.1
17.	# 2CMLNEY	0.60	2.0
18.	# 4CMLBRT	3.2	1.2
19.	# 11CMSTNEY	2.4	5.6
20.	# 17CMLNEY	0.96	2.4
21.	# 18CMLNEY	1.5	2.1
22.	# 23CMLNEY	2.4	5.1
23.	# 27CMLBRT	3.4	3.5
24.	# 29CMLNEY	0.89	2.3
25.	# 37CMLNEY	2.4	2.1
26.	# 40CMLBRT	0.10	3.1
27.	# # 1CMSTNEY	0.45	2.1
28.	# 13CMSTNEY	2.8	1.2
29.	# 26CMSTNEY	3.4	2.1
30.	# 35CMSTNEY	2.1	1.2
31.	# 36CMSTNEY	5.4	4.5
32.	# 43CMSTNEY	2.9	4.7
33.	# 44CMSTNEY	3.1	2.1
34.	# 45CMSTNEY	0.54	3.1
35.	# 49CMSTNEY	0.78	2.5
36.	# 55CMSTNEY	2.1	2.1
37.	#53AMSTWLS	1.2	5.6
38.	#1AMSTYEL	4.5	4.5
39.	#3AMSTYEL	3.4	2.5
40.	#5AMSTYEL	5.6	6.1

41.	#7AMSTYEL	2.8	7.1
42.	#8AMSTYEL	2.7	2.4
43.	#9AMSTYEL	3.6	3.5
44.	#1070AMSTITYEL	0.58	6.5
45.	#1118AMSTITYEL	0.91	5.5
46.	#1003MSTITYEL	0.78	5.7
47.	#28AMSTWLS	0.31	6.0
48.	#57AMSTWLS	2.5	3.4
49.	#47AMSTWLS	0.91	5.6
50.	#11AMBAWLS	6.4	7.1
51.	#59AMSTWLS	5.6	2.5
52.	#9(b)AMSTYEL	0.68	6.9
53.	#11AMSTYEL	0.40	3.2
54.	#17AMSTYEL	0.87	5.8
55.	#22 (b) AMSTYEL	0.98	4.6
56.	#23AMSTYEL	0.65	3.8
57.	#4AMSTYEL	6.5	5.8
58.	#9AMLBRT	2.7	6.1
59.	#1007AMLBRT	3.4	5.7
60.	#1016AMLBRT	2.5	4.1
61.	#10AMLBRT	2.3	5.8
62.	#23(P)VVLPM	6.38	5.2
63.	#51(P)VVLPM	4.5	5.2
64.	#129(P)VVLPM	2.1	4.8
65.	#14(P)VVLPM	1.2	6.2
66.	#57(P)VVLPM	2.3	5.4
67.	#140(P)VVLPM	3.5	4.5
68.	#117(P)VVLPM	8.8	6.2
69.	#11VSMP	4.5	5.6
70.	#43(P)VVLPM	7.51	6.36
71.	#9VVGSTL	6.6	5.8
72.	#131(P)VVLPM	3.4	4.1
73.	#133(P)VVLPM	2.1	5.1
74.	#113(P)VVLPM	1.2	6.6
75.	#5VV	4.2	5.8
76.	#12VV	3.9	3.2
77.	#20VVLMP	4.2	7.2
78.	#19(P)VVLPM	1.1	6.6
79.	#22(P)VVLPM	2.4	5.3
80.	#13(P)VVLPM	1.2	6.9
81.	#15(P)VVLPM	1.7	6.5
82.	#41(P)VVLPM	5.8	6.5
83.	#33(P)VVLPM	1.53	6.5
84.	#71(P)VVLPM	2.1	4.2
85.	#55(P)VVLPM	1.3	6.2
86.	#30(b)VVLPM	0.42	6.0

87.	#103(P)VVLPM	4.4	6.3
88.	#101(P)VVLPM	3.8	5.4
89.	#111VVLSWN	3.8	6.1
90.	# 20(b)VVLPM	4.5	5.6
91.	#31VVLPM	1.9	6.4

Figure 5.2: culture filtrates

5.3.1 Preliminary screening for tyrosinase inhibition

Out of 91 cultures, only 7 cultures namely #18CMBABRT, #42CMSTNEY, #1032AMSTITYEL, #1048 AMSTITYEL, #2158CZSTIT, #4CMBABRT, and #20CMBANEY exhibited the positive results in terms of tyrosinase inhibition (Table7) Out of 7 cultures, only # 20CMBANEY showed the maximum inhibition of 67% (figure 5.3) so it was taken for further processing.

Table7: Tyrosinase inhibitory activity of the culture filtrates of selected isolates

S.no.	Culture code	Zone of inhibition*
1.	#18CMBABRT	7.6±0.57
2.	#42CMSTNEY	8.6±0.57
3.	#1032AMSTITYEL	7.3±0.57
4.	#1048AMSTITYEL	6.6±0.57
5.	#2158CZSTIT	8.6±0.57
6.	#4CMBABART	8.6±1.15
7.	#20CMBANEY	5.0±0.57

(*indicates data represented as Mean±S.D.)

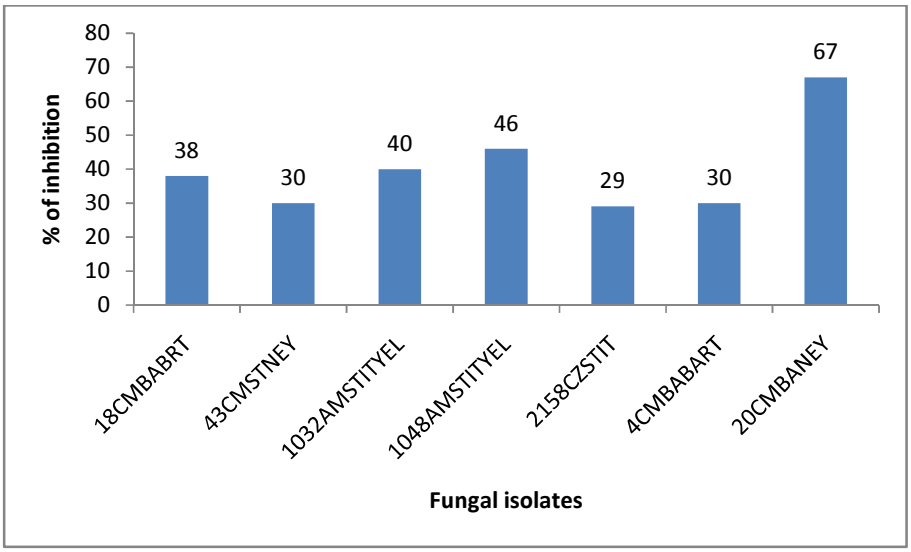

Figure 5.3: Tyrosinase inhibitory activity of the culture filtrates of selected isolates

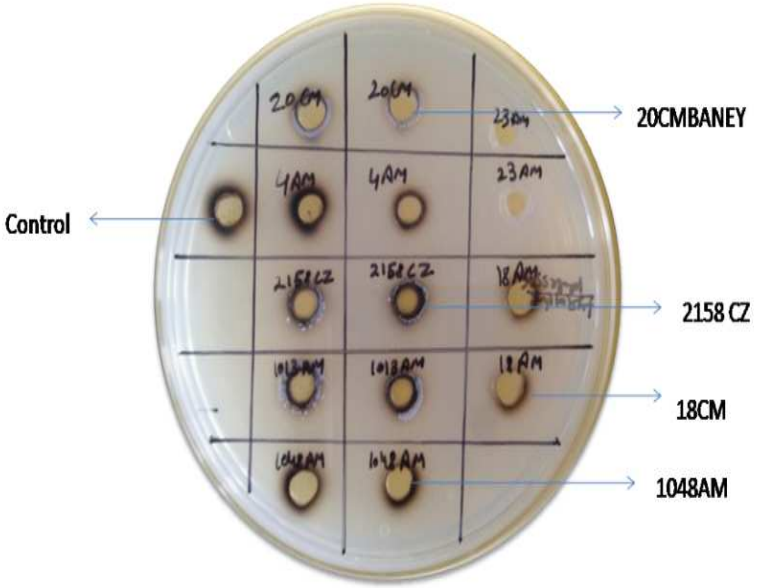

Figure 5.4: Preliminary screening for tyrosinase inhibition

5.3.2 Preliminary screening for laccase producer

Out of 91 cultures, only single culture namely 20(b) VVLPM exhibited a red halo of 10-12mm (Figure). Laccase oxidized the guaiacol and gave the red colour which proved the presence of the laccase activity. Kumar et la (2011) screened the 20 fungal isolates for laccase activity.

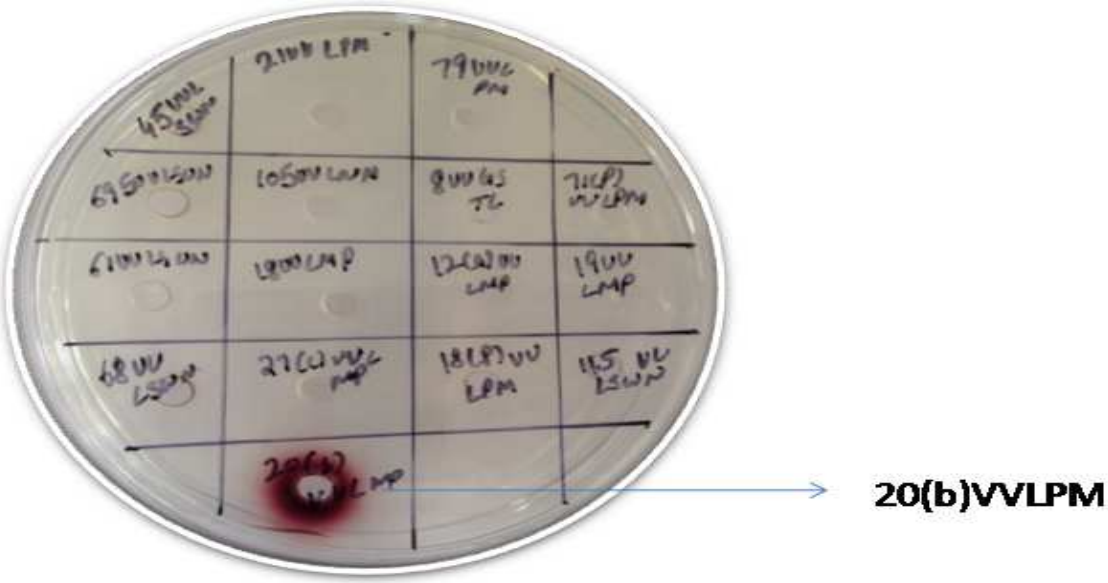

Figure 5.5: Red halo by #20(b) VVLPM showing production of laccase

5.4 Liquid-liquid extraction for partial isolation of tyrosinase inhibitor

The cell free filtrate of positive tyrosinase inhibitor i.e. #20CMBANEY was subjected to solvent extraction in increasing order of polarity viz: Hexane, Dichloromethane, Chloroform, Ethylactetate and Methanol. The obtained solvent fraction was further reconstituted into DMSO followed by storage at -20° C for further use.

Table 8: Yield of compound after liquid-liquid extraction

Extraction	Yield of compound (mg/ml)
Hexane	3
DCM	4
Chloroform	5
Ethyl acetate	3
Methanol	4

5.5 Optimization of mushroom tyrosinase activity

Optimization of the maximum activity of the enzyme was done to assess the potential of culture filtrate inducing maximum inhibition.

5.5.1 Optimization of substrate concentration

As the concentration of the substrate increased, there was proportionally increase in the absorbance. At particular concentration viz: 0.5mM, absorbance become constant (figure 5.6). It gave the information,enzyme reaches its saturation level after the substrate concentration of 0.5mM and cannot change the more substrate into product. It means, it is best concentration of substrate to achieve the maximum activity of enzyme.

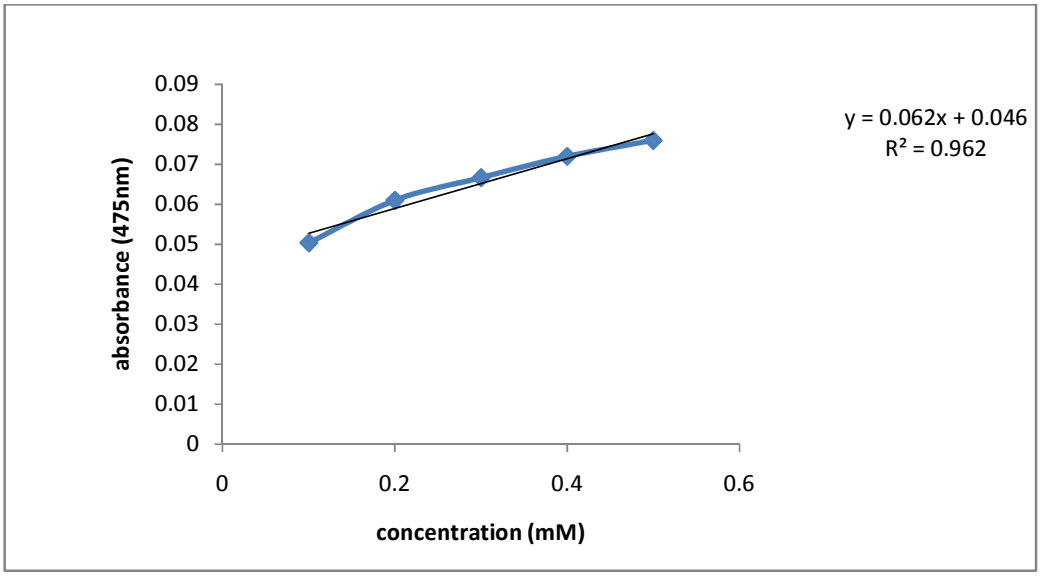

Figure 5.6: Optimized concentration of substrate for enzyme activity

5.5.2 Optimization of tyrosinase concentration

The optimal tyrosinase concentration was 0.8mg/ml as no significant change in absorbance at 475nm was observed (figure 5.7)

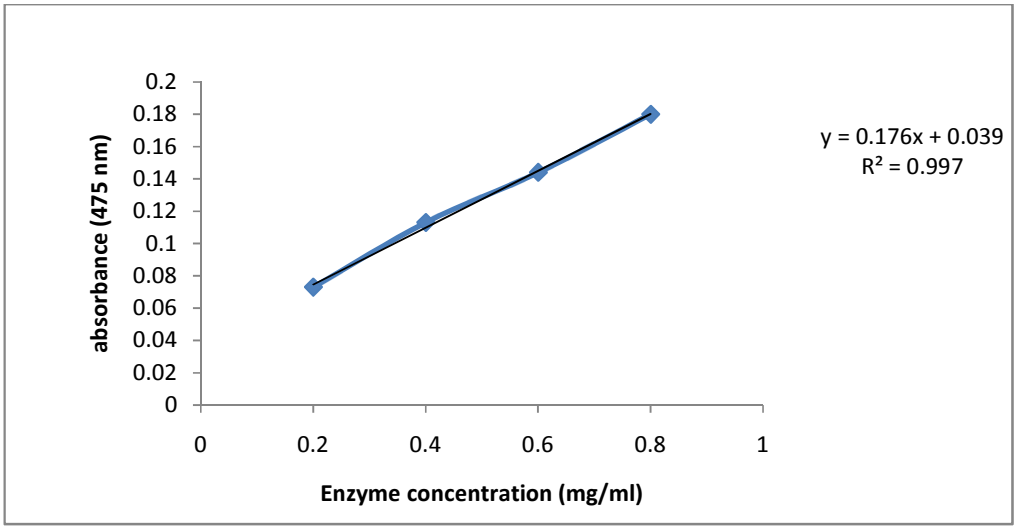

Figure 5.7: optimization of tyrosinase activity

5.5.3 Optimization of time for tyrosinase activity

The optimal time for tyrosinase activity was 90 minutes (figure 5.8). After the 90 minutes there was decline in the absorbance, which means enzyme denatured after particular time

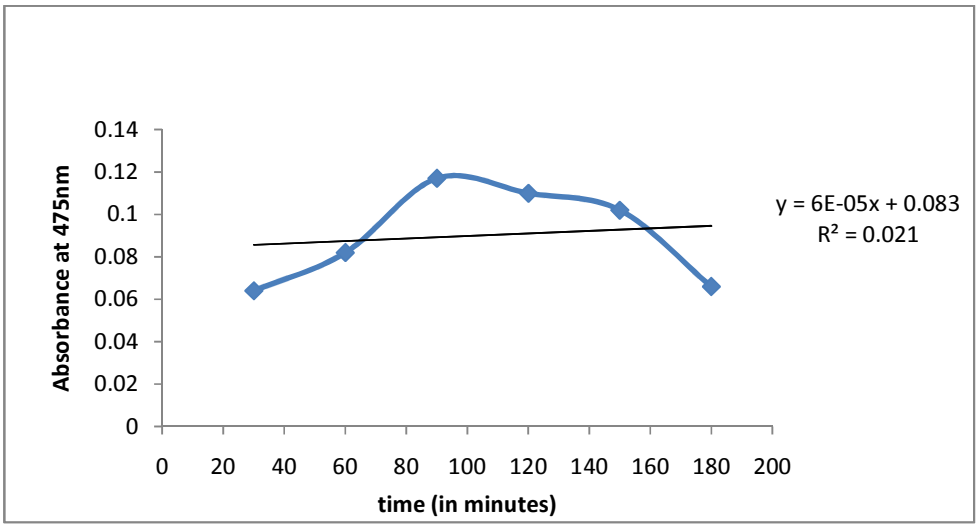

Figure 5.8: optimization of time for tyrosinase activity

5.6 Quantitative method of tyrosinase inhibitor

Out of the 5 different fractions of #20CMBANEY, only DCM fraction (Figure 6.0) exhibited the maximum tyrosinase inhibition activity of 84%. Rest all fractions exhibit the less activity as compare to the DCM.

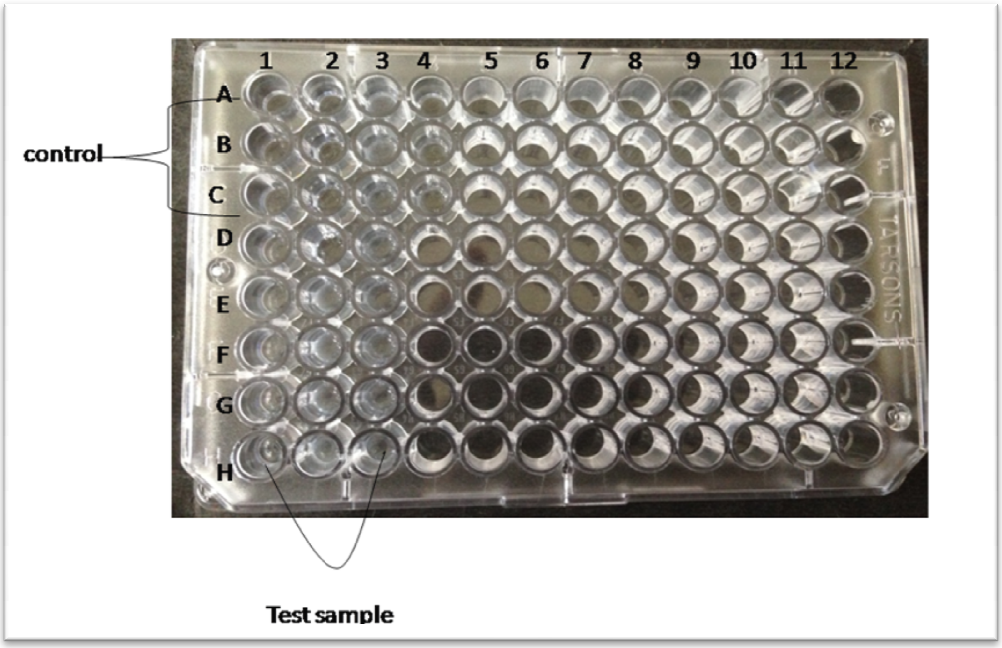

Figure 5.9: In vitro quantitative assay of tyrosinase inhibition of crude fractions of #20CMBANEY. Well A1-C1: control (DMSO instead of inhibitor); fractions of #20CMBANEY :(D1-F1: Hexane fraction) , (G1-A2: DCM fraction),(B2-D2: Chl fraction), (E2-G2 : Ethyl acetate fraction), (H2-B3: Methanol fraction), rest wells were empty.

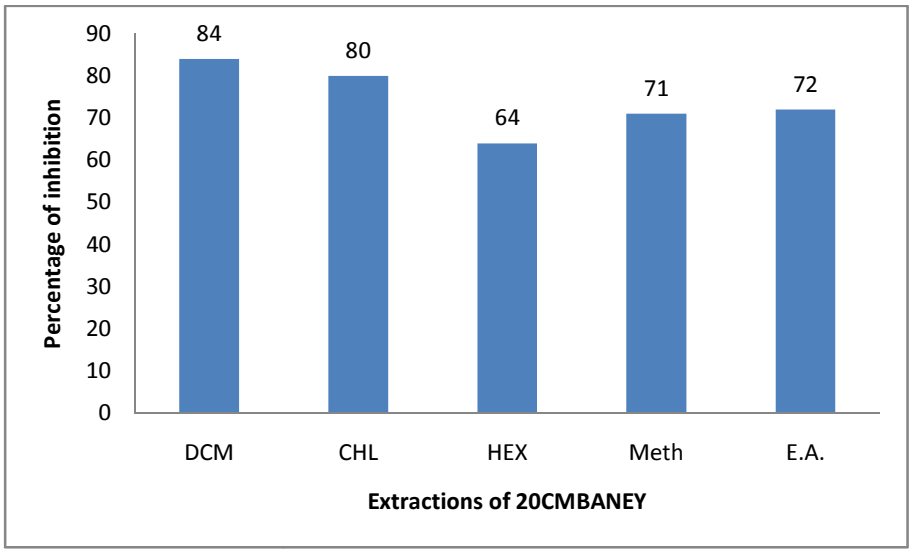

Figure 6.0: Fractions of the #20CMBANEY exhibiting tyrosinase inhibition.

5.7 Identification of tyrosinase inhibitor producing endophyte

Colony of #20CMBANEY is rapidly growing over PDA and MEA, matures in 5 to 6 days. Colour of colony was initially white and then become dark grey with black/brown which eventually turns into black from both front and reverse sides of the culture plate. Colony on Pine leaf agar and SNA was initially brown in colour and later becomes black. Its appearance was downy to wooly.

Among microscopic characteristics, conidia were shiny , black , single celled , egg shaped , spherical. Hypha were septate and hyaline. Conidiosphores were hyaline, short and inflated.

Hence, on the basis of morphological and microscopic characteristics, the potential endophytic fungi #20CMBANEY was tentatively identified as *Nigrospora sp.*

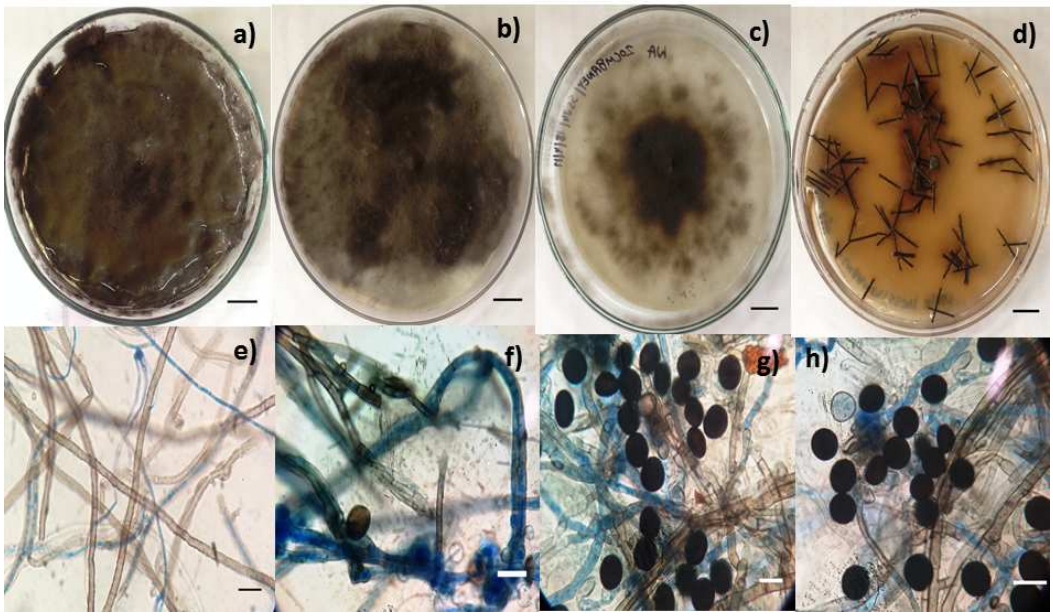

Figure 6.1: Colony morphology and microscopy characteristics of #20CMBANEY on different media a) MEA, b) PDA, c) Water agar, d) Pine agar (Bar : 10mm); e) septate hyphea f)-h) conidial arrangement on P.A. and W.A (Bar : 10µm).

5.7.2 Molecular identification

5.7.2.1 Genomic DNA isolation and PCR amplification

The genomic DNA isolation of potent tyrosinase inhibitor producing endophytic fungi was carried out (Figure 6.3). The concentration of DNA was estimated to be 40µg/ml at 260 nm. The size of PCR amplicon was estimated to be 500-600bp. The size easily compared to the ITS region, which was amplified to characterize the fungi at molecular level.

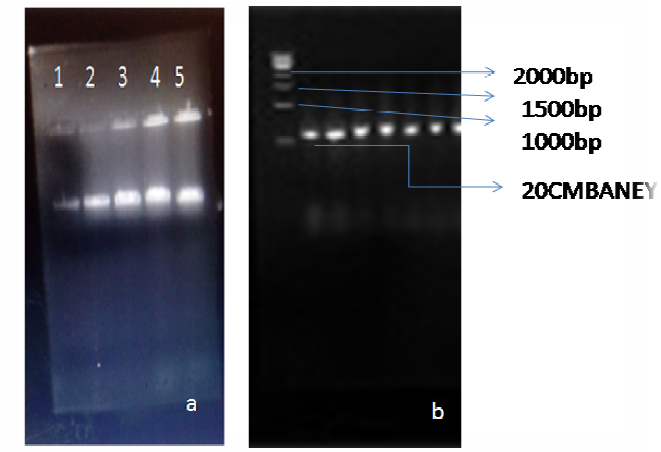

Figure 6.2: Molecular characteristics (a) genomic isolation of #20CMBANEY: Lane 1-5 contain DNA of #20CMBANEY (b) PCR product, L- DNA ladder, Lane 1- 20CMBANEY

Further, the sequencing of amplicon at Eurofins Genomics India Pvt. Ltd. , Banglore, is underway to ascertain its appropriate phylogenetic placement in *Nigrospora* genus.

5.8 In vitro anti microbial assay

Maximum inhibition was recorded by methanol fraction against E.coli whereas only ethyl acetate (E.A.) fraction exhibited inhibition of *Bacillus subtilis* in agar well diffusion assay (Figure 6.3 a),b)).

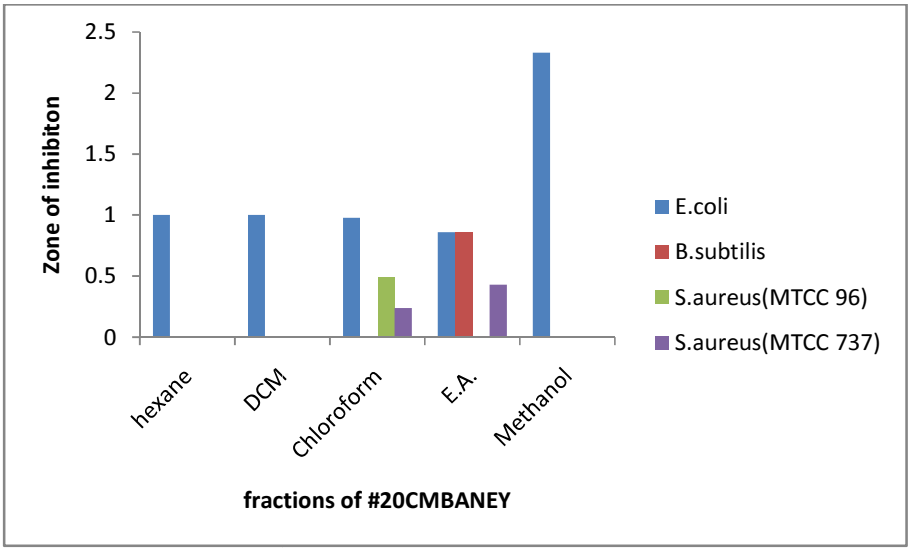

Figure 6.3: a). Anti microbial activity exhibited by different fractions of #20CMBANEY

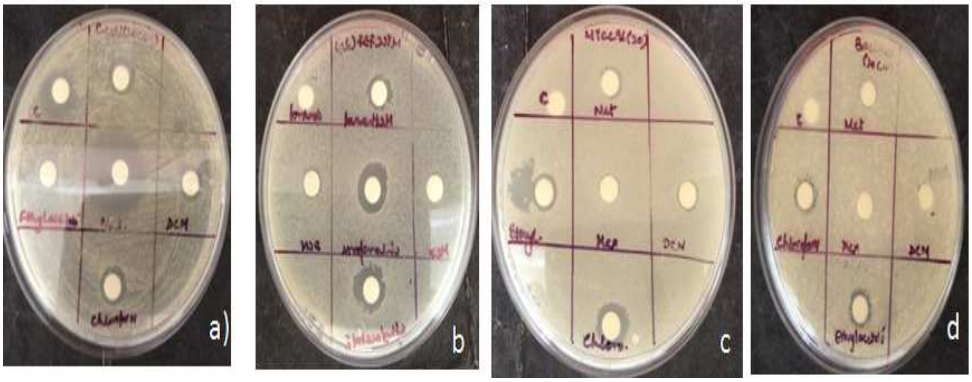

Figure 6.3: b). Agar well diffusion assay of fractions of #20CMBANEY a) *E.coli*, b) S.aureus (MTCC 737), c*) S. aureus(* MTCC 96), d) *Bacillus subtilis*

5.9 In vitro anti oxidant assay

Solvent fractions of #20CMBANEY were evaluated for their capacity of antioxidant activity using the DPPH free radical scavenging system. After the incubation, results depicted that scavenging activity of E.A. fraction exhibited maximum anti oxidant activity of 68% followed by Hexane fraction with 63%, Chloroform with 57% , Methanol with 53% and DCM with 51.2 % whereas in Quercetin which was used as standard, the scavenging activity was 82.58 ± 0.61% (50µg/ml).

Table 9: Anti oxidant activity of fractions of #20CMBANEY

S.no.	solvent fractions	%freeradical scavenging*(%)	equivalent quercetin activity (µg quercetin/mg extract)
1.	Chloroform	56.9 ± 1.1	29.4
2.	DCM	51.2 ± 1.39	25.3
3.	EA	68 ± 0.9	37.2
4.	Methanol	53.1 ± 1	26.7
5.	Hexane	63.2 ± 1.2	33.9

*** indicates data represented as Mean±S.D.**

5.10 Thin layer Chromatography of tyrosinase inhibitor producer

The DCM fraction of #20CMBANEY which exhibited maximum tyrosinase inhibition was subjected to TLC using different combinations of solvents to achieve good separation. The optimized solvent system at which best was achieved was Hexane: toluene (3:2). The DCM fraction of #20CMBANEY resolved into six bands.

Table 10: Separation of #20CMBANEY by using different solvent systems

s.no.	solvent system	ratio used	result
1	Hexane : Ethylacetate	2 : 1	No separation
2	Hexane : Ethylacetate	1 : 2	No separation
3.	Hexane : Chloroform	2 : 2	2 bands
4	Hexane : Chloroform	2 : 1	3 bands
5.	Toulene : Chloroform	1 : 1	No separation
6.	Toulene : DCM	1 : 2	3 bands
7.	DCM : Chloroform	2 : 1	No separation
8.	Toluene : DCM	3 : 1	4 bands
9.	Hexane : Toluene	3 : 2	6 bands

Table 11: Retention factor of DCM extraction of #20CMBANEY

s.no.	distance covered by solute(cm)	distance covered by solvent(cm)	retention factor
1.	2.5	14	0.18
2.	5	14	0.35
3.	6	14	0.42
4.	8	14	0.57
5.	11	14	0.8
6.	13	14	0.9

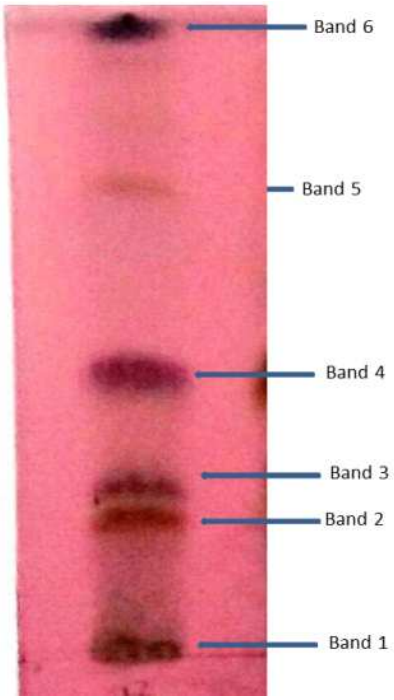

Figure 6.5: TLC analysis of #20CMBANEY

Chapter – 6

DISCUSSION

Discussion

Fairness is sign of beauty in some countries mainly among the youngsters. To get fairer skin, most of the people used the skin lightening agents. Even, these skin lightening agents has clinical purpose, they help in treatment of pigment disorders such as hyper-pigmentation. Hence, reduction in the melanin concentration is important and it can be achieved by blocking the melanin synthesis pathway (Ferrer et al.,1995).Till date, Kojic acid, hydroquinone and retinoids have been used for cosmeceutical purpose (Vasantha et al., 2014); however they have severe side effects which demand for new tyrosinase inhibitors and bio catalyst (enzyme) which will reduce the melanin level in the body and safer to use. In the present study, endophytic fungi has been exploited for tyrosinase inhibitors and laccase from different medicinal plants.

Endophytic fungi from last two decades have attend remarkable recognition in field of drug discovery and development as producers as well as sustainable source of unique and diverse bio active compounds which can be directly or indirectly can be used as potent drug for combating major global diseases (Strobel et al., 2003 ; Wang et al., 2015).Fungal endophytes produce a variety of compounds of different classes such as Polyphenols, alkaloids , terpenes etc. Exhibiting potent inhibitory action against various metabolic enzymes such as lipase, tyrosinase , α-amylase (Gupta et al., 2014,). There is possibility endophytic fungi might produce tyrosinase inhibitors and laccase as melanin is present in plants and it is in symbiotic relationship with their host.

In the present study preliminary screening of 91 isolates of endophytic fungi was performed by agar well diffusion method. The plate assay with slight modifications was used to screen the potent cultures for tyrosinase inhibition and laccase producer (Grund et al., 1986 , Kumar et al., 2011). Based on the preliminary screening, only those filtrates of fungi was selected whose percentage of inhibition was 50% or above. Among the 91 cultures, only #20CMBANEY exhibited 66% of tyrosinase inhibition activity whereas #20(b) VVLPM gave positive result for laccase activity. Hence on the basis of preliminary screening #20CMBANEY was found to be potent culture for Tyrosinase Inhibiting activity whereas #20(b) VVLPM was found to be Laccase Producer.

The quantitative estimation viz microtiter plate assay was further used for the identification of fungal endophytes possessing potent Tyrosinase inhibitory potential. Hence, optimization of

Tyrosinase activity using microtiter plate based spectrophotometric assay was carried out which comprised of optimal substrate concentration, enzyme concentration, pH and incubation time. in previous study Vasantha et al., 2014, used methanol fraction of fungal isolate which exhibited 84% of inhibitory activity against Tyrosinase where as in our study DCM fraction of #20CMBANEY exhibited the 84% of inhibitory activity against Tyrosinase.

Thus, the endophytic fungi exhibiting potential Tyrosinase inhibitory activity was #20CMBANEY. The organism was identified using morphological as well as molecular tools. Morphologically, the endophyte belongs to genus Nigrospora. The morphology was carried out by following the book by Barnett et al 1998 and the sequencing is under way.

Fungal endophytes have also been recognized as storehouse of novel secondary metabolites some of which have antibacterial, antifungal and anti-insecticidal activity (Zhao et al., 2012; Larran et al., 2007). The isolate # 20CMBANEY which is tentivtely identified as Nigrospora sp. possess best invitro-antimicrobial activity against E.coli, S.aureus (MTCC 96, MTCC 737), Bacillus subtilis.In previous studies Hady et al.,2014. carried out the anti microbial activity of *Amphimedonviridis against Staphylococcus aureus , Candida albicans ,Pseudomonas aeruginosa , Aspergillus niger by* agar disc method. In our study methanol fraction of #20CMBANEY exhibited the maximum anti microbial activity against *E.coli* and ethyl acetate fraction against *B.subtilis*.

The antioxidants capability of endophytic fungi isolated from different medicinal plants clearly showing that proper exploration of endophytic fungi from different medicinal plant may yields excellent secondary metabolite for health benefits. It is already proved in many studies that polyphenols are directly responsible for antioxidants activity (Khennouf et., al 2010; Baghiani et .,al 2010). In present study the in-vitro antioxidant assay of Hady et al., 2014 the fungal endophyte Hexane fraction of #20CMBANEY exhibited maximium 63.2% while minimum 51.2% of free radical scavenging activity in DCM fraction as compared to standard Quercitin83.9 ± 0.006% (50 µg/ml) . Isolated two endophytic fungi from sponges (Aspergillus sydowii and one is Unidentified) in which Aspergillus sydowii exhibited maximum free radical scavenging activity.

TLC analysis of DCM fraction of #20CMBANEY was performed according to Zhou et al.,2016. They have optimized the Methanol extract of *Rhodiola sacra* in mobile of chloroform: methanol: acetone (10 : 1: 1) and two bands of tyrosinase inhibitor compounds were observed

whereas in our study DCM fraction of #20CMBANEY separated into 6 bands in mobile phase of hexane : toluene in 3:2 ratio.

Thus, in present study we stabilized that endophytic fungus could be a novel source for Tyrosinase inhibitors and Laccases producers.

Chapter – 7

CONCLUSION

Conclusion

The present study concludes that #20CMBANEY, isolated from *Cinnamoum merbalicum* is potential producer of tyrosinase inhibition while #20(b) VVLPM isolated from *Vitis vinifera* is potential producer of laccase.

Hexane fraction exhibits the best antioxidant activity where as methanol and ethyl acetate fractions of #20CMBANEY exhibits the antimicrobial activity. The potential endophytic fungus, #20CMBANEY was tentatively identified as Nigrospora through morphological and microscopic characteristics. The species level identification of #20CMBANEY will be deduced after getting sequencing data ITS region. Further, purification of #20CMBANEY is needed to be carried out.

The potential 20(b)VVLPM producer's tentative identification is need to be done and enzyme purification will carried out.

Chapter – 8

BIBLIOGRAPHY

Bibliography

1. Agrawal, S., & Mmazhar, M. (2015). Adulteration of mercury in skin whitening creams-A nephrotoxic agent. *Current medicine research and practice, 5*, 172-175.
2. Arai, N., Shiomi, K ., Takamatsu, S., Komiyama, K ., Shinose, M., Takahashi, Y., Tanaka, Y., & Iwai, Y. (1997). Amphistin, A new melanogensis inhibitor, produced by Actinomycete. *The journal of Antibiotics*, *50(10),* 809.
3. Baghiani, A., Boumerfeg, S., Belkhiri, F., Khennouf, S., Charef, N., Harzallah, D., Arrar, L., & Abdel-Wahhab, M. A. (2010). Antioxidant and radical scavenging properties of *Carthamus caeruleus* L. extracts grow wild in Algeria flora. *Comunicata Scientiae*, *1*(2), 128–136.
4. Baldrian, P. (2005). Fungal laccases-occurence and properties. *FEMS microbial rev*, *30,* 215-242.
5. Barnett,H.L., Hunter, B.B. (1998). Ilustrated Genera of Imperfect *f*ungi *,4.*
6. Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Mdden, M., Brown, K.M., Halkier, T., Mondrof, K., & Xu, F. (1997). Characterization of the gene encoding an extracellular laccase of Myceliophthora thermophila and analysis of the recombinant enzyme expressed in Aspergillus oryzae. *Appl environ Microbiol, 63(8),* 3151-7.
7. Camarero, S., Ibarra, D., Martinez, A., Romero, J., Gutierrez, A., & Rio, J. (2007). Paper pulp delignification using laccase and natural mediators. *Enzyme and Microbiol technology ,40*, 1264-1271.
8. Chandra, S. (2012). Endophytic fungi: novel sources of anti cancer lead molecules. *Appl Microbiol Biotechnol*, *95(47).*
9. Chefetz, B., Chen, Y., & Hadar, Y. (1998). Purification and characterization of laccase from chaetomium thermophilium and its role in humification. *Applied and environmental microbiology, 64(9).*
10. Corinaldesi, C., Barone, G., Marcellini, F., Anno, A., & Danovara, R. (2017). Marine microbial derived molecules and their potential use in cosmeceutical and cosmetic products. *Marine drugs*, *15 (4)*, 118.

11. Dessinioti, C., Stratigos, A., Rigopoulos, D., & Katsambas, A. (2009). A review of genetic disorders of hypo pigmentation: lessons learn from biology of melanocytes. *Experimental dermatology*, *18,* 741-749.
12. Dorni, A., Amalraj, A., Gopi, S., Varma, K., & Anjana, S. (2016). Novel Cosmeceutical from plants-An industry guided review. *Journal of Applied research on Medicinal and Aromatic Plants.*
13. Dwivedi, U., Singh, P., Pandey, V., & Kumar, A. (2011). Structure-function relationship among bacterial, fungal and plant laccases. *Journal of molecular catalysis B: Enzymatic*, *68,* 117-128.
14. Faeth, S. (2002). Are endophytic fungi defensive plant mutualists. *OIKOS*, *98*, 25-36.
15. Fernandes, M., & Kerkar, S. (2017). Micro organisms as a source of tyrosinase inhibitors: a review. *Ann Mmicrobiol*, *67,* 343-358.
16. Ferrer, A., Lopez, J., Canovas, F.,& Carmona, F. (1995). Tyrosinase: a comprehensive review of its mechanism. *Journal of Biochimica et Biophysica acta, 1247*, 1-11.
17. Fisk, W., Agbai, O., Lev-tov, H., Sivamani, R., & Mather, A. (2014). The use of botanically derived agents for hyperpigmentation: A systematic review. *Journal of American Academy DERMATOL, 70(2),* 352-65.
18. Fujji, Y., Asahara, M., Ichinoe, M., & Nakajima, H. (2002). Fungal melanin inhibitor and related compounds from Pencillium decumbens. *Phytochemistry*, *60*, 703-708.
19. Gimeno, P., Bousquet, C., Lassu, N., Maggio, A., Civade, C., Brenier, C., & Lempereur, L. (2015). High performance liquid chromatography method for the determination of hydrogen peroxide present or released in teeth bleaching kits and hair cosmetic product. *Journal of Pharmaceutical and biomedical analysis*, *107,* 386-39.
20. Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M., & Bally, R. (1993). Polyphenol oxidase in Azospirillum lipoferum isolated from rice rhizosphere: Evidence for laccase activity in non-motile strains of Azospirillum lipoferum. *FEMS Microbiology Letters* , 108 , 205-210.
21. Grund, D., & Harrison, K. (1986). The taxonomic potential of laccase and tyrosinase spot test. *Mycologia, 78(2),* 169-184.
22. Gupta, M., Saxena, S., & Goyal, D. (2015). Potential pancreatic lipase inhibitory activity of an endophytic *Penicillium* species. *Journal of enzyme inhibition and medicinal chemistry, 30(1),* 15-21.

23. Hady,F., Mohamed,S., Aziz, A., Shaker, K., Shahid, Z. (2014). Tyrosinase, Acetylcholinesterase Inhibitory Potential, Antioxidant and Antimicrobial Activities of Sponge Derived Fungi with correlation to their GC/MS Analysis*. International journal of pharma science review and research , 26 (2),* 338-345.
24. Hearing, V. (2011). Determination of melanin synthetic pathways. *Cutaneous biology.*
25. Herrera, A . (2015). The biological pigments in plant physiology*. Agriculture sciences, 6*, 1262-1271.
26. HS, K., HR, K., DS, B., BW, S., TJ, N., & JS, C. (2004). Tyrosinase inhibitors isolated from the edible brown alga Ecklonia stolonifera. *Arch pharma res , 27(12)* ,1226-32.
27. http://www.strategyr.com/MarketResearch/Skin_Lighteners_Market_Trends.asp
28. Iozumi, K., Hoganson, G., Pennella, R., Everett, M., & Fuller, B. (1993). Role of Tyrosinase as the determinant of pigmentation in cultured Human Melanocytes. *The society of dermatology*, *100(6).*
29. JH, Y., JS, Shim., Y, Cho., NI, Baek., CW, Lee., HS, Kim., & JK, Hhwang. (2007). Depigmentation of melanocytes by Isopanduratin A and 4-Hydroxypanduratin A isolated from Kaempferia pandurata ROXB. *Biological and Pharmceutical Bulletin, 30 (11)* , 2141-5.
30. Jia, M., Chen, L., Xin, H., Zheng, C., Rahman, K., Han, T., & Qin, L. (2016). A friendly relationship between Endophytic fungi and medicinal plants: A systematic review, *Frontiers in Microbiology*, *906(7).*
31. Khennouf, S., Iratni, N., Baghiani, A., Harzallah, D., & Arrar, L. (2010). Antioxidant and antibacterial activities of extracts from Artemisia herba alba Asso leaves and some phenolic compounds. *Journal of Medicinal Plant Research*, *4* (13), 1273–1280.
32. Kim, J., Yeon, J., Yang, S., Choi, S., kwon, S., Cho, I., Jeong, M., Kim, Y., & Choi, G. (2017). Tyrosinase inhibitory components from Aloe vera and their antiviral activity. *Journal of enzyme ihhibition and medicinal chemistry, 32(1)* , 78-83.
33. Kim, S., Son, K., Chang, H., kang, S., & Kim, H. (2003). Tyrosinase inhibitory prenylated flavonoids from Sophora flavescens. *Archieves of Pharmaceutical research*, *26(9)* , 1348-1350.
34. Koroleva, O., Stepanova, E., Landesman, E., Vasilchenko, L., Khromonygina, V., Zherdev, A., & Rabinovich, M. (2002). Enzyme Immunoassay of Herbicide Decomposition by Soil and Wood Decay Fungi. *Applied biochemistry and microbiology, 38(4),* 355-360.

35. Kumar, V., Kirupha, S., Periyaraman, P., & Sivanesan, S. (2011). Screening and induction of laccase activity in fungal species and its application in dye decolorization. *African Journal of Microbiology Research, 5(11),* 1261-1267.
36. Kunamneni, A., Ballesteros, A., Plou,F., & Alcalde, M. (2007). Fungal laccase – a versatile enzyme for biotechnological applications. *Communicating current research and Educational topics and trends in Applied microbiology*, 233-245.
37. Larran, S., Perello, A., Simon, M. R., Moreno, V. (2007). The endophytic fungi from wheat (*Triticum aestivum* L.). *World Journal of Microbiology and Biotechnology*, *23*, 565–572.
38. Li, X., JH, J., KT, l., JR, R., HD, C., JS, K., & BW, S. (2003). Gmma-pyrone derivatives , Kojic acid methyl ethers from a marine-derived fungus Alternaria sp., *Archives of pharmacal Research, 26(7),* 532-4.
39. Lim, J., Ishiguro, K., & Kubo, I. (1999). Tyrosinase inhibitory p-coumaric acid from Ginseng leaves. *Phytotherapy Research.*
40. Lin, J., & Fisher, D. (2007). Melanocyte biology and skin pigmentation. *Nature,* 445.
41. Lu, R., Liu, X., Gao, S., Zhang, W., Peng, F., Hu, F., Huang, B., Chen,L., Bao, G., Li, C., & Li, Z. (2014). New tyrosinase inhibitors from Paecilomyces gunnii. *Journal of Agricultural and food chemistry*, *62*, 11917-11923.
42. Masamoto, Y., Ando, H., Murata, Y., Shimoishi, Y., Tada, M., & Takahata, K. (2002). Mushroom tyrosinase inhibitory activity of Esculetin isolated from seeds of Euphorbia lathyris L. *Bioscience , biotechnology and biochemistry* , *67(3),* 631-634.
43. Momtaz, S., Lall, N., & Basson, A. (2008). Inhibitory Activities of mushroom tyrosine and DOPA oxidation by plant extracts. *South African Journal of Botany*, *74*,577-582.
44. Morimura, K., Yamazaki, C., Hattori, Y., Makabe, H., Kamo, T., & Hirota, M. (2007). A tyrosinase inhibitor, Daedalin A, from mycelia culture of Daedalea dickinsii. *Bioscience, Biotechnology,Biochemistry, 71 (11)* , 2837-2840.
45. Muhammad, A., & Sirat, HM. (2013). Potent microbial and tyrosinase inhibitors from stem bark of Bauhinia rufescens (Fabaceae). *Natural product communications*, *8(10),* 1435-7.
46. Murase, D., Hachiya, A., Fullenkamp, R., Beck, A., Moriwaki, S., Hase, T., Takema, Y., & Manga, P. (2016). Variation in Hsp70-1A Expression Contributes to Skin Color Diversity. *Journal of Investigative dermatology, 136(8),*1681-1691.

47. Nakashima, T., Anzai, K., Kuwahar, N., & Ando, k. (2009). Physicochemical characters of tyrosinase inhibitor produced by streptomyces roseolilacinus NBRC 12815, *Biological and Pharmaceutical Bulletin* , *32(5*), 832-836.
48. Nerya, O., Vaya, J., Musa, R., Izrael, S., Ben, R., & Tamir, S. (2003). Glabrene and isoliquiritigenin as tyrosinase inhibitor from licorice roots. *Journal of Agriculture and Food Chemistry* ,*51(5),* 1201-7.
49. Nguyen, H., & Kim, S. (2012). Antioxidative , anticholinesterase and antityrosinase activities of red alga *Grateloupia lancifolia* extracts. *African journal of biotechnology*, *11(39)* , 9457-9467.
50. Pandya, A., & Guevara, I., (2000). Disorders of Hyperpigmentation . *Dermatologic Clinics*, *18(1)*.
51. Pietraszek, E., Kwiecien, I., Goldyn, A., & Pietraszek, J. (2016). HPLC-DAD analysis of arbutin produced from hydroquinone in a biotransformation process in Organum majorana L.shoot culture. *Phytochemistry letters.*
52. Ranocha, P., Chabannes, M., Chamayou, S., Danoun,. S., Jauneau, A., Boudet, A., & Goffner, D. (2002). Laccase down- regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Genetics, genomics and molecular evolution*.
53. Rodrigues, K.F., Manfred, H., & Christa, W. (2000). Antimicrobial activity of secondary metabolites produced by endophytic fungi from Spondias mombin. *Journal of basic microbiology*, *40(4),* 261-267.
54. RW, D., Chen, J., Sun, J., Ma, H., Dubert, J., Baria, J.L., Seeram, NP., Wang, H., & Rowley, D.C. (2016). N-Acyl Dehydrotyrosines, Tyrosinase inhibitors from Marine bacterium Thalassotalea sp. PP2-459. *Journal of Natural Products*, *79(2)* , 447-50.
55. Sasaki, L., & Yoshizaki, F. (2002). Nobiletin as tyrosinase inhibitor from the peel of citrus fruit, *Biological and Pharma Bulletin ,25(6)* , 806-8.
56. SH, C., SC, K., D, K., & YJ, J. (2011). Screening of marine algae for potential tyrosinase inhibitor: those inhibitors reduced tyrosinase activity and melanin synthesis in zebrafish, *Journal of Dermatology*, *38(4)*, 354-63.
57. Sharma, V., Jinsuk, C. , Sharma, N., Choi, M., & Seo, S. (2004). In vitro anti-tyrosinase activity of 5-(hydroxymethyl)-2-furfural isolated from Dictyophora indusiata , *Phytotherapy research* .

58. Shraddha, Shekher, R., Sehgal, S., Kamthania, M., & Kumar, A. (2011). Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications, *Enzyme research.*
59. Slominski, A., Tobin, D., Shibahara, S., & Wortsman, J. (2004). Melanin pigmentation in mammalian skin and its hormonal regulation, *The American Physiological Society , 84,* 1155-1228.
60. Strobel, G., & Daisy, B. (2003). Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiology and Molecular Biology Reviews*, *67*(4), 491–502.
61. Takahashi, S., Iwai, H., Kosaka, K., Miyazaki, T., Osanai, Y., Arao, N. , Tanaka, K. , Nagai, K., & Nakagawa, A. (2007). Byelyankacin: A novel melanogenesis inhibitor produced by Enterobacter sp. B20. *The journal of antibiotics*, *60(11),* 717-720.
62. Takamatsu, S., Rho, M., Hayashi, M., Komiyama, K., Tanaka, H., & Omura, S. (1993). New inhibitors of melanogenesis , OH-3984 k1 and K2, *The journal of Antibiotics*, *46(10),* 1526-9.
63. Thody, A., Higgins, E., Wakamatsu, K., Ito, S., Burchill, S , Marks, J. (1991). Pheomelanins as well as Eumelanin is present in Human Epidermis, *The society for Investigative Dermatology*.
64. Tsuchiya, T., Yamada, K., Minoura, K., Miyamoto, K., Usami, Y., Kobayashi, T., Sato, N., Imada, C., & Tsujibo, H. (2008). Purification and determination of the chemical structure of the Tyrosinase inhibitor produced by Trichoderma viridae H1-7 from a Marine Environment, *Biological and Pharmacal Bulletin*., *31(8),* 1618-1620.
65. Valipour, E., & Arikan, B. (2015). Optimization of Tyrosinase enzyme production from Native Bacillus sp. MV29 isolate, *Journal of Applied Biological Sciences*, *9(2),* 77-82.
66. Vasantha, K., Murugesh, C., & Sattur, A. (2014). A Tyrosinase inhibitor from Aspergillus niger, *Journal of food science technology*, *51(10),* 2877-2880.
67. Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A., & Narasimha, G. (2014). Fungal laccases and their applications in bio remediation, *Enzyme research*.
68. Wang, Zhang.X,Liu.L, Xiang.M, Wang.W, Sun,Che.Y, Guo.L, Liu.G, Guo.L, Wang.C, Yin.W, Stadler.M,Zhang.X. (2015). Genomic and transcriptomic analysis of the endophytic fungus Pestalotiopsis fici reveals its lifestyle and high potential for synthesis of natural products, *BMC Genomics*, 16:28.

69. White, T.J. , Bruns, T. , Lee, SJWT, Taylor, & J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics, *PCR protocols: a guide to methods and applications*, *18*, 315-322.
70. Yamaguchi, Y., Brenner, M., & Hearing.J.(2007). The regulation of skin pigmentation, *Journal of Biological Chem*istry. *282,* 27557-27561.
71. Yamauchi, K., Mitsunaga, T., & Batubara, I. (2011). Isolation , identification and Tyrosinase inhibitory activities of extractives from Allamanda cathartica, *natural resource,* 2, 167-172.
72. YM, K., J , Yun. , CK,L., Lee, H. , KR, M. , Kim. (2002). Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect on tyrosine and mechanism of action , *Journal of Biology Chemistry*, *277(18)*, 16340-4.
73. Yoshida, H. (1883). Chemistry of Lacquer part 1, *Journal of chem. Sol*., *43,* 472-486.
74. Yoshimura, M., Watanabe, Y., Kasai, K., Yamakoshi, J., & Koga,T. (2005). Inhibitory effect of an ellagic acid rich pomegranate extract on tyrosinase activity and ultraviolet-induced pigmentation, *Bioscience Biotechnology Biochemistry ,69(12),* 2368-73.
75. Zhao, J., Mou, Y., Shan, T., Li, Y., Lu, S., & Zhou, L. (2012). Preparative separation of helvolic acid from the endophytic fungus *Pichia guilliermondii* Ppf9 by high-speed counter-current chromatography. *World Journal of Microbiology and Biotechnology*, *28*, 835-840.
76. Zhou, J., Tang, Q., Wu, T., Cheng, Z. (2016). Improved TLC bioautographic assay for Qualitative and quantitative estimation of tyrosinase inhibitors in Natural products ,*Phytochem anal*. *28*, 115-124.
77. Zhu, W., & Gao, J . (2008). The use of botanical extracts as topical skin-lightening agents for the improvement of skin pigmentation disorders. *Journal of Investigative dermatology symposium proceedings*, *13(1)*, 20-24.

APPENDIX

Media

1. **Potato dextrose agar**

   Potato dextrose agar – 39gm

   Distilled water – 1L

   Final pH (at 26°C)- 5.6±0.2

   Autoclave at 121ºC for 15 min

2. **Malt extract agar**

   Malt extract – 30gm

   Mycological peptone – 5gm

   Agar – 15gm

   Distilled water – 1L

   Final pH - 7.6 ± 0.2 at 37°C

   Autoclave at 121ºC for 15 min

3. **Synthetic nutrient deficient agar**

   Glucose – 0.2gm

   Sucrose – 0.2gm

   Pot. dihydrogen phosphate – 1gm

   Potassium nitrate – 1gm

   Magnesium sulphate – 0.25gm

   Potassium chloride – 0.5gm

   Agar – 15gm

   Distilled water – 1L

   Final pH – 5.4 ± 0.2 at 26°C

   Autoclave at 121ºC for 15 min

4. **Water agar**

   Agar – 15gm

   Distilled water – 1L

   Autoclave at 121ºC for 15 min

5. **Pine leaf agar**

   Pine leaves

   Agar – 15gm

   Distilled water – 1L

   Autoclave at 121ºC for 15 min

Buffers

1. **50XTAE**

   Tris base – 242g

   Glacial acetic acid – 57.1ml

   0.5M EDTA – 10ml

   Distilled water – 1L

2. **1X TE Tris-HCl (pH 8.0)**

   10 mM EDTA - 0.1 mM

   Distilled water - 100ml

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