SCREENING ENDOPHYTIC FUNGI FOR PRODUCTION OF PHENYLALANINE AMMONIA LYASE, FOR USE AS A THERAPEUTIC INTERVENTION TO TREAT PHENYLKETONURICS

A THESIS

In partial fulfillment for the award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

Submitted by

Anshu Singh

(Roll no. 602004003)

Under the supervision of

Dr. Sanjai Saxena

Professor

Department of Biotechnology

Thapar Institute of Engineering & Technology

Deemed to be university

Patiala-147004

June, 2022

CERTIFICATE

This is to certify that the thesis entitled **"Screening endophytic fungi for production of phenylalanine ammonia lyase, for use as a therapeutic intervention to treat phenylketonurics"** being submitted by Ms. Anshu Singh (Roll No. 602004003) in the partial fulfillment of the requirements for the award of degree of Masters of Technology in Biotechnology, Thapar University, Patiala is a bonafide work carried under supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

Dr. Sanjai Saxena
Professor / Supervisor
Department of Biotechnology
Thapar Institute of Engineering and Technology
Patiala, Punjab

DECLARATION

I, hereby declare that the work being presented in the thesis entitled **"Screening endophytic fungi for production of phenylalanine ammonia lyase, for use as a therapeutic intervention to treat phenylketonurics"** in the partial fulfillment of requirements for the award of degree of Masters in Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of August 2021 to June 2022, under the conception and supervision of Dr. Sanjai Saxena, Professor, Department of Biotechnology (DBT), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Anshu Singh

Anshu Singh

Roll No. 602004003
Place: Patiala
Date: 12/09/22

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

Dr. Sanjai Saxena
Professor / Supervisor
Department of Biotechnology
Thapar Institute of Engineering and Technology
Patiala, Punjab

ACKNOWLEDGEMENT

Firstly, I would like to thank the god for determination and patience, for letting me through all the difficulties.

I would like to acknowledge and give my sincere regards to my supervisor **Dr. Sanjai Saxena, Professor**, Department of Biotechnology, Thapar University, who made this work possible. His guidance and advice carried me through all the stages of my project.

I thank **Professor M.S. Reddy**, Head of Department for providing the necessary facilities to carry out my work efficiently.

I would like to thank all my research scholars for their continuous guidance and encouragement during my project work.

I would like to express my special gratitude to Ms. Gurleen Kaur for her constant guidance and supervision.

I would like to thank my classmates for making my project journey memorable and enjoyable.

Finally, I would like to thank my parents and brother for continuous support and prayers.

Anshu Singh

Anshu Singh

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PAL	Phenylalanine ammonia lyase
PKU	Phenylketonuria
PAH	Phenylalanine hydroxylase
AWD	Agar Well Diffusion
PDA	Potato Dextrose agar
FC	Folin-Ciocalteu's
FDA	Food and Drug Administration

Executive summary

Phenylketonuria is a rare inborn autosomal recessive disorder in which an accumulation of an amino acid, phenylalanine and receded levels of a hepato-cellular enzyme phenylalanine hydroxylase (PAH) which is accountable for regulation of phenylalanine levels in the plasma. Generally seen in neonates, the outcome of enhanced level of phenylalanine can lead to the symptoms such as brain dysfunction, eczema, hyperactivity, abnormal electroencephalogram, microcephaly and autism. The sustainable interventions such as enzyme phenylalanine ammonia lyase (PAL) that can cure phenylketonuria are in high demand. PAL catalyzes the deamination of L-phenylalanine into trans-cinnamic acid and trace amount of ammonia. PAL is widely apportioned in plants, cyanobacteria, algae and fungi. Various biological entities have been studied for the production of PAL. However, the research is limited for the production of PAL using endophytic fungi in the context of therapeutic intervention. Endophytic fungi inhabit within the living tissues of host plant in a symbiotic association without causing any overt disease symptoms to the host plant. The association between endophytic fungi and host plant enables the endophytic fungi to produce of bioactive compounds and secondary metabolites having therapeutic applications. In this study, we screened 43 endophytic fungi out of which isolate #2CCBD and #61MKLTh02 exhibited highest PAL activity using agar well diffusion (AWD) assay. Further on direct nesslerization assay isolate #2CCBD exhibited highest ammonia production closely followed by #61MKLTh02. On protein estimation isolate #2CCBD exhibited 1.93±0.02 and 1.87±0.01 mg/mL protein content using Bradford and Folin-Lowry assay whereas isolate #61MKLTh02 exhibited 1.18±0.06 and 1.17±0.05 mg/mL protein content respectively. The UV-Vis spectroscopy-based enzyme activity of crude protein of isolate #2CCBD was 0.85 U/min/mL whereas #61MKLTh02 exhibited activity of 0.42 U/min/mL of crude protein. The isolate #2CCBD also exhibited better results in DPPH activity with IC_{50}485.213±1.204 µg/mL, total phenolic content of 594.73 ± 1.91 µg/mL and total flavonoid content 877.07 ± 20.94 µg/mL. Thus, the isolate #2CCBD was tentatively identified as a *Penicillium* sp. using morpho-taxonomic tools.

Keywords: Phenylketonuria, phenylalanine ammonia lyase, L-phenylalanine, trans-cinnamic acid, endophytic fungi

CHAPTER 1

INTRODUCTION

1. Introduction

Phenylketonuria, also known as phenylalanine hydroxylase deficiency and Folling disease, is a rare inborn autosomal recessive disorder in which an out-of-action accumulation of an amino acid, phenylalanine occurs in the blood (Lichter-Konecki et al., 2019; Spronsen et al., 2021). However, the amino acid should be taken through diet. Phenylalanine accumulation occurs when the activity of the enzyme phenylalanine hydroxylase (PAH) gets reduced (Ashe et al., 2019). This ailing accumulation of phenylalanine causes brain dysfunction, eczema, hyperactivity, abnormal electroencephalogram, and microcephaly (Vliet et al., 2018; Quinn et al., 2022).In 1949, Dr. Woolf and Dr. Vulliamy evinced that phenylalanine or one of its metabolites responsible for brain intoxication and caused mental retardation in the patients and also suggested that phenylalanine concentration reduction in the blood can cure mental retardation along with three ideas to cure this condition out of three the idea includes dietary restriction chosen to be accomplishable (Woolf et al., 2020).

The critical early age to show disease symptoms is between the third and sixth months. Most cases of PKU occur in Turkey with 1 in 4000 reasons behind highest occurrence is union between close people (consanguinity). The least cases of PKU occur in Finland with 1 in every 100,000 new born. In Caucasians PKU occur 1 in every 10,000 new births. Approximately 25 new born examined with PKU every year in Australia (Hafidet al., 2015; Hillert et al., 2020; Shoraka et al., 2020). However, the occurrence of PKU differs in worldwide population. According to World health organization (WHO), 140 million children were born every year in developing countries and 5 million dies in the first month. 4% of the population in India are mentally retarded and metabolic disorder occurs in 5-15% of sick newborns. According to previous, result indicated that the ratio of PKU disorder in India is1:18300 (Devi et al., 2004). In 1952 an enzyme system was identified which converts phenylalanine to tyrosine, and in the same year, a restricted diet of phenylalanine for the first time was used. In the 1960s, mass screening for PKU in newborn babies was done using microbial inhibition assay (Williams et al., 2008). In the 1980s, a gene responsible for mental retardation named phenylalanine hydroxylase was mapped and cloned, and this resulted in the identification of the first mutation (Scriver et al., 2007).

Phenylalanine ammonia lyase was first discovered by Koukol and Conn in barley seedlings. Phenylalanine ammonia lyase (PAL) is an important enzyme in Phenylpropanoid metabolism and catalyzes the conversion of phenylalanine to trans-cinnamic acid, the first step

in the biosynthesis of Phenylpropanoid and plant Phenylpropanoid plays crucial biological roles (Guo et al., 2009). Plant enzyme Phenylalanine ammonia-lyase exhibited potential in depleting the phenylalanine in the gut. In 1999 patients suffering from mild PKU were treated by giving BH4 therapy to the patients to lower phenylalanine concentration in blood, and few individuals responded to this therapy (Strisciuglio et al., 2014). Reports on human trials with PEGylated PAL of phase I came around in 2014 after this Palynziq^{TM}, AvPAL double mutant drug was approved by the United States Food and Drug Administration (FDA) (Kawatra et al., 2020). A diet in which phenylalanine is completely avoided in PKU is quite challenging of a task especially in the adulthood and alterations in blood phenylalanine levels with any treatment with uncertain approaches can cause grave outcomes (Hanley et al., 1970).

A propitious approach used to cure PKU which includes enzyme substitution therapy using an enzyme called phenylalanine ammonia lyase to degrade the elevated levels of phenylalanine which causes neurological intoxication and leads to PKU. PAL basically converts phenylalanine into harmless trans cinnamic acid and ammonia (Moffit et al., 2007). Phenylalanine ammonia lyase is extensively apportioned in plants (Bernardsand et al., 1991; Havir et al., 1973; Rosler et al., 1997; Xiang et al., 2005), cyanobacteria, algae and fungi (Fiske and Kane, 1984; Gilbert et al., 1982; Marusich et al., 1981; Wick and Willis, 1982).

Various assorted tissues such as shoots, leaf-sheath, seedlings, cell culture, mycelium, prokaryotic cells and fruits are used for isolation of phenylalanine ammonia lyase (Hyun et al.,2011). PALs obtained from plants are usually not very thermally stable at high temperature which is required for thawing and freezing. The temperature reaches upto 60°C and enzyme obtained from plants become sensitive and loses its activity (Kalghatgi et al., 1975). As for cyanobacterial PALs crystal structures, the unsuccessful binding of PALs of the substrateL-Phe, the product cinnamic acid, and a potent PAL-specific inhibitor (2-aminoindan-2-phosphonic acid) caused due to the active-site cleft seems to be very disclosed to the external solvent and the disordered inner loop leads to the absence of stabilizing interactions (Moffitt et al., 2008). As for Yeast PALs engineered protease-protected variants of yeast PAL, the mutant enzymes have notably lessened specific activity and a repeated dose of the engineered (PEGylated) PAL is required to alter depletion of plasma L-Phe levels (Moffitt et al., 2008). Both un-PEGylated and PEGylated cyanobacterial PALs have some drawbacks in the treatment of PKU (Bell et al., 2017).

However, PAL obtained from fungi can withstand repeated freezing and thawing and shows better thermal stability than plant PALs. For instance**,** PAL obtained from *Rhodotorula* can be kept at -60°C for about six months in a stable state (Fritz et al., 1976). Alternatives for treatmentof PKU are still inadequate even after a new drug was consented lately so for the unfulfilled medical demands of PKU substitute sources and more modified therapies must be explored (Sousa et al., 2020).

A novel strategy involves utilization of endophytic fungi for the production of PAL for therapeutic exploitation and fulfillment of medical needs as endophytic fungi itself have various advantages over other PAL producing biological entities. Endophytic fungi inhabit within the living tissues of host plant and populate within living tissues in a symbiotic association without causing any overt disease symptoms to the host plant. This kind of association between host plant and endophytic fungi referred as holobiont concept (Vandenkoornhuyse et al., 2015). The colonization of tissues of host by endophytic fungi takes place via transmission named as vertical and horizontal transmission (Arnold et al., 2007). The synergy between endophytic fungi and host plant turns out in the generation of bioactive compounds and secondary metabolites (Liarzi et al., 2016; Schulz et al., 2002). These compounds have wide range of applications in industry- primarily in agriculture and pharmaceutical sectors (Zhao et al., 2010;Gouda et al., 2016; Manganyi and Ateba 2020).

Medicinally necessary natural products can be synthesized by some capable endophytes from plants, utilizing such organisms can be anticipated to meet the clinical needs of these substances as conventional and sustainable sources (Singh et al., 2022; Mishra et al., 2022). An endophytic fungi *Penicillium brasilianum* associated with plant *Melia azadirachta* was investigated to check the PAL activity by Fill et al., (2009) and it was found that fungal endophyte possesses PAL activity as it catalyzed L-phenylalanine into trans-cinnamic acid. Considering the above and crucial importance of PAL, endophytic fungi associated with *Cinnamomum camphora* (Camphor tree), *Murraya koenigii* (Curry leaf tree), *Cinnamomum zeylanicum* (Cinnamon), *Tinospora cordifolia* (Guduchi) plants were qualitatively and quantitatively screened for PAL production. Further, *in-vitro* antioxidant potential of the potent isolates was evaluated and the isolates were tentatively identified using morpho-taxonomic tools.

CHAPTER 2

REVIEW OF LITERATURE

2. Review of Literature

2.1 Phenylketonuria

Phenylketonuria is a rare recessive autosomal inherited disorder seen in neonates caused by the elevated levels of an amino acid named phenylalanine and receded levels of a hepato-cellular enzyme phenylalanine hydroxylase (PAH) which is accountable for regulation of phenylalanine levels in the plasma (Surtees et al., 2000; Mitchell et al., 2011; Waisbren et al., 2007). The outcome of upraised level of phenylalanine can lead to the symptoms such as brain dysfunction, eczema, hyperactivity, abnormal electroencephalogram, microcephaly and autism (Sousa et al., 2020).

In early 1934 two mentally disabled siblings were examined byAsbjornFolling, a pediatric resident (Centerwall et al., 2000). At that time Folling identified and recognized the condition as phenyl pyruvic oligophrenia caused by elevated level of pyruvic acid a derivative of dietary phenylalanine and this discovery was renamed as phenylketonuria (PKU) (Woolf et al., 2020). Guthrie's screening test, and dietary treatment discovery plays crucial role in next explored treatments of PKU (Levy et al., 2021).

2.2 Phenylalanine Hydroxylase

The hepato-cellular enzyme phenylalanine hydroxylase (PAH) is specifically catalyzing the stereospecific hydroxylation of an amino acid, phenylalanine. BH4, a cofactor and molecular oxygen are requisite for PAH to function the catalytic activity of L-phenylalanine (Lidsky et al., 1985).

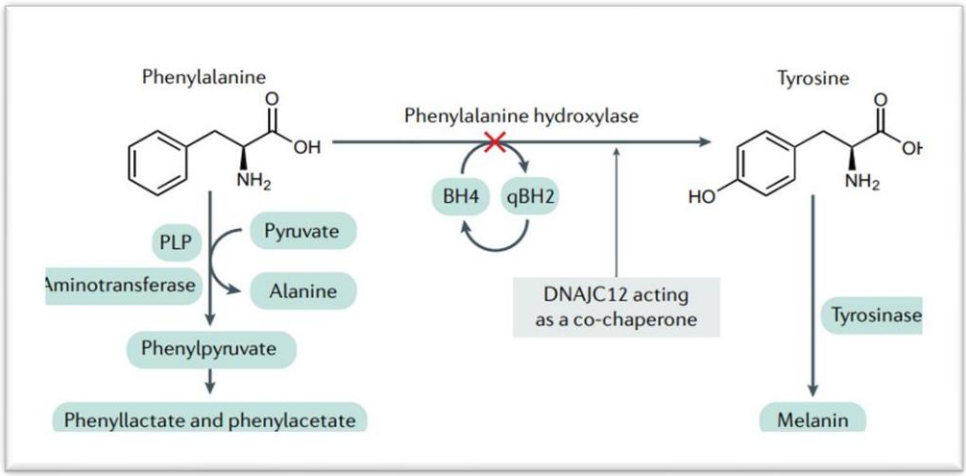

Figure 1: Reaction mechanism of PAH showing BH4 deficiency (Spronsen et al., 2021)

However, phenylalanine hydroxylase deficiency occurs due to the defect in tetrahydrobiopterin or BH4 synthesis (synthesis by *de novo* pathway and salvage pathway) or

it's related to its recycling (when *de novo* pathway is lacking in biosynthesis) results in uncontrolled and elevated phenylalanine (Thony et al., 2006; Moens et al., 2006).

2.3 Phenylalanine Hydroxylase mutations and elevated levels of L-phenylalanine

In case of PKU genetics of PAH gene plays important role, which is positioned on chromosome 12q23.2, spans about 171 kb and contains 13 exons. Mutation at the PAH locus can lead to HPA which later turn out to be PKU. Non-PKU HPA occur as the result of mutations in a number of loci which effect BH4 synthesis and regeneration (Eisensmith et al., 1992; Chakraborty et al., 1987).

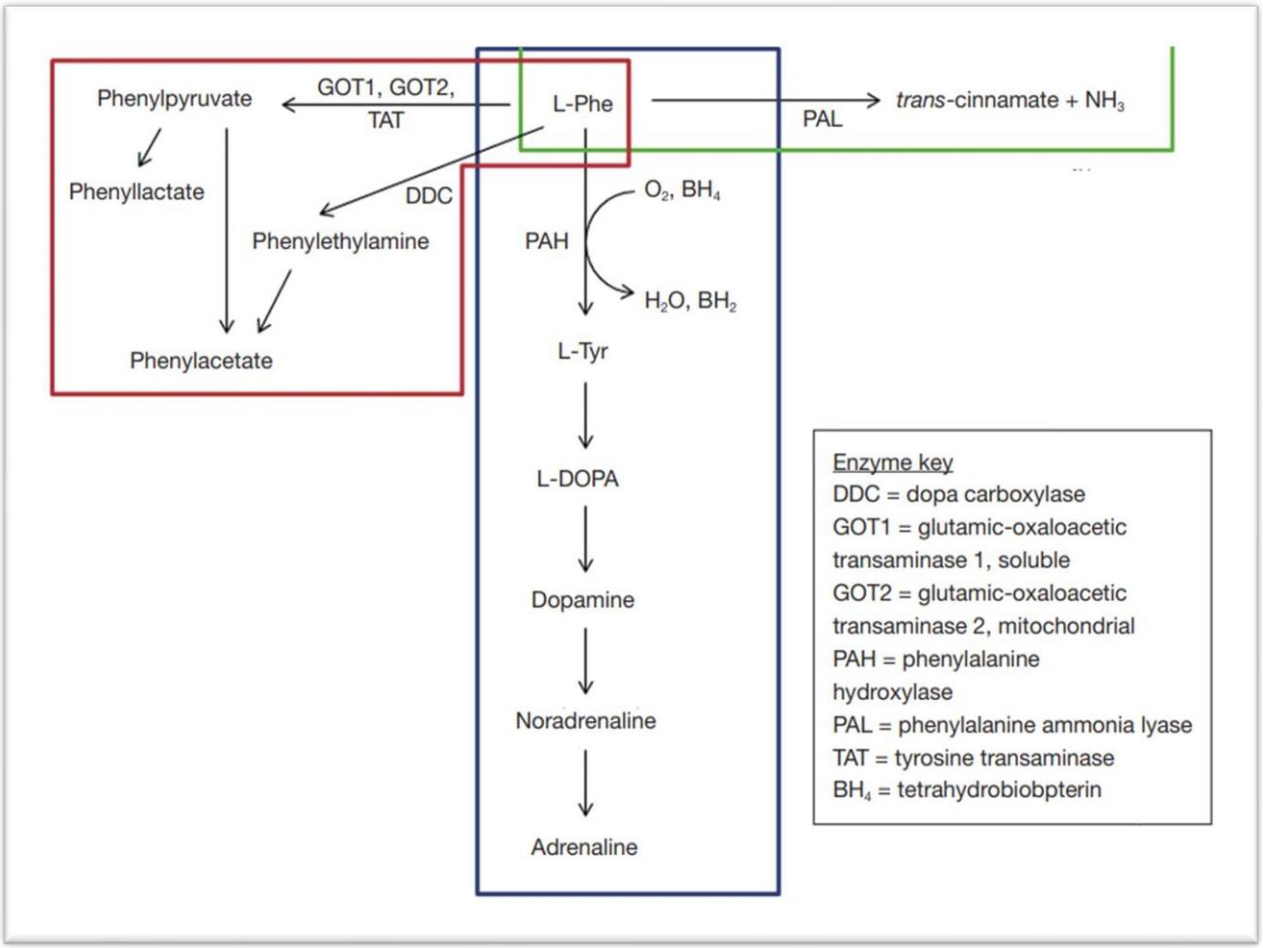

Figure 2: L-phenylalannine pathway showing the correct pathway of L-phenylalanine deamination, defected pathway of L-phenylalanine deamination and corrected pathway of L-phenylalanine deamination using PAL enzyme. (Hafid et al., 2015)

More than 500 disease-causing mutations have been identified in patients with PKU or HPA and recorded on the mutation database for PAH (Scriver et al., 2007). Mutations in the PAH gene result in decreased catalytic activity affecting the catabolic pathway of phenylalanine (Phe).

2.4 Phenylalanine Toxicity

Inadequacy of phenylalanine hydroxylase enzyme induce the rise in the levels of blood L-phenylalanine and phenyl ketones which are excreted in urine are the result of elevated phenylalanine and turn out in phenylketonuria (Harding et al., 2010; Spronsen et al., 2021; Sidell et al., 2009). Brain is the main organ affected by elevated level of L-phenylalanine and deficit levels of tyrosine. In other words, PKU is the reason of disruption of essential cellular processes in the brain such as protein synthesis and myelination, in due course leads to severe intellectual disability (Kukil et al., 2022). Increase in the phenylalanine levels in the blood cause inhibition of the cerebral uptake of other large neutral amino acids such as branched-chain amino acids, tyrosine, and tryptophan, impairing brain protein synthesis and saturate the transport system across the blood-brain barrier (Andersen et al., 1976).

2.5 Oxidative stress of phenylketonuria

Oxidative stress in PKU occur due to production in reactive oxygen species (ROS) which ultimately cause the injury and death to cell (Bortoluzziet al., 2019; Sirtori et al., 2005; Stepien et al., 2017). Antioxidant defense mechanisms are used to reduce and avoid the cell damage through enzymatic and non-enzymatic reactions by the reduction of free radicals.

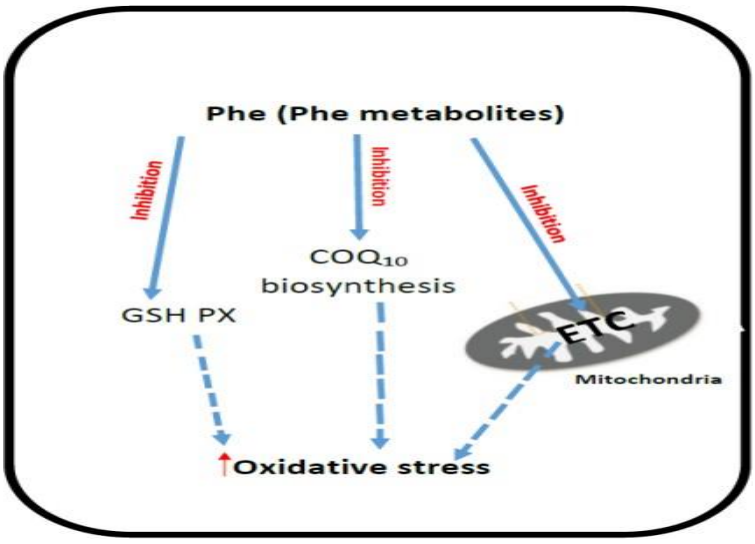

Figure 3: Oxidative stress in PKU due to free radicals (Stepien et al., 2017)

The data obtained were in correlation with micro and macro nutrients dietary restriction with antioxidant properties leads to decrease of antioxidant defenses In PKU neurological damage, studies speculate that oxidative stress may be involved in the pathophysiology as prior mentioned discoveries damage occur in brain (Raussell et al., 2018).

It may be predicated to utilize the association of antioxidant along with therapy given to PKU patients (Ribas et al., 2011).

2.6 Medicaments of PKU

2.6.1 Dietary therapy

In the beginning, treatment of PKU was based on the dietary restriction of L-phenylalanine came into picture and recommended in no or very low L-phenylalanine in the diet (Sarkissian et al., 1998; Knox et al., 1960). The Drawback of this intervention was lack in proper growth as the major protein sources were excluded from the diet. Hence, it was recommended to take commercially available L-amino acids barring L-phenylalanine as supplements on daily basis The imbalance diet and daily testing of children make this intervention a bit challenging (MacDonald et al., 1997).

2.6.2 BH4 (Tetrahydrobiopterin) therapy

This therapy involves the defects in BH4 synthesis or recycling due to the mutations in the patients suffering from atypical PKU (Williams et al., 2008). An orally active synthetic form of BH4 named as Sapropterin dihydrochloride (Kuvan) has been developed for the treatment of PKU. Kuvan has received Orphan Drug status and Fast Track approval. Patients with hyperphenylalaninemia and mild-to-moderate PKU who responded to a BH4 loading test in Phase II and III clinical trials can depend on Kuvan. The drawback of BH4 therapy is that it can be used by some selected patients suffering from mild-to-moderate PKU only (Hafid et al., 2015).

2.6.3 LNAA (Large neutral amino acids) therapy

LNAA therapy in which provided LNAAs competitively reduce the L-Phe levels helpful in treating BH4 regeneration disorders. However, LNAA formulations prepared for this therapy only benefits the adults suffering from PKU (Matalon et al., 2006).

2.6.4 Gene therapy

Somatic gene therapy was used for complete correction of HPA in the PKU mouse model (Pah^{enu2} mouse) have been reported. To maximize the therapeutic effect the therapy includes administration of immunosuppressant agents to block the immune response. This was considered as inappropriate treatment regimen for children suffering with PKU. Leukemia-like disorders may occur with the involvement of the recombinant adeno vectors and recombinant

retroviral vectors in trials were abandoned. Trial involves the PAH-deficient (Pah^{enu2}) mouse models for gene transfer including human PAH gene with promising results via a recombinant adeno-associated virus vector (Ding et al., 2006).

2.6.5 Enzyme replacement therapy

In early years various attempts were made to treat PKU using enteral (enteral delivery by gastrointestinal (GI) tract) and parenteral (a method of PAL isolation in the porous shell of a multi-tubular enzyme reactor) PAL. After a while engineered PAL came into picture PEGylated and engineered mutant form of PAL studied in vivo and in vitro and a PEGylated double mutant PAL make way in the human clinical trials which was obtained from Anabaena variabilis (Av), a filamentous cyanobacteria (Blau et al., 2015). In 2018, Palynziq, Av PAL double mutant drug was approved by the FDA as an enzyme therapy in the US. The discovery of enzyme Phenylalanine ammonia lyase from various microbial entities plays a very critical role in the treatment of PKU (Levy et al., 2018)

2.7 Phenylalanine ammonia lyase

Phenylalanine ammonia lyase enzyme was disclosed by Koukol and Conn in 1961.PAL catalyzes the deamination of L-phenylalanine into trans-cinnamic acid and trace amount of ammonia (Cui et al., 2013).

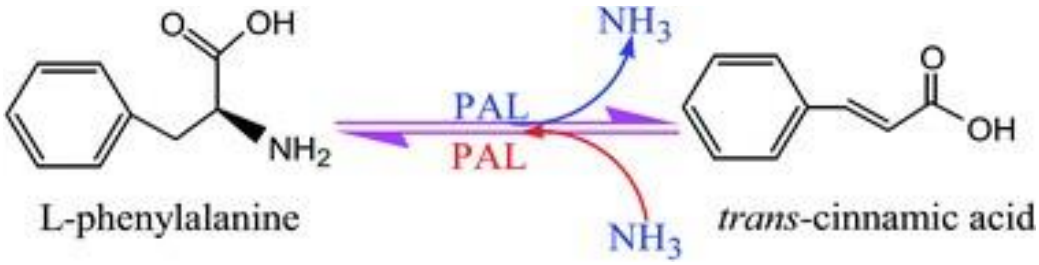

Figure 4: Catalysis of trans-cinnamic acid by PAL (Kong et al., 2015)

The native molecular mass of PAL ranges between 300 to 340 kDa for most of the PALs. There are some exceptions also in PALs which differ in molecular mass. Generally, Iso-electric points for PAL lies in the acid range, from 2.5 to 6.3. Usually, pH optimum for PAL lies in the range from 8.2~9.0 (Hyun et al., 2011). With identical subunits PAL is tetrameric. Each of the four identical active sites is formed within a homo-tetramer by three distinct monomeric subunits. An amino-acid transforming enzyme PAL contain uncommon prosthetic group dehydroalanine instead of cofactor pyridoxal 5-phosphate. L-Phenylalanine serves as the specific substrate, to a certain extent, L-tyrosine is also catalyzed by PAL. PAL catalysis is specific to certain level to a specific substrate L-phenylalanine and can also catalyze L-tyrosine.

Other L-amino acids remain uncatalyzed by PAL. For catalysis D-phenylalanine and D-tyrosine acts as competitive inhibitors of PAL and do not function as PAL substrates. In different species specificity for the substrate varies for PAL (Ogata et al., 1967; MacDonald et al., 2007).

2.8 The formation of trans-cinnamic acid by PAL involves two acknowledged mechanisms

First mechanism (E1cB): PAL is one of the enzymes which is dependent on the MIO (4-methylideneimidazole-5-one) cofactor, an autocatalytic condensation reaction of a conserved Ala-Ser-Gly motif led to the formation of MIO cofactor and this prosthetic group is responsible for the deamination of L-Phe. After amine–MIO intermediate formation a mechanism E1cB takes place in which elimination of amine occur to form cinnamic acid (Kawatra et al., 2020).

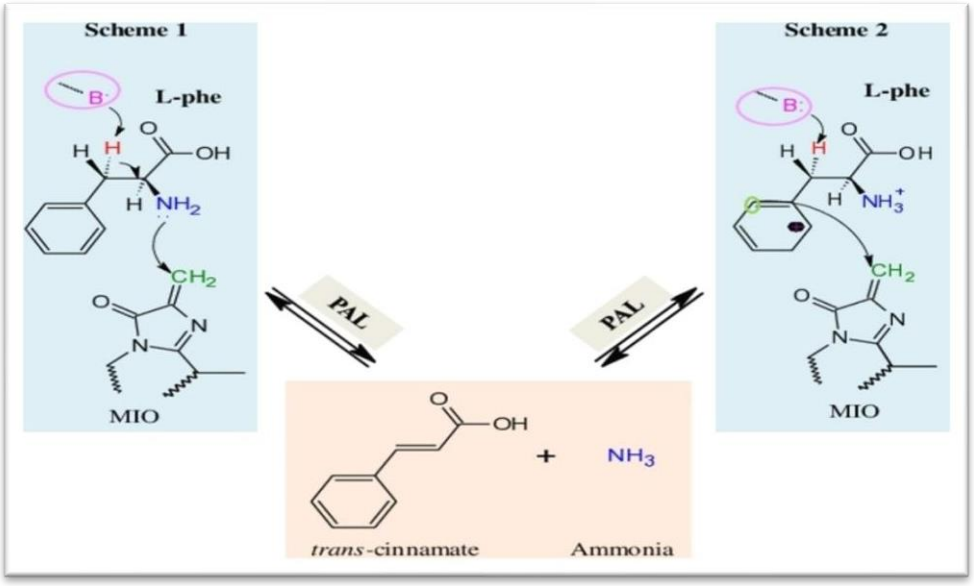

Figure 5: Reaction mechanisms involved in the formation trans-cinnamic acid by PAL (Kawatraet al., 2020)

Second mechanism: This elimination mechanism is based on the Friedel–Crafts mechanism which involves the detachment of C-3 (pro-3S)-proton, and C-2 amino group from the β-position. Trans-cinnamic acid releases at the completion of the reaction mechanism (Kawatra et al., 2020; Schuster et al., 1995).

Therapeutic applications of PAL include the treatment of phenylketonuria, treatment of Tyrosinemia, applicable in cancer therapy and also applicable in the production of antimicrobials and health supplements (MacDonald et al., 2007; Kawatra et al., 2020).PAL is widely distributed in plants, bacteria, cyanobacteria, algae, fungi. Microbial PALs have such properties which make these PALs compatible for the therapeutic exploitation (Kong et

al.,2015). Most known examples include their therapeutic application (Table 1) in the treatment of PKU using PEGylated Av PAL from cyanobacteria-PAL and Tc-PAL, protein sequences of two fungal enzymes used to treat Tyrosinemia.

Table 1: Timeline showing clinical applications of PAL in PKU and other diseases (Kawatra et al., 2020)

Year	Clinical Applications of PAL	References
1973	PAL was utilized to target L-phenylalanine to control thelymphoblasts proliferation.	Abell et al., 1973
1980	In the treatment of phenylketonuria an enteral PAL formulation wasutilized as the introductory treatment.	Hoskin et al., 2003
1994-2001	Formulations named as enteral and parenteral for PKU treatment-result of recombinant form of PAL. Mouse model was used to test the effects of formulations.	Sarkissian et al., 1995
2005-2007	PAL optimized by utilizing gene manipulation and PEGylation fortreating PKU.	Longo et al., 2014
2008	Av-PAL, an engineered PAL chosen for coming clinical trials for treating PKU.	Longo et al., 2014
2013	PAL activity explored against tumors and showed supreme activityagainst prostate tumors and breast tumors.	Babich et al., 2013
2018	CDX-6114, novel Av-PAL mutant-based drug enlisted for clinicaltrials for PKU.	Hydery et al., 2019
2018-2019	Palynziq^{TM}, Av-PAL double mutant drug was approved by the USFDA and EC.	Hydery et al., 2019
2019	Av-PAL used in the reduction of proliferation of colorectal tumors.	Yang et al., 2019
2020	More effective PAL variant was developed against tyrosine related metabolic disorders	Hendrikse et al., 2020

2.9 Endophytic Fungi

Microbial PALs exploitation in medical field presents the idea of utilizing diversified microorganisms such as endophytic fungi. Endophytic fungi area salient unit of fungal biodiversity lives within the living tissues of host plant without harming the co-host and do not show any disease symptoms representing mutual symbiont relationship between two

entities (Sieber et al., 2007). As the result of association between endophytic fungi and plants or their co-evolution, these microorganisms can exchange the genetic information with plants which leads to them becoming producers of host plant's putative phytochemicals, generally mediated via horizontal gene transfer (Liu et al., 2021). Fungi can fit with host plant more efficiently and can easily survive the environmental conditions with the exchange of the genetic information. In the process of co-evolution fungi contribute to perform protective functions against insects and pathogens and keep the host safe and healthy (Strobel et al., 2003; Gunatilaka et al., 2006). According to co-evolution hypothesis, bioactive secondary metabolites produced by endophytes in order to assist plant in chemical defense (Ji et al., 2009; Carroll et al., 1993).

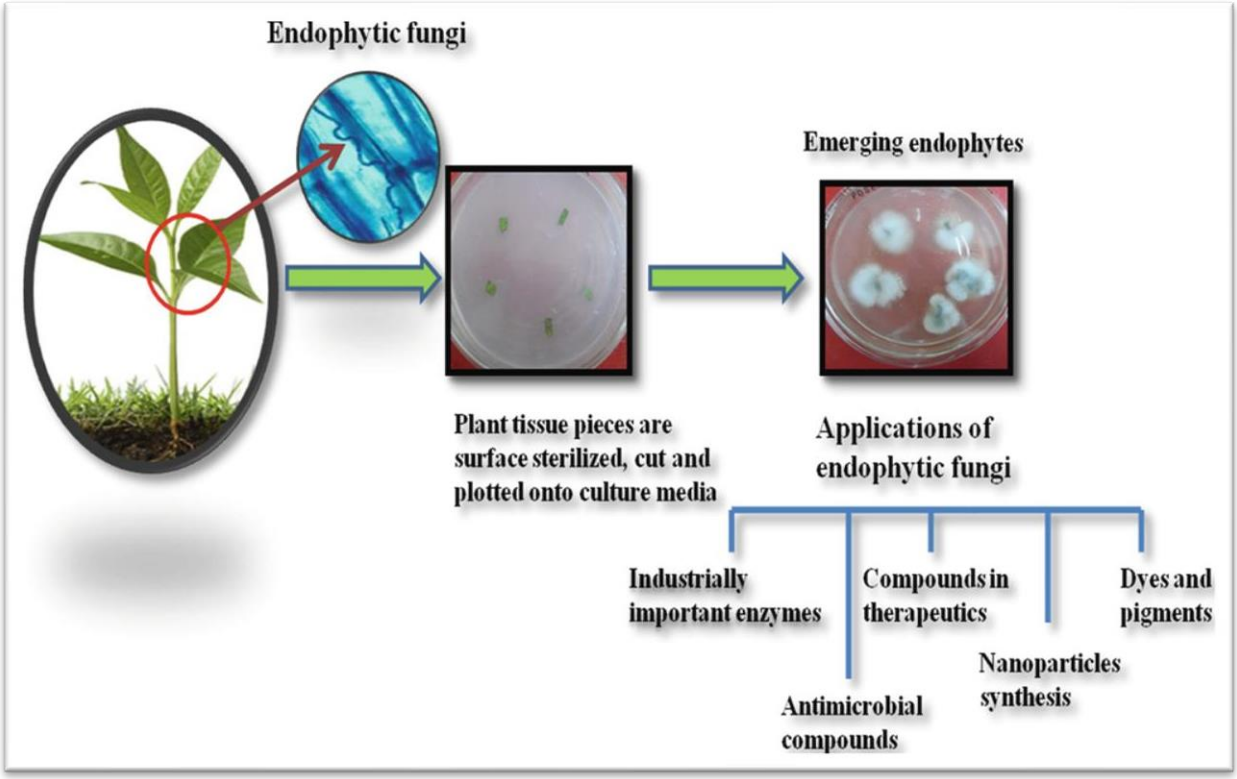

Figure 6: Endophytic fungi and its applications (Nishadet al., 2019)

The secondary metabolites produced by endophytic fungi such as terpenoids, flavonoids, steroids, phenol, phenylpropapanoids, quinines, alkaloids, amides, pyrrolizidines, sesquiterpenes, diterpenes, lignans, indole derivatives, isocoumarin derivatives, peptides, phenolic acids, chlorinated metabolites, aliphatic compounds, etc. possess crucial role in the field of medicine, agriculture industry. Bioactive compounds production is mediated by systematic chemical reaction chains controlled by significant precursors and their important enzymes, known as

biosynthetic pathways. (Meena et al., 2019; Sharma et al., 2016). Endophytic fungi *Penicillium brasilianum* isolated from *Melia azedarach* (Meliaceae) was explored for its PAL activity. The result research indicates that after the incubation of L-phenylalanine in the enzymatic culture extracts brought about the production of cinnamic acid, LC/MS was used for identification of cinnamic acid. The conversion time taken by substrate was very short which shows the high activity of PAL (Fill et al., 2009). Considering the importance of PAL and the limited studies carried out using endophytic fungi, we intend to explore the PAL activity using endophytic fungi.

CHAPTER 3

AIM OF THE STUDY

3. Aim of the study

The main aim of this research study is to screen endophytic fungi for production of phenylalanine ammonia lyase, for its possible use as a therapeutic intervention to treat phenylketonurics.

The objectives are as follows:

1. Qualitative screening and selection of phenylalanine ammonia lyase producing endophytic fungi
2. Quantification of PAL production by potent isolate(s)
3. To check *in-vitro* antioxidant potential of PAL producing endophytic fungi

CHAPTER 4

MATERIALS AND METHODS

4. Material and Methods

4.1 Culture procurement from repository

A total of 43 endophytic fungal cultures of *Cinnamomum camphora* (Camphor tree), *Murraya koenigii* (Curry leaf tree), *Cinnamomum zeylanicum* (Cinnamon), *Tinospora cordifolia* (Guduchi) were procured from pre-existing repository maintained by Dr. Sanjai Saxena, Professor, Thapar Institute of Engineering and Technology, Patiala.

4.2 Preparation of Potato Dextrose Agar (PDA) plates

Potato dextrose agar was prepared by boiling 200 g potato, the potato infusion was filtered using 8-fold muslin cloth. Further, 2% dextrose (w/v) and 1.5% agar (w/v) were added and the volume was made up to 1000 mL using distilled water. The pH was set to 5.3 ± 2˚C and the media was autoclaved at 121˚C, 15 psi for 15 minutes (Westphal et al., 2021).

4.3 Sub culturing and preservation

Hyphal tips of 43 procured cultures were inoculated aseptically on Potato Dextrose Agar plates and incubated at 28˚C in the BOD incubator (Bio-oxygen demand, Metrex Scientific Instruments, India) for 7 days until ample growth of fungal growth observed. Active fungal cultures were point inoculated into the PDA slants containing 10% glycerol and kept at 4°C for preservation (Talukdar et al., 2021).

4.4 Culture filtrate production

For culture filtrate production of the endophytic fungi 5 mm plug of actively dividing fungal culture using sterile cork-borer were inoculated in the pre-sterilized Potato Dextrose Broth (PDB) medium in the 50 ml Erlenmeyer flasks under aseptic conditions. Subsequently, these were incubated in shaker incubator at 120 rpm, 28°C for 7-10 days. Filtrates collected using Whatman filter paper No. 1 along with the centrifugation at 10,000 rpm for 10 minutes and stored at -20°C to use in future (Mehta et al., 2000).

4.5 Preliminary screening of phenylalanine ammonia lyase using agar well diffusion assay

Agar well diffusion (AWD) assay was used for qualitative screening for phenylalanine ammonia lyase production using selected fungal endophyte culture. Agar plates were prepared containing L-Phenylalanine as substrate along with the indicator phenol red and pH adjusted to 7 to check the formation of ammonia, the co-product of trans-cinnamic acid

Other indicator congo red was also used in the same manner pH adjusted to 7 to check the formation of trans-cinnamic acid as the main product of PAL catalysis or L-phenylalanine

deamination. Plates were prepared by dividing the plates into four quadrants and 5mm wells were punched using sterile cork-borer in each quadrant. Filtrate (30 µL) loaded into the three wells and fourth well remain unloaded and act as control for the assay. Plates were then incubated at 26°C for few days. After the completion of incubation period, pink zone was observed around the wells in the plates with indicator phenol red and violet zone was observed around the wells in the plates with indicator congo red. The zone diameter was recorded in triplicate and mean and standard deviation was calculated.

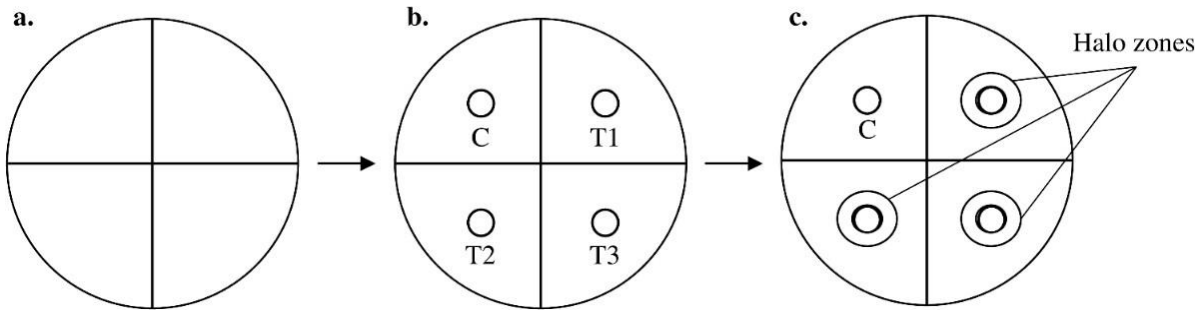

Figure 7: Procedure of AWD assay (a) showing division of petri plate into four quadrants (b) wells punched (c) halo zone observation around the wells

4.6 Secondary screening of phenylalanine ammonia lyase using Nesslerization.

To quantify the activity of Phenylalanine ammonia lyase direct nesslerization were performed in the secondary screening. First and foremost, standard curve of ammonium chloride was created in pursuance of estimation of the ammonia liberated by protein sample. Nesslerization was performed according to Chand et al., (2020) with some modifications. Supernatant (2mL) was taken into the test tube and Nessler's reagent (1mL) was added to determine the ammonia production by the culture filtrates. The change in color to faint yellow that indicated that the minimum ammonia production and color change into deep yellow to the brownish color indicated the maximum ammonia production by fungal endophyte culture filtrates. The absorbance was taken at 450nm.

4.7 Total protein estimation by Bradford and Folin-lowry method

Bradford assay for determination of protein carried out according to Kruger et al., (2010) with some modifications. 1 mL BSA (Bovine serum albumin) was mixed with 3mL 1 X Bradford reagent (prepared by dissolving Coomassie Brilliant Blue G-250 95% ethanol and 85% (w/v) phosphoric acid), followed by the incubation for 45 minutes at room temperature. The absorbance was measured at 595 nm.

Folin-Lowry protein determination method was carried out according Stauffer et al., (1975), with some modifications. Solution A, B, C was prepared in the ratio (v/v) of 100:1:1 to make solution D (where solution A contained NaOH, Na_2CO_3; solution B contained $CuSO_4.5(H_2O)$ and solution C contained Na_2 Tartrate). To 1 mL BSA 5 mL Solution D was added followed by the incubation for 10 minutes at room temperature. After incubation, 1.5 mL of 1N Folin-Ciocalteu (FC) reagent was added. The absorbance was measured at 750 nm. BSA standard curve was constructed to estimate the protein.

4.8 Estimation of PAL using UV-Vis spectroscopy

UV-Vis spectroscopy was used to carry out the qualitative screening of phenylalanine ammonia lyase.

4.8.1 Calibration curve preparation of trans-cinnamic acid

First and foremost, standard curve of trans-cinnamic acid were constructed in pursuance of estimation of the formation of trans-cinnamic acid by protein sample. Trans-cinnamic-acid was weighed and dissolved in methanol and stock solution (1.0mg/mL) was prepared. Curve calibration prepared from standard stock solution of trans-cinnamic acid containing concentration 0.5-2.5 µg/mL. The absorbance was measured at 270 nm (Wardatun et al.,2017).

4.8.2 PAL activity determination

The test tube's reaction mixture contained 100µM borate buffer (pH 8.8), 0.2 mL or 200 µL of culture extract and 15µM L-phenylalanine. The mixture was incubated at 32°C for 15 minutes. After incubation, absorbance was measured at 290 nm using UV-Vis spectrophotometer. The obtained concentrations were determined in corresponding with the standard curve prepared of trans-cinnamic acid (Zucker et al., 1969).

4.9 *In-vitro* antioxidant potential of selected fungal isolates

4.9.1 Culture filtrate production

The culture filtrate production of endophytic fungi was carried out as mentioned in section 4.3 at 120 rpm, 28°C for 7-10 days for further utilization in liquid-liquid extraction.

4.9.2 Liquid-liquid extraction

Liquid-liquid extraction was carried out according to Kjer et al., (2010) with slight modifications. Samples were mixed with the ethyl acetate in the separating funnel extracted three times in a ratio of 1:3. Separating funnel with the mixture of sample and ethyl acetate were shaken vigorously for some time. After shaking two layers were formed and the ethyl acetate layer was collected and transferred to crucibles before that initial weight of crucibles was recorded

and kept in the BOD incubator at 50°C for the evaporation. After evaporation, the final weight of crucibles was recorded and the remaining extract was diluted with methanol and kept for further use.

4.9.2.1DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) assay

DPPH scavenging activity of extracts was measured according to Rustaiyan et al., (2011) with some modifications. Summarily, five different diluted concentrations (viz. 0.2-1.0mg/ml) of extracts were dissolved in methanol, incubated in 96-well microplates with a methanolic solution of 100µM DPPH. Incubation was carried out at room temperature in the dark for 30 minutes, after the incubation absorbance was measured at 490 nm by a microplate reader.

4.9.2.2 Total phenolic content determination

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent as per the method ofArora et al., ((2010) with some modifications. 50 µL sample was mixed with 750 µL of distilled water and 50 µL of 1N FC reagent and incubated for 10 minutes in this 100 µL of 6% sodium carbonate was mixed followed by 1 hour incubation. After incubation, absorbance was measured at 760 nm by a microplate reader. Gallic acid served as standard. Gallic acid (1mg/mL) was weighed and dissolved in methanol. Five different concentrations (0.2-1.0mg/mL) were prepared for the construction of standard curve.

4.9.2.3 Total flavonoid content determination

Total flavonoid content was determined as per the method of Diaz et al., (2012). Brief, 100 µL of sample and 400 µL and 30 µL of 5% sodium nitrite were pipetted out and kept for incubation for 5 minutes. After this, 30µL of 10% $AlCl_3$were added kept for 1 minute, 200 µL of 1N NaOH were added to the reaction mixture and the absorbance was measured at 510 nm. Quercetin served as standard and concentrations ranging from 0.2 to 1.0mg/mL were prepared and a standard calibration curve was constructed.

4.10 Morphological identification of top isolates

To identify the selected endophytic fungus morphological characteristics would be recorded. Colony characteristics of endophytic fungal isolates such as colony shape, color, pigment production, growth rate etc., were recorded on different media (potato dextrose agar, corn meal agar and water agar media). The slide was prepared by taking a small amount of culture and teasing it using a fine tip needle. For staining, Lacto-phenol cotton blue was used and covered using a cover slip (18 ×10 mm). For visualizing the morphological characteristics light compound microscope (Nikon E200, Tokyo, Japan) was used (Meshram et al., 2013).

CHAPTER 5

RESULTS

5. Results:

5.1 Sub-culturing and preservation endophytic isolates

A total of 43 endophytic fungi were procured from existing repository of Dr. Sanjai Saxena, DBT, TIET Patiala, and screened for phenylalanine ammonia lyase production. The isolates were sub-cultured on PDA media at an interval of 10-15 days to keep the cultures in active condition.

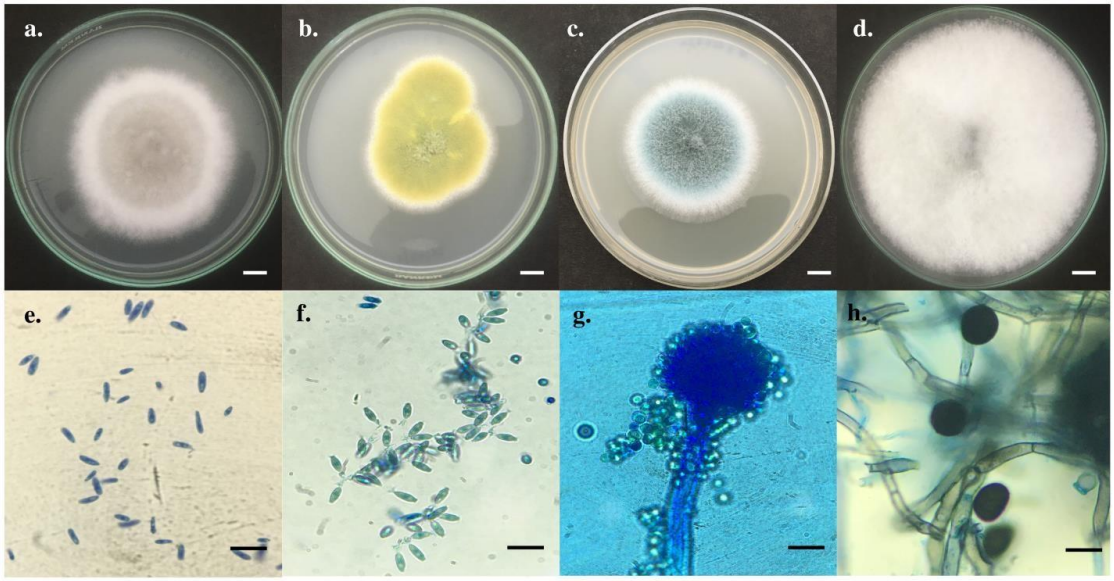

Figure 8: a-d) Some of the endophytic isolates screened during the study (Bar 10 mm). Morphological features of some of the isolates screened during the study e-f) *Fusarium* sp. g) *Aspergillus* sp. h) *Nigrospora* sp. (Bar 10 µm)

Of the 43 isolates, 1 isolate was from *Cinnamomum camphora*, 28 isolates were from *Murraya koenigii*, one isolate was from *Tinospora cordifolia* and 10 isolates were from *Cinnamomum zeylanicum*. Some of the isolates screened during the study are shown in Figure 8 (a-d) and Figure 8 (e-h) shows morphological features of some of the isolates screened during this study. Further the revived isolates of endophytic fungi were preserved (as shown in Figure 9) in PDA-glycerol slants containing 10% glycerol. The slants were stored at 4°C for long term preservation.

Figure 9: Picture showing the slants of fungal cultures

5.2 Preliminary screening of phenylalanine ammonia lyase

The endophytic isolates were tested qualitatively to check whether isolates utilize the L-phenylalanine to produce trans-cinnamic and co-product ammonia. Preliminary screening was conducted utilizing agar well diffusion assay.

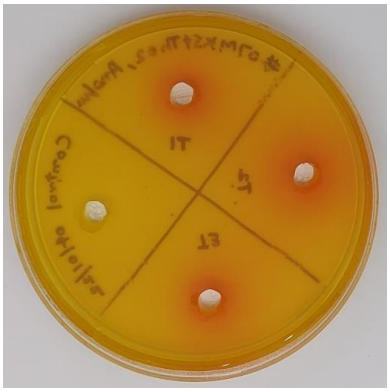

Figure 10: Picture showing the pink zone around the wells (Phenol red).

The water agar medium with phenol red as pH indicator was utilized and the isolates were selected on the basis of the pink color halo zone observed around the wells. The change in color exhibited the accumulation of ammonia a co-product of trans-cinnamic acid (as shown in Figure 10). Out of 43 isolates, 17 isolates exhibited positive activity on using phenol red indicator. The diameter of pink zone formation was recorded, post-hoc analysis revealed that isolate #61MKLTh02 exhibited the highest activity followed by #2CCBD, #65MKLTh03, #29CZSTBRT, #07MKStTh02, #23MkStTh03, #65MKStTh03 (Table 2).

Further, the water agar medium with congo red as pH indicator were utilized and the isolates were selected on the basis of the violet halo zone observed around the wells. The change in color exhibited the production of trans-cinnamic acid (as shown in Figure 11). The zone diameter is noted in triplicate. Of the 43 cultures, 9 were found to be positive extracellular production of phenylalanine ammonia lyase.

Figure 11: Picture showing violet zone around the wells (Congo red).

The highest activity was observed in isolate #2CCBD followed by #61MKLTh02, #65MKLTh03, #29CZSTBRT, #07MKStTh02 and #23MkStTh03. On the basis of post-hoc analysis top six isolates were selected for further evaluation.

Table 2: List of isolates screened qualitatively for PAL production using phenol red and congo red indicator

Sr. No.	Culture code	Host plant	Zone diameter* (mm)	
			Phenol red	**Congo red**
1.	#2CCBD	*Cinnamomum camphora*	15.66 ± 0.58^{ab}	34.5 ± 2.65^{a}
2.	#61MKLTh02	*Murraya koenigii*	15.83 ± 0.76^{a}	29.33 ± 2.57^{b}
3.	#65MKLTh03	*Murraya koenigii*	15.5 ± 0.87^{ab}	12.33 ± 0.58^{c}
4.	#29CZSTBRT	*Cinnamomum zeylanicum*	14.83 ± 0.76^{abc}	11.33 ± 0.58^{cd}
5.	#07MKStTh02	*Murraya koenigii*	14.66 ± 0.58^{abcd}	9.33 ± 0.58^{def}
6.	#23MkStTh03	*Murraya koenigii*	14.83 ± 0.76^{abc}	10.33 ± 0.58^{cde}
7.	#01MKStTh03	*Murraya koenigii*	14 ± 0.50^{bcde}	ND
8.	#65MKStTh03	*Murraya koenigii*	14.16 ± 1.26^{abcde}	8.67 ± 0.58^{ef}
9.	#43MKLTh02	*Murraya koenigii*	14 ± 1.00^{bcde}	7.67 ± 0.58^{f}
10.	#22MKLTh03	*Murraya koenigii*	13.5 ± 0.50^{cde}	ND
11.	#64MKLTh02	*Murraya koenigii*	11.66 ± 0.58^{f}	ND
12.	#02MKLTh03	*Murraya koenigii*	12.5 ± 0.50^{ef}	ND
13.	#36MKStTh02	*Murraya koenigii*	13 ± 1.00^{def}	ND
14.	#33MKStTh03	*Murraya koenigii*	13.33 ± 1.04^{cdef}	ND
15.	#105TICSTITPLM	-	$15ab\pm0.50^{c}$	$9.670.58^{def}$
16.	#52CZSTITBRT	*Cinnamomum zeylanicum*	3.33 ± 1.53^{g}	ND

17.	#34CZSTITBRT	*Cinnamomum zeylanicum*	13.33 ± 0.58^{cdef}	ND
18.	#47MKStTh03	*Murraya koenigii*	ND	ND
19.	#23CZSTITG	*Cinnamomum zeylanicum*	ND	ND
20.	#40MKStTh03	*Murraya koenigii*	ND	ND
21.	#45TCSTPLM	*Tinospora cordifolia*	ND	ND
22.	#34MKStTh03	*Murraya koenigii*	ND	ND
23.	#4TICSTITPLM	**-**	ND	ND
24.	#27MKStTh03	*Murraya koenigii*	ND	ND
25.	#05MKStTh03	*Murraya koenigii*	ND	ND
26.	#51MKLTh03	*Murraya koenigii*	ND	ND
27.	#26MKStTh02	*Murraya koenigii*	ND	ND
28.	#03MKLTh03	*Murraya koenigii*	ND	ND
29.	#74MKLTh02	*Murraya koenigii*	ND	ND
30.	#2106CZSTITG	*Cinnamomum zeylanicum*	ND	ND
31.	#33TICSTITPLM	**-**	ND	ND
32.	#09MKStTh03	*Murraya koenigii*	ND	ND
33.	#78MKLTh02	*Murraya koenigii*	ND	ND
34.	#2116CZSTITBRT	*Cinnamomum zeylanicum*	ND	ND
35.	#41CZSTITBRT	*Cinnamomum zeylanicum*	ND	ND
36.	#22MKStTh03	*Murraya koenigii*	ND	ND
37.	#2107CZSTITG	*Cinnamomum zeylanicum*	ND	ND

38.	#41MKStTh02	*Murraya koenigii*	ND	ND
39.	#56MKLTh02	*Murraya koenigii*	ND	ND
40.	#31CZBAG	*Cinnamomum zeylanicum*	ND	ND
41.	#55MKStTh03	*Murraya koenigii*	ND	ND
42.	#2CZBAWLS	*Cinnamomum zeylanicum*	ND	ND
43.	#13MKStTh02	*Murraya koenigii*	ND	ND

*The data represents mean ± SD; values with different superscript letters are different by Tukey's post-hoc test ($p<0.05$). ND denotes not determined.

5.3 Secondary screening of phenylalanine ammonia lyase using nesslerization

Phenylalanine ammonia lyase activity were estimated when the pink color zone was formed around the wells indicating the accumulation of ammonia as a co-product of trans-cinnamic acid catalyzed by phenylalanine ammonia lyase. This was evaluated by using direct nesslerization method (Figure 12).

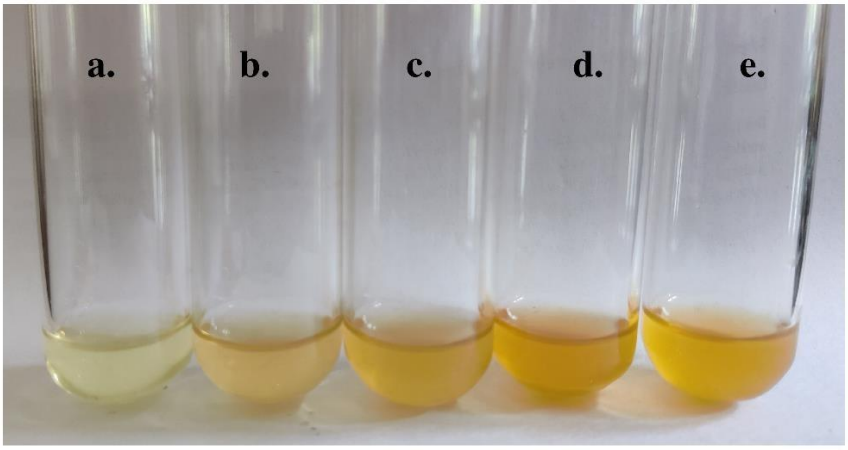

Figure 12: a-e) Color intensity of different concentrations of ammonium chloride

By quantifying the amount of ammonia released the activity of phenylalanine ammonia lyase were checked. The crude culture filtrate of #2CCBD followed by #61MKLTh02 exhibited the maximum activity. Whereas the culture filtrate of #23MKStTh03 exhibited the lowest activity (Figure 13).

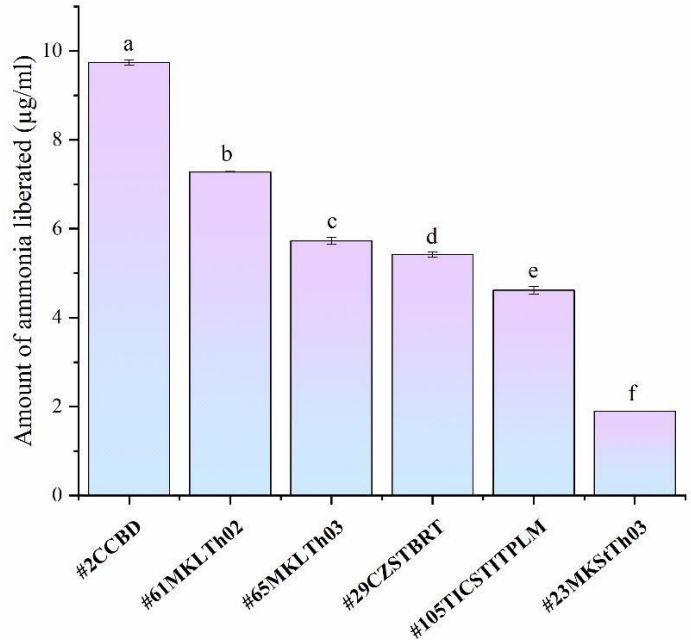

Figure 13: The amount of ammonia production seen in top six isolates

On the post-hoc analysis it was observed that the isolate #2CCBD exhibited highest ammonia production closely followed by #61MKLTh02. To further quantify the amount of PAL production using UV-vis spectroscopy the top two isolates were selected.

5.4 Total protein estimation by Bradford and Folin-Lowry

The crude protein of the top two was estimated. The total protein content was obtained per mL of crude culture filtrate.

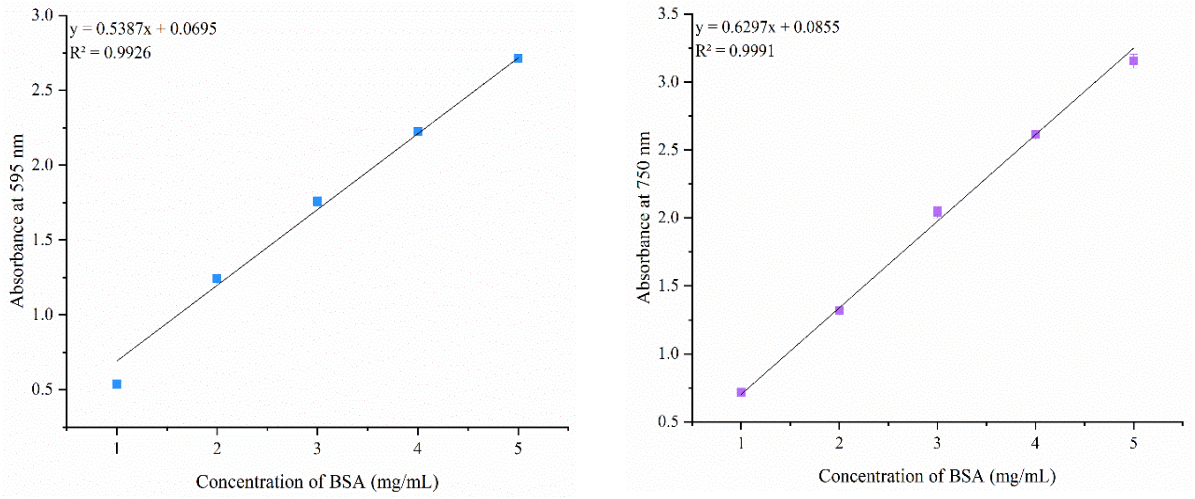

Figure 14: Standard curve of BSA using Bradford and Folin Lowry test

The protein estimation of top selected isolates was done by Bradford's and Lowry's method and the amount of protein obtained using these methods next utilized in the further quantitative screening. In Bradford method, the protein concentration of crude cultures:

#2CCBD (1.93±0.02) and #61MKLTh02 (1.18±0.06). In Folin-Lowry method, the protein concentration of crude cultures: #2CCBD (1.87±0.01) and #61MKLTh02 (1.17±0.05).

5.5 Estimation of PAL using UV-Vis spectroscopy

The activity of phenylalanine ammonia lyase were estimated according to the production of trans-cinnamic acid as a result of the conversion of L-phenylalanine to trans-cinnamic acid by using UV-Vis spectroscopy. To find the activity of phenylalanine ammonia lyase standard curve of trans-cinnamic acid were constructed. The equation of straight-line y = 0.0739x - 0.0634 (where y is the absorbance and x is the concentration of cinnamic acid), obtained from the standard curve was then used to calculate the amount of cinnamic acid formed in the test sample. One unit (IU or U) is the amount of enzyme required to convert 1 µM of substrate into product in one minute. The enzyme activity was expressed in U/min/mL of the crude protein. Isolate#61MKLTh02 exhibited 0.42 U/min/mL of crude protein whereas isolate #2CCBD exhibited nearly double i.e., 0.85 U/min/mL of crude protein.

Table 3: Total protein content of isolates and test activity of PAL in U/min/mL of crude protein

Sr. No.	Culture code	Protein content (mg/mL)		U/min/mL of crude protein
		Bradford	Folin-lowry	
1.	#2CCBD	1.93 ± 0.02	1.87 ± 0.01	0.85
2.	#61MKLTh02	1.18 ± 0.06	1.17 ± 0.05	0.42

5.6 *In-vitro* antioxidant potential of selected fungal isolates

Both the isolate exhibited PAL production on quantitative UV-Vis based analysis thus their *in-vitro* antioxidant potential was determined.

5.6.1 DPPH antioxidant assay

The crude fungal culture #2CCBDin the DPPH assay exhibited fine scavenging activity as compared to standard. The change in color from purple to yellow during scavenging reaction were quite noticeable. Crude fungal culture #2CCBD exhibited 85.306±0.368% free radical scavenging activity at 1 mg/mL concentration whereas standard quercetin exhibited

92.520±0.123% free radical scavenging at same concentration. Further, isolate #61MKLTh02 exhibited 9.305±0.301% free radical scavenging activity.

The crude fungal culture #2CCBD exhibited IC_{50} 485.213±1.204 µg/mL whereas standard quercetin exhibited an IC_{50} value of 369.976±2.730 µg/mL and #61MKLTh02 exhibited IC_{50} (5442.281±60.182 µg/mL).

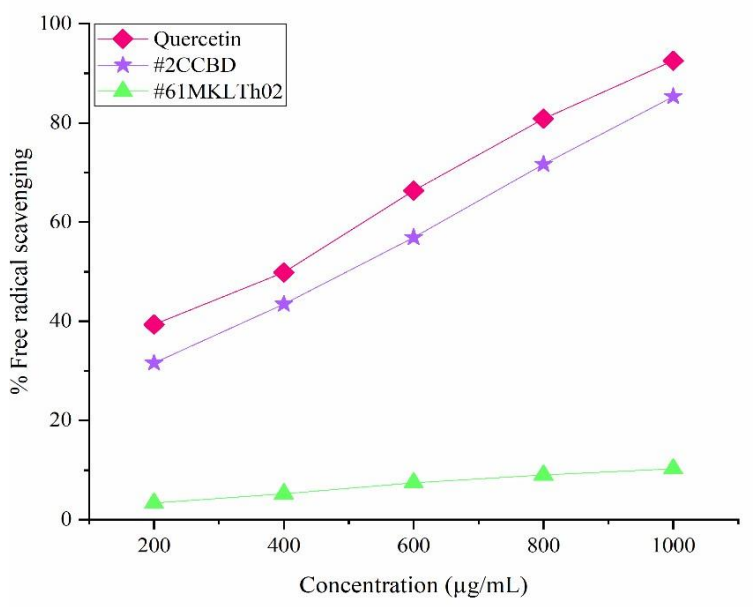

Figure 15: Picture showing %FRS of culture filtrate #2CCBD and 61MKLTh02 as compared to quercetinat different concentrations.

5.6.2 Total phenolic content

The reduction of the Folin–Ciocalteu reagent in the presence of phenolics resulting in the production of molybdenum–tungsten blue indicating the antioxidant activity. The total phenolic content concentrations of crude culture #2CCBD (594.73 ± 1.91) and #61MKLTh02 (139 ± 11.98) and the color change of culture into molybdenum–tungsten blue was quite noticeable.

5.6.3 Total flavonoid content

The change in color into yellow as a result of aluminum ion complex formation demonstrated the flavonoids content presence. The total flavonoids content concentrations of crude culture #2CCBD (877.07 ± 20.94) and #61MKLTh02 (514.48 ± 1.96).

Table 4: IC_{50} value, TPC and TFC of #2CCBD and #61MKLTh02

Sr. No.	Culture code	DPPH IC_{50} (µg/mL)	Total phenolic content (µg Gallic acid equivalent/mg of sample)	Total flavonoid content (µg Quercetin equivalent/mg of sample)
1.	Quercetin	369.976±2.730	-	-
2.	#2CCBD	485.213±1.204	594.73 ± 1.91	877.07 ± 20.94
3.	#61MKLTh02	5442.281±60.182	139.98 ± 11.98	514.48 ± 1.96

5.7 Morpho-taxonomic identification of selected isolate

Based on statistically significant results exhibited in *in-vitro* antioxidant potential by the endophytic fungus #2CCBD, it was tentatively identified using morpho-taxonomic tools. The isolate was grown on three different media named as potato dextrose agar, corn meal agar and water agar. The morphological characteristics of *Penicillium*: *Penicillium* were composed of filaments called hyphae contained internal cross walls dividing the hyphae into separate cells. The color of fungi was green and the texture was powdery to velvety and dense brush structures bearing spores on to them. Based on the morphological characteristics the isolate was identified tentatively identified as *Penicillium* sp.

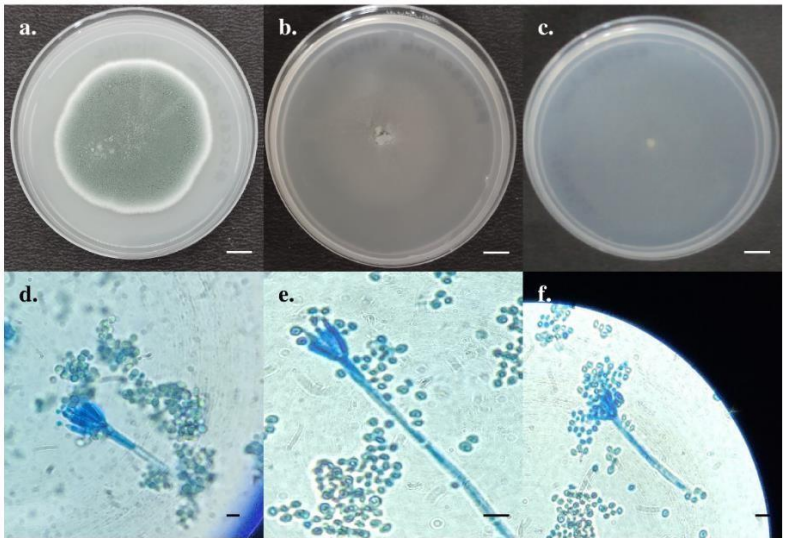

Figure 16: a-c) Plate morphology of #2CCBD on PDA, CMA and WA respectively. d-f) Microscopic features of #2CCBD (Hyphae, dense brush like structure having spores)

CHAPTER 6

DISCUSSION

6. Discussion

Phenylketonuria, is a rare inherited disease that occurs due to the elevated levels of an amino acid phenylalanine. An enzyme Phenylalanine hydroxylase is responsible for the accumulation of phenylalanine as it fails to hydrolyze the phenylalanine into the tyrosine (Bosch et al., 2015). The severity of PKU includes the L-phenylalanine intolerance on daily basis in patients. The regulation of a L-phenylalanine deficient diet is quite difficult and impractical alternative. A given range in which blood L-phenylalanine should be maintained and it is bit challenging to do so (Brown et al., 2016). Hence, the regulation of the treatment of PKU is bit complex.

Enzyme substitution therapy have been utilized with the help of phenylalanine ammonia lyase an enzyme which converts the toxic phenylalanine into trans-cinnamic acid *via* different route as a substitution of phenylalanine hydroxylase (Levy et al., 2018). Phenylalanine ammonia lyase is involved in the amino acid metabolism and natural product biosynthetic pathways and acts as connector between primary metabolism and secondary metabolism. Phenylalanine ammonia lyase plays an important role in metabolic pathways and this fact make this enzyme more crucial and suitable in biotechnological, industrial and medical applications (Dixon et al.,1995; Wang et al., 2013)

Microbial PAL have been utilized for therapeutic applications of phenylketonuria as a non-dietary treatment. A formulation as an oral PEGylated form to treat phenylketonuria (PKU) patients have been manufactured and microbial PAL substantiate itself as a better therapy for PKU patients than restricted phenylalanine diet therapy or any other therapy. However, considering the drawbacks associated with the use prokaryotic enzymatic system the exploration of other potential biological entities such as endophytic fungi could be a better therapeutic approach to deal with such diseases or disorders.

Endophytic fungi reside within the intracellular spaces of the plants generally root, bark and leaf sheaths without showing any harmful symptoms. Endophytic fungi produce the same secondary metabolites as plants as a result of co-evolution between host plant and endophytic fungi. These endophytic fungi mimic the plant secondary metabolites which have been utilized for human health. For instance, Taxol, camptothecin and its structural analogs, jasmonic acid, ginkgolide, azadirachtin etc., (Stierle et al., 1993; Patial et al., 2016).

In this study, 43 endophytic fungi were screened for PAL production. Out of the 43 isoates, only 17 isolates were found to be positive among which 6 isolates exhibited maximum activity when indicator phenol red was used to check the production of ammonia and only 9

isolates were found to be positive among which 6 isolates exhibited maximum activity when indicator congo red was used to check the production of trans-cinnamic acid. The above-mentioned screening was performed by using agar well diffusion (AWD) assay. These six isolates were further evaluated using direct nesslerization assay. Out of six isolates, two isolates were identified as potent producer of ammonia, #2CCBD followed by #61MKLTh02 was showing maximum activity in nesslerization test and thus chosen for the further study.

The total protein determination was carried out by using Bradford and Folin-Lowry method of the two selected isolates. The Bradford assay based on the dye Coomassie Blue G250 binding to protein and Folin-Lowry method depends on the interaction of copper ions to the peptides under alkaline conditions. In this way, the protein concentration of samples was measured where out of 2 selected samples one #2CCBD shows better protein concentration than #61MKLTh02. For the accurate measurement of the PAL, UV-Vis spectrometry were used as proteins absorbs light at a particular wavelength and concentration of proteins can be measured by using spectrophotometer. In another study, the activity of the crude and partially purified PAL from *Spirulina* CPCC-6$_{95}$ were found in the range: crude PAL activity (80.96 ± 0.064 U/l) and partial purified PAL activity (105±0.118U/l) (Ahmad et al., 2022). In this study, crude culture #2CCBD (0.85 U/min/mL) exhibited the higher activity than #61MKLTh02 (0.42 U/min/mL) in UV-Vis spectroscopy assay.

For the *in vitro* estimation of antioxidant activity DPPH has been used for rapid and dependable estimation. The purple color turned into yellow as the concentration of radical decreased when the concentration of extract increases the absorbance shows the correlation in reducing power and activity. In previous study, antioxidant activity of the endophytic fungal culture *Penicillium* sp. was evaluated by its ability to scavenge DPPH free radicals. The crude extract of *Penicillium* sp. exhibited high antioxidant activity with IC50 value, 54.72±2.19µg/ml (Devi et al., 2014). In present study, the crude culture filtrate #2CCBD exhibited the antioxidant activity (IC_{50} 485.213±1.204 µg/mL) and #61MKLTh02 exhibited the antioxidant activity (IC_{50} 5442.281±60.182 µg/mL). In earlier study, the endophytic fungi *Phyllosticta* sp. were subjected to check antioxidant activity and total phenol found was 18.33±0.68mg GAE/g dry weight and flavonoid content was 6.44±1.24 µg/mg of quercetin equivalent (Srinivasan et al., 2010). In present study, the crude culture filtrate #2CCBD were found to possess the highest phenolic (594.73 ± 1.91) and flavonoids (877.07 ± 20.94) content. Considering these findings, the isolate #2CCBD was tentatively identified as *Penicillium* sp. using morpho taxonomic tools.

CHAPTER 7

CONCLUSION

7. Conclusion

In the present study, isolate #2CCBD tentatively identified as *Penicillium* sp. was found to be the top potent PAL producer. The results were confirmed by rigorous screening using different indicators, direct nesslerization assay and quantitative UV-Vis spectroscopy. The isolate also exhibits promising antioxidant potential with IC_{50} of 485.21 ± 1.20 as compared to standard quercetin. High TPC (594.73 ± 1.91) and TFC content (877.07± 20.9) was also observed. Further work on molecular identification of the isolate followed by optimization of PAL production is warranted to convene the industrial demand of PAL.

CHAPTER 8

REFERENCES

References:

Abell C W, Hodgins D S, Stith W J (1973): An in vivo evaluation of the chemotherapeutic potency of phenylalanine ammonia-lyase. Cancer research, 33(10): 2529-2532.

Ahmad, Rakhshan, (2022): Biochemical Characterization of Novel Phenylalanine Ammonia-Lyase from *Spirulina* CPCC-695. The Protein Journal: 1-10. https://doi.org/10.1007/s10930-022-10063-8

Al Hafid N, Christodoulou J (2015): Phenylketonuria: a review of current and future treatments. Translational pediatrics, 4(4): 304–317. https://doi.org/10.3978/j.issn.2224-4336.2015.10.07.

Andersen AE, Avins L (1976): Lowering brain phenylalanine levels by giving other large neutral amino acids: a new experimental therapeutic approach to phenylketonuria. Archives of neurology, 33(10): 684-686. https://doi.org/10.1001/archneur.1976.00500100018008.

Arnold AE (2007): Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal biology reviews, 21(2-3): 51-66. https://doi.org/10.1016/j.fbr.2007.05.003.

Arora DS, Chandra P (2010): Assay of antioxidant potential of two *Aspergillus* isolates by different methods under various physio-chemical conditions. Brazilian Journal of Microbiology, 41: 765-777. https://doi.org/10.1590/S1517-83822010000300029.

Ashe K, Kelso W, Farrand S, Panetta J, Fazio T, De Jong G, Walterfang M (2019): Psychiatric and cognitive aspects of phenylketonuria: the limitations of diet and promise of new treatments. Frontiers in Psychiatry, 10: 561. https://doi.org/10.3389/fpsyt.2019.00561.

Babich OO, Pokrovsky VS, Anisimova NY, Sokolov NN, Prosekov AY (2013): Recombinant l-phenylalanine ammonia lyase from *Rhodosporidium toruloides* as a potential anticancer agent. Biotechnology and Applied Biochemistry, 60(3): 316-322. https://doi.org/10.1002/bab.1089

Bernards MA, Ellis BE (1991): Phenylalanine ammonia-lyase from tomato cell cultures inoculated with *Verticillium albo-atrum*. Plant physiology, 97(4): 1494-1500. https://doi.org/10.1104/pp.97.4.1494.

Blau N, Longo N (2015): Alternative therapies to address the unmet medical needs of patients with phenylketonuria. Expert opinion on pharmacotherapy, *16*(6): 791-800. https://doi.org/10.1517/14656566.2015.1013030.

Blau N, Duran M, Gibson KM, Dionisi-Vici C (Eds) (2014): Physician's guide to the diagnosis, treatment, and follow-up of inherited metabolic diseases (pp. 247-264). Berlin/Heidelberg: Springer. https://doi.org/10.1007/978-3-642-40337-8.

Bortoluzzi VT, Brust L, Preissler T, de Franceschi ID, Wannmacher CMD (2019): Creatine plus pyruvate supplementation prevents oxidative stress and phosphotransfer network disturbances in the brain of rats subjected to chemically-induced phenylketonuria. Metabolic Brain Disease, 34(6): 1649-1660. https://doi.org/10.1007/s11011-019-00472-7.

Bosch AM, Burlina A, Cunningham A, Bettiol E, Moreau-Stucker F, Koledova E, Regnault A (2015): Assessment of the impact of phenylketonuria and its treatment on quality of life of patients and parents from seven European countries. Orphanet journal of rare diseases, 10(1): 1-14. https://doi.org/10.1186/s13023-015-0294-x

Brown CS, Lichter-Konecki U (2016): Phenylketonuria (PKU): A problem solved?. Molecular genetics and metabolism reports, 6: 8-12. https://doi.org/10.1016/j.ymgmr.2015.12.004

Carroll GC (1991): Beyond pest deterrence—alternative strategies and hidden costs of endophytic mutualisms in vascular plants. In Microbial ecology of leaves (pp. 358-375). Springer, New York, NY. https://doi.org/10.1007/978-1-4612-3168-4_18.

Centerwall SA, Centerwall WR (2000): The discovery of phenylketonuria: the story of a young couple, two retarded children, and a scientist. Pediatrics, 105(1): 89-103. https://doi.org/10.1542/peds.105.1.89.

Chakraborty R, Lidsky AS, Daiger SP, Güttler F, Sullivan S, Dilella AG, Woo SL (1987): Polymorphic DNA haplotypes at the human phenylalanine hydroxylase locus and their relationship with phenylketonuria. Human genetics, 76(1): 40-46. https://doi.org/10.1007/BF00283048.

Chand K, Shah S, Sharma J, Paudel MR, Pant B (2020): Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata*. Plant signaling and behavior, 15(5): 1744294. https://doi.org/10.1080/15592324.2020.1744294.

Cui JD, Qiu JQ, Fan XW, Jia SR, Tan ZL (2014): Biotechnological production and applications of microbial phenylalanine ammonia lyase: a recent review. Critical reviews in biotechnology, 34(3): 258-268. https://doi.org/10.3109/07388551.2013.791660.

Devi A, Naushad SM (2004): Newborn screening in India. The Indian Journal of Pediatrics, 71(2): 157-160. https://doi.org/10.1007/BF02723099

Devi NN, Prabakaran JJ (2014): Bioactive metabolites from an endophytic fungus *Penicillium* sp. isolated from *Centella asiatica*. Curr Res Environ Appl Mycol, 4(1): 34-43. Doi 10.5943/cream/4/1/3

Diaz P, Jeong SC, Lee S, Khoo C, Koyyalamudi SR (2012): Antioxidant and anti-inflammatory activities of selected medicinal plants and fungi containing phenolic and flavonoid compounds. Chinese Medicine, 7(1): 1-9. https://doi.org/10.1186/1749-8546-7-26.

Ding Z, Georgiev P, Thöny B (2006): Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer. Gene therapy, 13(7): 587-593. https://doi.org/10.1038/sj.gt.3302684.

Dixon, RA, Paiva NL (1995): Stress-induced phenylpropanoid metabolism. The plant cell, 7(7): 1085. https://doi.org/10.1105/tpc.7.7.1085

Eisensmith RC, Woo SL (1992): Molecular basis of phenylketonuria and related hyperphenylalaninemias: mutations and polymorphisms in the human phenylalanine hydroxylase gene. Human mutation, 1(1): 13-23. https://doi.org/10.1002/humu.1380010104.

Fill TP, Silva BFD, Rodrigues-Fo E (2010): Biosynthesis of phenylpropanoid amides by an endophytic *Penicillium brasilianum* found in root bark of *Melia azedarach*. Journal of Microbiology and Biotechnology, 20(3): 622-629. https://doi.org/10.4014/jmb.0908.08018.

Fiske MJ, Kane JF (1984): Regulation of phenylalanine biosynthesis in *Rhodotorula glutinis*. Journal of bacteriology, 160(2): 676-681. https://doi.org/10.1128/jb.160.2.676-681.1984.

Fritz RR, Hodgins DS, Abell CW (1976): Phenylalanine ammonia-lyase. Induction and purification from yeast and clearance in mammals. Journal of Biological Chemistry, 251(15): 4646-4650. https://doi.org/10.1016/S0021-9258(17)33251-9.

Gautam AK, Avasthi S (2019): Fungal endophytes: potential biocontrol agents in agriculture. In Role of plant growth promoting microorganisms in sustainable agriculture and nanotechnology (pp. 241-283). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-817004-5.00014-2.

Gilbert HJ, Tully MICHAEL (1982): Synthesis and degradation of phenylalanine ammonia-lyase of *Rhodosporidium toruloides*. Journal of bacteriology, 150(2): 498-505. https://doi.org/10.1128/jb.150.2.498-505.1982.

Gouda S, Das G, Sen S K, Shin HS, Patra JK (2016): Endophytes: a treasure house of bioactive compounds of medicinal importance. Frontiers in microbiology, 7: 1538. https://doi.org/10.3389/fmicb.2016.01538.

Gunatilaka AL (2006): Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. Journal of natural products, 69(3): 509-526. https://doi.org/10.1021/np058128n.

Guo J, Wang MH (2009): Characterization of the phenylalanine ammonia-lyase gene (SlPAL5) from tomato (*Solanum lycopersicum* L.). Molecular biology reports, 36(6): 1579-1585. https://doi.org/10.1007/s11033-008-9354-9

Gupta S, Chaturvedi P, Kulkarni MG, Van Staden J (2020): A critical review on exploiting the pharmaceutical potential of plant endophytic fungi. Biotechnology advances, 39: 107462. https://doi.org/10.1016/j.biotechadv.2019.107462.

Hanley WB, Linsao L, Davidson W, Moes CAF (1970): Malnutrition with early treatment of phenylketonuria. Pediatric research, 4(4): 318-327. https://doi.org/10.1203/00006450-197007000-00002.

Harding CO, Blau N (2010): Advances and challenges in phenylketonuria. Journal of inherited metabolic disease, 33(6): 645-648. https://doi.org/10.1007/s10545-010-9247-7.

Havir EA, Hanson KR (1973): L-phenylalanine ammonia-lyase (maize and potato). Evidence that the enzyme is composed of four subunits. Biochemistry, 12(8): 1583-1591. https://doi.org/10.1021/bi00732a019.

Hillert A, Anikster Y, Belanger-Quintana A, Burlina A, Burton BK, Carducci C, Blau N (2020): The genetic landscape and epidemiology of phenylketonuria. The American Journal of Human Genetics, 107(2): 234-250. https://doi.org/10.1016/j.ajhg.2020.06.006.

Hoskins J, Jack G, Peiris RD, Starr DT, Wade H, Wright E, Stern J (1980): Enzymatic control of phenylalanine intake in phenylketonuria. The Lancet, 315(8165): 392-394. https://doi.org/10.1016/S0140-6736(80)90944-7

Hydery T, Coppenrath VA (2019): A comprehensive review of pegvaliase, an enzyme substitution therapy for the treatment of phenylketonuria. Drug target insights, 13: 1177392819857089. https://doi.org/10.1177%2F1177392819857089

Hyun MW, Yun YH, Kim JY, Kim SH (2011): Fungal and plant phenylalanine ammonia-lyase. Mycobiology, 39(4): 257-265. https://doi.org/10.5941/MYCO.2011.39.4.257.

Ji HF, Li XJ, Zhang HY (2009): Natural products and drug discovery: can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? EMBO reports, 10(3): 194-200. https://doi.org/10.1038/embor.2009.12.

Kalghatgi KK, Subba Rao PV (1975): Microbial L-phenylalanine ammonia-lyase. Purification, subunit structure and kinetic properties of the enzyme from *Rhizoctonia solani*. Biochemical Journal, 149(1): 65-75. https://doi.org/10.1042/bj1490065.

Kawatra A, Dhankhar R, Mohanty A, Gulati P (2020): Biomedical applications of microbial phenylalanine ammonia lyase: current status and future prospects. Biochimie, 177: 142-152. https://doi.org/10.1016/j.biochi.2020.08.009.

Keller NP, Turner G, Bennett JW (2005): Fungal secondary metabolism—from biochemistry to genomics. Nature Reviews Microbiology, 3(12): 937-947. https://doi.org/10.1038/nrmicro1286.

Kjer J, Debbab A, Aly AH, Proksch P (2010): Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. Nature protocols, 5(3): 479-490. https://doi.org/10.1038/nprot.2009.233.

Knox WE (1960): An evaluation of the treatment of phenylketonuria with diets low in phenylalanine. Pediatrics, 26(1): 1-11. https://doi.org/10.1542/peds.26.1.1.

Kong JQ (2015): Phenylalanine ammonia-lyase, a key component used for phenylpropanoids production by metabolic engineering. RSC advances, 5(77): 62587-62603. https://doi.org/10.1039/C5RA08196C.

Kruger NJ (2009): The Bradford method for protein quantitation. The protein protocols handbook, 17-24. https://doi.org/10.1007/978-1-59745-198-7_4.

Kukil K, Lindberg P (2022): Expression of phenylalanine ammonia lyases in *Synechocystis sp*. PCC 6803 and subsequent improvements of sustainable production of phenylpropanoids. Microbial cell factories, 21(1): 1-16. https://doi.org/10.1186/s12934-021-01735-8.

Levy HL (2021): Robert guthrie and the trials and tribulations of newborn screening. International Journal of Neonatal Screening, 7(1): 5. https://doi.org/10.3390/ijns7010005.

Levy HL, Sarkissian CN, Scriver CR (2018): Phenylalanine ammonia lyase (PAL): From discovery to enzyme substitution therapy for phenylketonuria. Molecular Genetics and Metabolism, 124(4): 223-229. https://doi.org/10.1016/j.ymgme.2018.06.002

Liarzi O, Bucki P, Braun Miyara S, Ezra D (2016): Bioactive volatiles from an endophytic Daldinia cf. concentrica isolate affect the viability of the plant parasitic nematode Meloidogyne javanica. PLoS One, 11(12): e0168437. https://doi.org/10.1371/journal.pone.0168437

Lichter-Konecki U, Vockley J (2019): Phenylketonuria: current treatments and future developments. Drugs, 79(5): 495-500. https://doi.org/10.1007/s40265-019-01079-z.

Lidsky AS, Law ML, Morse HG, Kao FT, Rabin M, Ruddle FH, Woo S. (1985):Regional mapping of the phenylalanine hydroxylase gene and the phenylketonuria locus in the human genome. Proceedings of the National Academy of Sciences, 82(18): 6221-6225. https://doi.org/10.1073/pnas.82.18.6221

Liu X, Zhou ZY, Cui JL, Wang ML, Wang JH (2021): Biotransformation ability of endophytic fungi: from species evolution to industrial applications. Applied Microbiology and Biotechnology, 105(19): 7095-7113. https://doi.org/10.1007/s00253-021-11554-x

Longo N, Harding CO, Burton BK, Grange DK, Vockley J, Wasserstein M, Sile S (2014): Single-dose, subcutaneous recombinant phenylalanine ammonia lyase conjugated with polyethylene glycol in adult patients with phenylketonuria: an open-label, multicentre, phase 1 dose-escalation trial. The Lancet, 384(9937): 37-44. https://doi.org/10.1016/S0140-6736(13)61841-3

Lu C, Shen Y (2004): Harnessing the potential of chemical defenses from antimicrobial activities. BioEssays, 26(7): 808-813. https://doi.org/10.1002/bies.20060

MacDonald A, Harris G, Rylance G, Asplin D, Booth I W (1997): Abnormal feeding behaviours in phenylketonuria. Journal of Human Nutrition and Dietetics, 10(3): 163-170. https://doi.org/10.1046/j.1365-277X.1997.00050.x

Manganyi MC, Ateba CN (2020): Untapped potentials of endophytic fungi: A review of novel bioactive compounds with biological applications. Microorganisms, 8(12): 1934. https://doi.org/10.3390/microorganisms8121934.

Marusich WC, Jensen RA, Zamir LO (1981): Induction of L-phenylalanine ammonia-lyase during utilization of phenylalanine as a carbon or nitrogen source in *Rhodotorula glutinis*. Journal of bacteriology, 146(3): 1013-1019. https://doi.org/10.1128/jb.146.3.1013-1019.1981

Matalon R, Michals-Matalon K, Bhatia G, Grechanina E, Novikov P, McDonald JD, Guttler F (2006): Large neutral amino acids in the treatment of phenylketonuria (PKU). Journal of inherited metabolic disease, 29(6): 732-738. https://doi.org/10.1007/s10545-006-0395-8

Meena H, Hnamte S, Siddhardha B (2019): Secondary metabolites from endophytic fungi: chemical diversity and application. In Advances in Endophytic Fungal Research (pp. 145-169). Springer, Cham. https://doi.org/10.1007/978-3-030-03589-1_7.

Mehta YR, Brogin RL (2000): Phytotoxicity of a culture filtrate produced by *Stemphylium solani* of cotton. Plant disease, 84(8): 838-842. https://doi.org/10.1094/PDIS.2000.84.8.838

Meshram V, Kapoor N, Saxena, S. (2013): *Muscodor kashayum* sp. nov.–a new volatile anti-microbial producing endophytic fungus. Mycology, 4(4): 196-204. https://doi.org/10.1080/21501203.2013.877990.

Mishra S, Bhardwaj P, Sharma S (2022): Metabolomic insights into endophyte-derived bioactive compounds. Frontiers in microbiology, 416. https://doi.org/10.3389/fmicb.2022.835931.

Mitchell JJ, Trakadis YJ, Scriver CR (2011): Phenylalanine hydroxylase deficiency. Genetics in medicine, 13(8): 607-617. https://doi.org/10.1097/GIM.0b013e3182141b48

Moens AL, Kass DA (2006): Tetrahydrobiopterin and cardiovascular disease. Arteriosclerosis, thrombosis, and vascular biology, 26(11): 2439-2444. https://doi.org/10.1161/01.ATV.0000243924.00970.cb

Moffitt MC, Louie GV, Bowman ME, Pence J, Noel JP, Moore BS (2007): Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. Biochemistry, 46(4): 1004-1012. https://doi.org/10.1021/bi061774g

Nishad JH, Singh A, Gautam VS, Kumar D, Kumar J, Kharwar RN (2019): Endophytic fungi: A cryptic fountainhead for biodiversity, functional metabolites, host stress tolerance, and myco-mediated nanoparticles (Nps) synthesis. In Endophytes and Secondary Metabolites (pp. 541-569). Springer, Cham. https://doi.org/10.1007/978-3-319-90484-9_18.

Ogata K, Uchiyama K, Yamada H (1967): Metabolism of aromatic amino acid in microorganisms: part I. Formation of cinnamic acid from phenylalanine. Agricultural and Biological Chemistry, 31(2): 200-206. https://doi.org/10.1080/00021369.1967.10858795

Patil RH, Patil MP, Maheshwari VL (2016): Bioactive secondary metabolites from endophytic fungi: a review of biotechnological production and their potential applications. Studies in natural products chemistry, 49: 189-205. https://doi.org/10.1016/B978-0-444-63601-0.00005-3

Pereira de Sousa I, Gourmel C, Berkovska O, Burger M, Leroux JC (2020): A microparticulate based formulation to protect therapeutic enzymes from proteolytic digestion: phenylalanine ammonia lyase as case study. Scientific reports, 10(1): 1-11. https://doi.org/10.1038/s41598-020-60463-y

Pieters L, Vlietinck AJ (2005): Bioguided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds? *Journal* of ethnopharmacology, 100(1-2): 57-60. https://doi.org/10.1016/j.jep.2005.05.029.

Quinn J, Georgiadis A, Lewis HB, Jurecki E (2022): Measuring Burden of Illness in Phenylketonuria (PKU): Development of the PKU Symptom Severity and Impacts Scale as a Robust Patient-Reported Outcome. Advances in Therapy, 39(2): 971-991. https://doi.org/10.1007/s12325-021-01986-2.

Rausell D, García-Blanco A, Correcher P, Vitoria I, Vento M, Cháfer-Pericás C (2019): Newly validated biomarkers of brain damage may shed light into the role of oxidative stress in the pathophysiology of neurocognitive impairment in dietary restricted phenylketonuria patients. Pediatric research, 85(2): 242-250. https://doi.org/10.1038/s41390-018-0202-x.

Ribas G S, Sitta A, Wajner M, Vargas CR (2011): Oxidative stress in phenylketonuria: what is the evidence?. Cellular and molecular neurobiology, 31(5): 653-662. https://doi.org/10.1007/s10571-011-9693-2.

Rodriguez RJ, White Jr JF, Arnold AE, Redman ARA (2009): Fungal endophytes: diversity and functional roles. New phytologist, 182(2): 314-330. https://doi.org/10.1111/j.1469-8137.2009.02773.x.

Rosler J, Krekel F, Amrhein N, Schmid J (1997): Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. Plant physiology, 113(1): 175-179. https://doi.org/10.1104/pp.113.1.175.

Rustaiyan A, Masoudi S, Eghbali H, Salehi HR (2011): Phenological Variation on Essential Oil Composition of Stachys inflataBenth. From Iran. Journal of Essential Oil Bearing Plants, 14(6): 679-683. https://doi.org/10.1080/0972060X.2011.10643989.

Sarkissian CN, Shao Z, Blain F, Peevers R, Su H, Heft R, Scriver CR (1999): A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase. Proceedings of the National Academy of Sciences, 96(5): 2339-2344. https://doi.org/10.1073/pnas.96.5.2339.

Schulz B, Boyle C, Draeger S, Römmert AK, Krohn K (2002): Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological research, 106(9): 996-1004. https://doi.org/10.1017/S0953756202006342.

Schuster B, Retey J (1995): The mechanism of action of phenylalanine ammonia-lyase: the role of prosthetic dehydroalanine. Proceedings of the National Academy of Sciences, 92(18): 8433-8437. https://doi.org/10.1073/pnas.92.18.8433.

Scriver CR (2007): The PAH gene, phenylketonuria, and a paradigm shift. Human mutation, 28(9): 831-845. https://doi.org/10.1002/humu.20526.

Sharma D, Pramanik A, Agrawal PK (2016): Evaluation of bioactive secondary metabolites from endophytic fungus *Pestalotiopsis neglecta* BAB-5510 isolated from leaves of *Cupressus torulosa* D. Don. 3 Biotech, 6(2): 1-14. https://doi.org/10.1007/s13205-016-0518-3

Shoraka HR, Haghdoost AA, Baneshi MR, Bagherinezhad Z, Zolala F (2020): Global prevalence of classic phenylketonuria based on Neonatal Screening Program Data: systematic review and meta-analysis. Clinical and experimental pediatrics, 63(2): 34. https://doi.org/10.3345/kjp.2019.00465.

Sidell N, Hao L, Pasquali M, McDonald JD (2009): Carcinogenic effects in a phenylketonuria mouse model. Plos one, 4(1): e4292. https://doi.org/10.1371/journal.pone.0004292.

Sieber TN (2007): Endophytic fungi in forest trees: are they mutualists?. Fungal biology reviews, 21(2-3): 75-89. https://doi.org/10.1016/j.fbr.2007.05.004.

Singh SK, Singh VK, Kumar D, Gond DP, Kumar A (2022): Endophytes of Medicinal Plants: Diversity and Bioactivity. In Bacterial Endophytes for Sustainable Agriculture and Environmental Management (pp.117-128). Springer, Singapore. https://doi.org/10.1007/978-981-16-4497-9_6.

Sirtori LR, Dutra-Filho CS, Fitarelli D, Sitta A, Haeser A, Barschak AG, Vargas CR (2005): Oxidative stress in patients with phenylketonuria. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1740(1): 68-73. https://doi.org/10.1016/j.bbadis.2005.02.005.

Srinivasan K, Jagadish LK, Shenbhagaraman R, Muthumary J (2010): Antioxidant activity of endophytic fungus Phyllosticta sp. isolated from Guazuma tomentosa. Journal of Phytology, 2(6).

Stauffer CE (1975): A linear standard curve for the Folin Lowry determination of protein. Analytical biochemistry, 69(2): 646-648. https://doi.org/10.1016/0003-2697(75)90172-4.

Stepien KM, Heaton R, Rankin S, Murphy A, Bentley J, Sexton D, Hargreaves IP (2017): Evidence of oxidative stress and secondary mitochondrial dysfunction in metabolic and non-metabolic disorders. Journal of clinical medicine, 6(7): 71. https://doi.org/10.3390/jcm6070071.

Stierle A, Strobel G, Stierle D (1993): Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science, 260(5105): 214-216. https://doi.org/10.1126/science.8097061

Strisciuglio P, Concolino D (2014): New strategies for the treatment of phenylketonuria (PKU). Metabolites, 4(4): 1007-1017. https://doi.org/10.3390/metabo4041007

Strobel G, Daisy B (2003): Bioprospecting for microbial endophytes and their natural products. Microbiology and molecular biology reviews, 67(4): 491-502. https://doi.org/10.1128/MMBR.67.4.491-502.2003.

Surtees R, Blau N (2000): The neurochemistry of phenylketonuria. European journal of pediatrics, 159(2): S109-S113. https://doi.org/10.1007/PL00014370.

Talukdar R, Padhi S, Rai AK, Masi M, Evidente A, Jha DK, Tayung K (2021): Isolation and Characterization of an Endophytic Fungus *Colletotrichum coccodes* Producing Tyrosol From *Houttuynia cordata* Thunb. Using ITS2 RNA Secondary Structure and Molecular Docking Study. Frontiers in Bioengineering and Biotechnology, 9: 650247. https://doi.org/10.3389/fbioe.2021.650247.

Thöny B, Blau N (2006): Mutations in the BH4-metabolizing genes GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, sepiapterin reductase, carbinolamine-4a-dehydratase, and dihydropteridine reductase. Human mutation, 27(9): 870-878. https://doi.org/10.1002/humu.20366.

van Spronsen FJ, Blau N, Harding C, Burlina A, Longo N, Bosch AM (2021): Phenylketonuria. Nature Reviews Disease Primers, 7(1): 1-19. https://doi.org/10.1038/s41572-021-00267-0.

Van Vliet D, van Wegberg AM, Ahring K, Bik-Multanowski M, Blau N, Bulut FD, van Spronsen FJ (2018): Can untreated PKU patients escape from intellectual disability? A systematic review. Orphanet journal of rare diseases, 13(1): 1-6. https://doi.org/10.1186/s13023-018-0890-7.

Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A (2015): The importance of the microbiome of the plant holobiont. New Phytologist, 206(4): 1196-1206. https://doi.org/10.1111/nph.13312.

Waisbren S E, Noel K, Fahrbach K, Cella C, Frame D, Dorenbaum A, Levy H (2007): Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis. Molecular genetics and metabolism, 92(1-2): 63-70. https://doi.org/10.1016/j.ymgme.2007.05.006.

Wang S, Zhang S, Zhou T, Zeng J, Zhan J (2013): Design and application of an in vivo reporter assay for phenylalanine ammonia-lyase. Applied microbiology and biotechnology, 97(17): 7877-7885. https://doi.org/10.1007/s00253-013-5122-4

Wardatun S, Rustiani E, Alfiani N, Rissani D (2017): Study effect type of extraction method and type of solvent to cinnamaldehyde and trans-cinnamic acid dry extract cinnamon (*Cinnamomum burmanii* Blume). Journal of Young Pharmacists, 9(1s): S49. https://dx.doi.org/10.5530/jyp.2017.1s.13.

Wen J, Okyere SK, Wang S, Wang J, Xie L, Ran Y, Hu Y (2022): Endophytic fungi: an effective alternative source of plant-derived bioactive compounds for pharmacological studies. Journal of Fungi, 8(2): 205. https://doi.org/10.3390/jof8020205.

Westphal KR, Heidelbach S, Zeuner EJ, Riisgaard-Jensen M, Nielsen ME, Vestergaard SZ, Sondergaard TE (2021): The effects of different potato dextrose agar media on secondary metabolite production in Fusarium. International Journal of Food Microbiology, 347: 109171. https://doi.org/10.1016/j.ijfoodmicro.2021.109171.

Wick JF, Willis JE (1982): Phenylalanine-dependent de novo synthesis of phenylalanine ammonia-lyase from *Rhodotorula glutinis*. Archives of Biochemistry and Biophysics, 216(2): 385-391. https://doi.org/10.1016/0003-9861(82)90226-0.

Williams RA, Mamotte CD, Burnett JR (2008): Phenylketonuria: an inborn error of phenylalanine metabolism. The Clinical biochemist. Reviews, 29(1): 31–41.

Woolf LI, Adams J (2020): The Early History of PKU. International Journal of Neonatal Screening, 6(3): 59. https://doi.org/10.3390/ijns6030059.

Xiang L, Moore BS (2005): Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. Journal of bacteriology, 187(12): 4286-4289. https://doi.org/10.1128/JB.187.12.4286-4289.2005.

Yang J, Tao R, Wan LI, Song L, Wang Y, Gong C, Wu Q (2019): Thermosensitive micelles encapsulating phenylalanine ammonia lyase act as a sustained and efficacious therapy against colorectal cancer. Journal of biomedical nanotechnology, *15*(4): 717-727. https://doi.org/10.1166/jbn.2019.2734

Curiginal

Document Information

Analyzed document	Anshu_Final draft 04 (2).docx (D141390702)
Submitted	2022-06-28 14:09:00
Submitted by	Shreya Gupta
Submitter email	sgupta_msc18@thapar.edu
Similarity	7%
Analysis address	sgupta_msc18.thapar@analysis.urkund.com

Sources included in the report

W	URL: https://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-13-S3-S1 Fetched: 2022-06-28 13:13:00	1
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2423317/ Fetched: 2019-10-11 15:07:10	9
W	URL: https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.652.8056&rep=rep1&type=pdf Fetched: 2022-01-17 17:08:37	3
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3385129/ Fetched: 2020-12-21 00:18:29	6
W	URL: https://pubmed.ncbi.nlm.nih.gov/17240984/ Fetched: 2022-06-28 09:06:54	1
W	URL: https://pubmed.ncbi.nlm.nih.gov/34826353/ Fetched: 2022-06-28 14:09:37	1
W	URL: https://www.sciencedirect.com/science/article/abs/pii/S0300908420301954 Fetched: 2022-04-15 03:32:24	4
W	URL: https://www.nature.com/articles/s41598-020-60463-y Fetched: 2020-09-01 09:26:32	2
W	URL: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0173269 Fetched: 2020-10-13 13:40:02	4
W	URL: https://www.nature.com/articles/gim2011114 Fetched: 2020-03-24 06:25:36	1
W	URL: https://en.wikipedia.org/wiki/Phenylalanine_hydroxylase Fetched: 2020-03-20 14:42:24	1
W	URL: https://www.jmb.or.kr/journal/download_pdf.php?doi=10.4014/jmb.0908.08018 Fetched: 2022-06-28 14:09:56	1