Epigenetic modifications of *Xylaria psidii* for enhancing Resveratrol production

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled **"Epigenetic modifications of *Xylaria psidii* for enhancing Resveratrol production"** is being submitted by SHREYA KALIA, Roll no.-301601022 in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

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DECLARATION

I hereby declare that the work being presented in the thesis entitled **"Epigenetic modifications of *Xylaria psidii* for enhancing Resveratrol production"** in partial fulfilment of the requirements for the award of degree of Master in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala is my own laboratory work carried out during the period of January 2018 to June 2018, under the supervision of **Dr. Sanjai Saxena,** Professor, Department of Biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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ABBREVIATIONS

S.No.	Abbreviation	Full form
1.	µg	Micro Gram
2.	µl	Micro Litre
3.	µM	Micro Molar
4.	AZA	5-Azacytidine
5.	DNA	Deoxyribonucleic Acid
6.	dNTP	Deoxynucleotide Triphosphate
7.	DPPH	1,1-Diphenyl-2-Picrylhydrazyl
8.	EDTA	Ethylene Diamine Tetra Acetic Acid
9.	Et Br	Ethidium Bromide
10.	FRAP	Ferric Ion Reducing Assay
11.	HPLC	High Performance Liquid Chromatography
12.	ITS	Internal Transcribed Spacer
13.	L	Litre
14.	LSU	Large Subunit
15.	MEA	Malt Extract Agar
16.	mg	Milli Gram
17.	MHA	Muller Hinton Agar
18.	MHB	Muller Hinton Broth
19.	ml	Millilitre
20.	ng	Nano Gram
21.	PCR	Polymerase Chain Reaction
22.	PDA	Potato Dextrose Agar
23.	PDB	Potato Dextrose Broth
24.	pH	Potential Of Hydrogen
25.	PLA	Pine Leaf Agar
26.	rpm	Revolutions per minute
27.	RT	Room Temperature

28.	SAHA	Suberoylanilide Hydroxamic Acid
29.	TAE	Tris Acetate EDTA
30.	TFC	Total Flavanoid Content
31.	TPC	Total Phenoliccontent
	TEAC	Trolox Equivalent Antioxidant Capacity Assay
32.	UV	Ultra Violet
33.	WA	Water Agar

Nowadays endophytic fungi are known for their ability to produce various industrially important molecules such as resveratrol which has brought in notice the need to employ strategies that can help increase the yield of resveratrol. Resveratrol is naturally occurring stilbenes, which has high demand due to its multifarious applications in medicinal, nutraceutical and cosmeceutical industries.

Endophytic fungi lives in a symbiotic relationship with the host plants and adopts various mechanisms, analogous to the host plants for example, endophytic fungi exhibit an interesting property of producing compounds analogous to those present in the host plant. Endophytic fungi help host plant to combat abiotic stresses by enhancing its defence mechanism and by secreting secondary metabolites and are thus an essential source for the production of various biologically important molecules. This brought in notice the need to employ strategies that can help increase the yield of these molecules.

The current study mainly focuses on strategies to enhance the fungal potential to produce resveratrol via the activation of the cryptic biosynthetic pathway with their particular interest in antioxidant application. The endophytic fungus *Xylaria psidii* was isolated from the surface sterilized leaf of *Vitis vinifera*. With the help of HPLC analysis it is found that resveratrol concentration was maximum and enhanced in case of treatment with 5 µm SAHA (52.32µg/ml) and by 10 µm AZA (48.94 µg/ml) followed by 10 µm SAHA (41.10 µg/ml) and 5 µm AZA (37.72µg/ml). After treatment with different concentration of epigenetic modifiers such as HDAC inhibitors (SAHA) and DMNTs (AZA) inhibitors a significant increase in antioxidant potential was obtained. In case of DPPH increase in scavenging potential was found as compared to wild strain. Treatment of fungal culture with 5 µm SAHA and by 10 µm AZA was showing strong antioxidant potential among all the epigenetic variants as compared to wild strain. In case of TEAC also same trend as in case of DPPH was obtained, whereas in case of FRAP, TPC and TFC the increment was observed in case of modification done using 3 µm AZA and SAHA , 5 µm AZA and SAHA, 10 µm AZA and SAHA only as compared to #22(P)VVLPM control.

All these finding advocate the future use of these epigenetic modifiers for enhancement of secondary metabolite (resveratrol), discovery of new bioactive natural cryptic metabolite as well as fungal strain improvement.

Keywords: endophytic fungi, resveratrol, epigenetic modification, HPLC, antioxidant activity

Chapter – 1

INTRODUCTION

Resveratrol or 3,5,4'-trihydroxy-trans-stilbene (RESV) is a natural polyphenol of stilbene family which is characterised by two benzene rings linked via isopropyl moiety separated by a double bond (Kasiotis *et al.*,2013). Resveratrol was first discovered in 1940's from the roots of *Veratrum grandifolium* (White helleborne) and subsequently in 1960's from *Polygonum cuspidatum* traditionally used as a component of Chinese and Japanese medicines (Lee *et al.*, 2012; Zhuang *et al.*, 2008). However the medicinal properties of Resveratrol came into limelight when it was observed that the French people exhibited very less symptoms of coronary heart diseases despite French cusine containing large amount of saturated fats. This observation was termed as "French paradox". Subsequently the presence of Resveratrol in the red wine was attributed for the cardioprotective action (Bradamante *et al.*, 2004). Further studies found that Resveratrol posses a wide range of pharmacological & medicinal properties like anti-cancerous (Jang *et al.*, 1997; Kundu and Surh, 2008), anti-inflammatory (Donnelly *et al.*, 2004), anti- diabetic (Sameer *et al.*, 2007), anti-ageing (Pearson *et al.*, 2008), anti-microbial (Nawrocki *et al.*, 2014) and vasodilatory properties (Beher *et al.*, 2000). RESV posses a strong antifungal activity against *Botrytis cinerea* (Langcake and Pryce,1976). Therefore Resveratrol has been a molecule of high appeal to the researchers since its discovery. The other sources of Resveratrol comprises of grapes, peanuts, chocolate, tea and other berry species (Burns *et al.*, 2002; Tokusoglu *et al.*, 2005; Counet *et al.*, 2006). Currently RESV is being commercially extracted from the roots of *Polygonum cupidatum*, which is expensive as well as time consuming process. Moreover, this may also have aecological impact since extensive annihilation of the plant is carried out. Many techniques have been employed for enhancing secondary metabolite production like plant cell culture technology wherein cells are cultivated in bioreactors to produce secondary metabolite (Donnez *et al.*, 2011). As there is global demand for RESV molecule, it has become imperative to explore alternative methods of bulk production of RESV.

An endophyte is an organism like bacteria or fungi which resides asymptomatically in the host plant tissues and derives many benefits by living in mutualistic relationship with plants (Strobel *et al.*, 1996). Endophyte is a novel and abundant microorganism resource, possessing the unique ability to manufacture bioactive compounds analogous to their host plants. In the past two decades researchers have focused on investigating diverse nature of endophytic fungi, relationship between endophytic fungi and their host plants, natural bioactive compounds produced by the endophytic fungi. Endophytic fungi exhibit an interesting property of producing compounds analogous to those present in the host plant as a result endophytes are today recognised as an essential source for the production of various medicinal molecules of plant origin for direct use as drug (Zhuang *et al.*, 2004). Various techniques are these days employed on endophytes to enhance the production of secondary molecule like pathogen attack, UV irradiation, genetic modification using *STS* genes or *rol* genes (Kobayashi *et al., 2000;* Palazon *et al., 1998*), using biosynthetic precursor or chemical elicitors.

Epigenetic regulation of gene transcription using chemical elicitors can be applied to wide range of fungi without any prior knowledge of the genome sequence. Epigenetic modification like histone deactivation and DNA methylation control the putative biosynthesis of gene clusters (Aghchech *et al.*, 2015). Therefore epigenetics can be utilised as a strategy for modulating the gene expression and enhancing the production of desired secondary metabolite. Chemical elicitors like 5-azacytidine (AZA) and suberoylanilide hydroxamic acid (SAHA) which causes hypomethylation and histone deacetylation respectively, have been used to target biosynthetic pathways. AZA induces hypomethylation of the DNA by inhibiting DNA methyltransferase (DNMT) enzyme which catalyse the transfer of methyl group to the DNA apart from getting incorporated into genetic material which leads to the disassembly of polyribosomes, defective methylation and acceptor function of tRNA thereby inhibiting production of certain proteins (Cherblanc *et al.*, 2013). SAHA acts as histone deacetylase inhibitor which by acting as a chelator for zinc ions present in active site of the histone deacetylase leads to the accumulation of the acetylated histones and acetylated protein further inhibiting the gene expression (Richon, 2006).

We have previously reported that *Xylaria psidii* has been found to produce RESV. In present study we intent to develop a stable epigenetic variant of *Xylaria psidii* having high titre value of RESV.

Chapter – 2

REVIEW OF LITERATURE

3,5,4'-trihydroxy-trans-stilbene commonly known as Resveratrol is a phytoalexin molecule found in plants, belonging to stilbene family of polyphenols. Resveratrol is characterised by two benzene rings linked via isopropyl moiety separated by a double bond (Kasiotis *et al.*, 2003*).* It exists in *cis* and *trans* isomeric forms (*Figure-2.1*). Condensation of *p*-courmaroyl-CoA and malonyl-CoA leads to the formation of Resveratrol in Shikimate pathway (Figure-2.2).

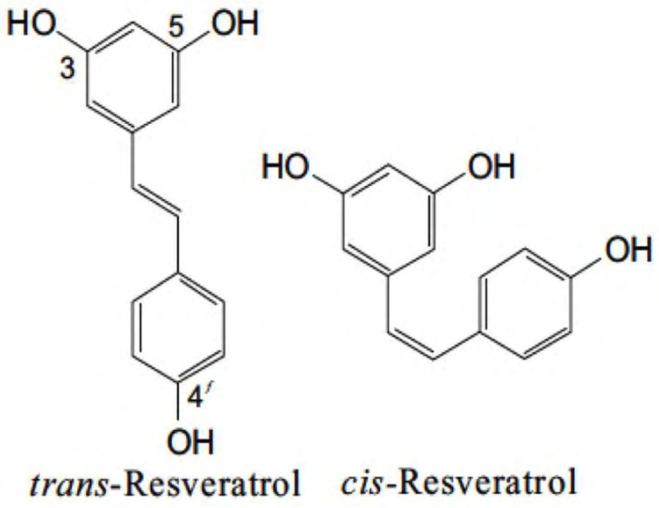

Figure-2.1: *cis* and *trans* Resveratrol isomers (Rege *et al.*,2014)

Resveratrol was first isolated from *Veratrum grandiflorum*, a medicinal plant in 1940's (Lancon *et al.*, 2007) and *Polygonum capsidatum* has also been reported as a source of production of Resveratrol in 1960's. Both these plants are traditionally used in Chinese and Japenese medication. Various families of plant kingdom have been reported to be prolific producers of Resveratrol viz. *Vitaceae, Pinaceae, Liliaceae, Cyeraceae*, *Gnetaceae*, *Moraceae* (Langcake and Pyre, 1976). Grape skin (Langcake and Pyre, 1976), seeds (Pezet and Cuenat, 1996), stem (Bavaresco *et al.*,1997) and leaves of grape plant have a resveratrol content but mainly grape skin has the highest resveratrol content. The resveratrol content in grape skin can be related to the grape variety and the stage of ripening. The first use of grape as a medicine can be dated back to as long as 2000 years, an ayurvedic preparation "darakchasava" which acts as cardiotonic is prepared from *Vitis vinifera* (Paul *et al.*, 1999).

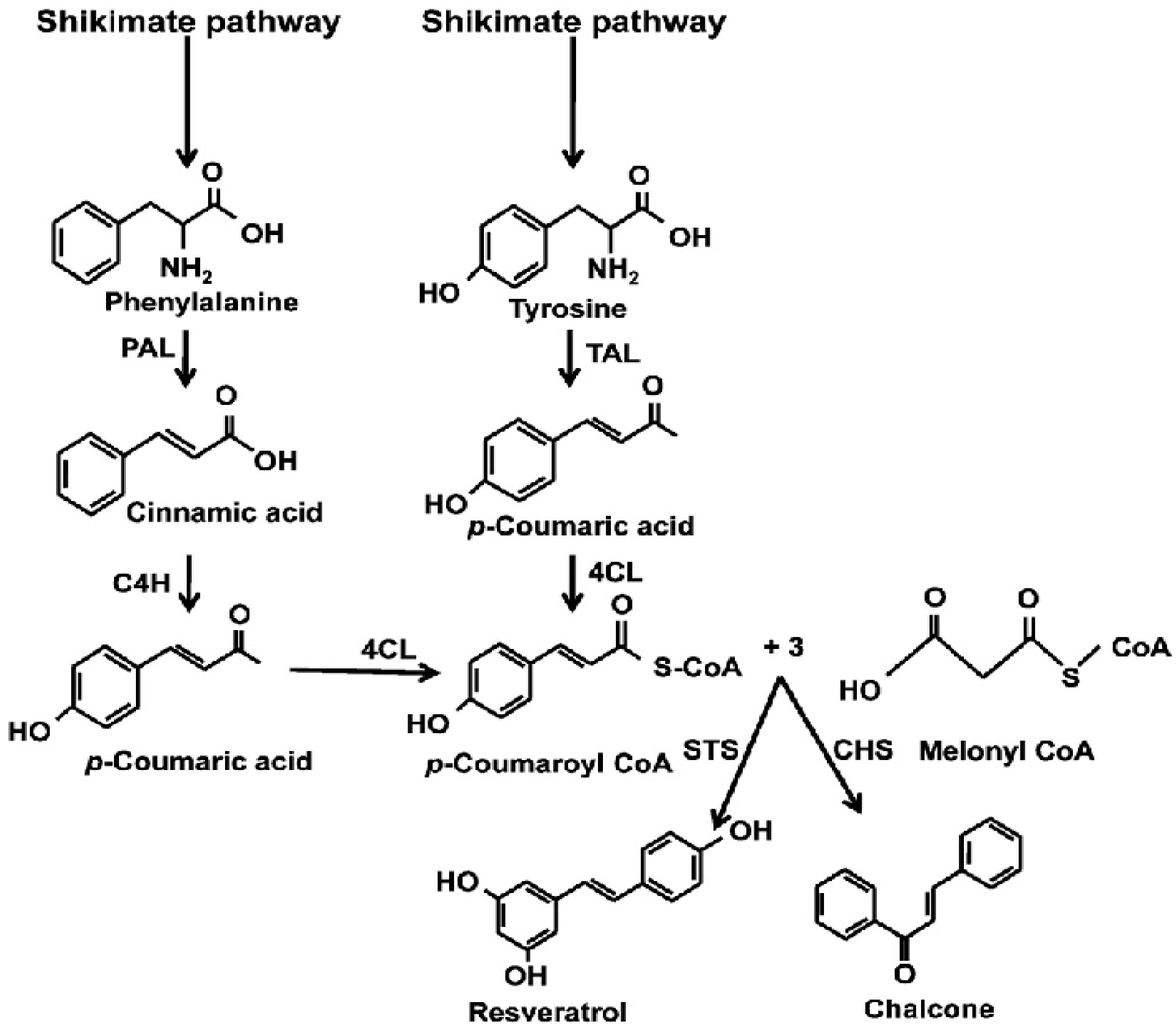

Figure-2.2: Biosynthesis of Resveratrol by Shikimate pathway (Hasan *et al.*, 2013)

Resveratrol protects plants against the attack of pathogenic fungi *Botrytis cinerea* by acting as a strong anti-fungal agent. The production of resveratrol in plants has seen a marked increase under the conditions like environmental stress or attack by pathogen. According to the famous "French paradox" people consuming French cuisine has lower mortality due to CVD since they have adequate intake of red wine containing RESV (Constant, 1997). Since then the research on RESV caught momentum for the inspection of its multi faceted biological properties (*Figure-2.3*).

Table-2.1: Natural sources of Resveratrol

SOURCES	Percentage of Resveratrol	REFRENCE
PLANTS		
Polygonum cuspidatum	73.8%	Wang *et al.*, 2013
Arachis hypogeal	0.09 to 0.30µg/gm	Lee *et al.*, 2004
Vitis rotundifolia	In seed 30.1 %	Ector *et al.*, 1996
FOOD PRODUCT		
Red wine	5.8 mg/L	Gu *et al.*, 1999
Peanut Butter	0.27 to 0.70 µg/gm	Lee *et al.*, 2003
FUNGI		
Alternaria sp from *Vitis vinifera*	123 µg/ml	Lee *et al.*, 2003
Arcopillus from *Vitis vinefera*	89.1 µg/ml	Dwibedi *et al.*, 2018

2.1. Medicinal uses of Resveratrol

2.1.1. Antioxidant activity

Exogenous and endogenous free radicals have an important role in development of many diseases. Antioxidants are the molecules which can act as reducing agents, hydrogen donors, chelators of metal ions, singlet oxygen quencher, prevention of metal catalyzed formation of free radical species. Formation of reactive oxygen species (ROS) can be inhibited in the presence of RESV (Aggarwal *et al.*, 2005). As a natural antioxidant RESV has many beneficial effects on human health.

2.1.2. Anticancer activity

Cancer is a chronic life style disease with high mortality rates. The development of cancer involves many disease and multiple steps (Aziz *et al.*, 2003). Resveratrol leads to apoptosis, cell arrest and inhibition of GFR involving pathway (Fresco *et al.*, 2006). It also exhibits the potency to inhibit the activity of COX enzyme and therefore affect tumor initiation and promotion (Aggarwal *et al.*, 2004). It works as a chemopreventive agent by producing effects on many cell apoptosis and anti proliferation of tumors. It inhibits ERK-1/2 gene suppressing the levels of estrogen and insulin growth factors-1 (IGF-1). It can also suppress PKG signalling and decreases the expression of cell survival protein (Wong and Fiscus, 2015).

2.1.3. Neuroprotective activity

Resveratrol has been reported to be effective in neuroprotective activity. It acts against oxidative stress and inflammation which is attributed to the antioxidant properties of Resveratrol (Okawara *et al.*, 2007). It has been found effective against ischemic brain injury and brain seizure (Peng *et al.*, 2016). It exhibits the property to delay onset of beta amyloids formation on the onset of neurodegenration leading to oxidative stress and thus modulating the status of health by inducing cell death and detoxification of RON and ROS (Marambaud *et al.*, 2005).

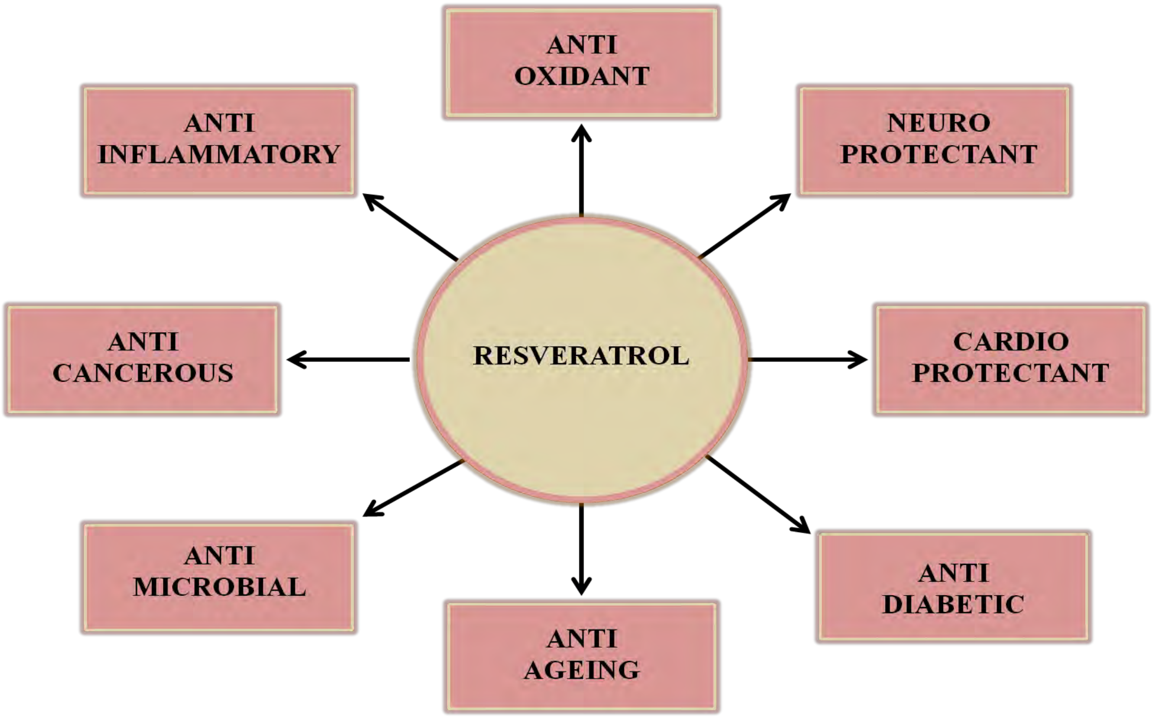

Figure -2.3: Therapeutic properties of Resveratrol

2.1.4. Cardioprotective activity

The interaction of RESV with multiple molecular targets and many intracellular pathways such as SIR through transcriptional factors that induces autophagy (Sharma *et al.*, 2011). Resveratrol targets at molecular level to reduce oxidative stress by ROS & RNS (Petrovski *et al.*, 2011). In 1980 a close observation towards eating habits and disease related mortality leads to the conclusion that despite their famous French cuisine rich in saturated fat components the French people were less affected by the cardiovascular diseases - the phenomenon popularly known as French paradox (Constant, 1997).

2.1.5. Anti-ageing property

RESV has been proved to enhance the lifespan of yeast cells by activating the SIRT1 genes leading to modulation of proteins including peroxisome proliferator, protein kinase B and NFκβ. It also has been found to activate human SIRT1 gene (Borra *et al.*, 2005). Accelerated formation of human mesenchymal stem cells or delay in senescence of human cells can be achieved by over expressing the *SIRT1* gene (Yuan *et al.*, 2015). This property of RESV has increased its demand in cosmetic and nutacuetical industry.

2.1.6. Anti-microbial activity

Initially identified as phytoalexin resveratrol shows antifungal activity against Botrytis cinerea. The antioxidant property of Resveratrol have retarding effect on the growth of microbe (Chan, 2002). RESV has proved to exhibit retarding effect on the growth of various pathogenic organisms like *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Trichophyton rubrum and Microsporum gypsum* (Aslam *et al.*, 2009)

2.1.7. Anti-diabetic activity

According to World Health Organisation (WHO) diabetes will be the seventh leading cause of death in 2030. Diabetes is a group of metabolic disease which potrays the symptoms of hyperglycemia elicted due to defective insulin secretion, insulin action or both. Anti-diabetic property of RESV has been studied on animal models and RESV has proved to regulate the level of insulin in blood and also helps to protect pancreatic cells from oxidative stress (Minakawa *et al.*, 2011; Sharma *et al.*, 2011; Palsammy *et al.*, 2008).

2.1.8. Anti-inflammatory activity

RESV can enhance the immune response against tissue injury by chemically modulating the inhibition of release of leukotriene-B4, Tumor Necrotic Factor-α, Inter leukine factor-1. Dietary supplements of RESV increase the amount of superoxide dismutase, catalase and Inter Leukin-2 factor evidently protects brain during infractions (Prabhakar, 2013). RESV leads to down regulation of Protaglandin E synthase-1 by inhibiting COX-2 enzyme activity and thereby reducing inflammation.

2.2. Endophyte : Gold Mine of Secondary metabolites

Endophyte was the term coined by the scientist de Barry in 1866 referring to organisms such as bacteria, fungi, actinomycetes etc occupying the internal tissues of the host plants without displaying any identifiable symptoms of disease or stress to the host plants (*Figure-2.4*). Endophytes and host system prospers through mutalistic relationship in which endophytes derives nutrients while residing inside the host (Walton *et al.*, 2000). Also endophytes regulates the enhancement of the defence mechanism against a spectra of pathogens and also the helps host plants to combat environmental stress (Verma *et al.*, 2009).

Fungi are today recognised as an essential source for the production of various biologically important molecules since the discovery of fungal Taxol an anticancer compound derived from endophytic fungi *Taxomyces andreance* (Stierle *et al.*, 1993). Further researchs were dedicated to explore the potential endophytic fungi from the biological niches and their future prespective to produce various bioactive molecules (Keller, 1997). This brought in notice the need to employ strategies that can help to increase the yield of these biologically important molecules from endophytic fungi (Kusari *et al.*, 2012; Chithra *et al.*, 2014; Su *et al.*, 2014). In the filamentous fungi gene cluster are responsible for the biosynthesis of secondary metabolites (Keller and Hohn, 1997; Walton, 2000). Some methods exploit the fact that the cells can undergo heritable change in the gene expression whereas the DNA sequence is conserved. These phenotypic changes are termed as epigenetics (Weinhold 2006). Recent studies on fungal genome indicate that epigenetic modification like histone deacetylation and DNA methylation is transcriptionally control the putative biosynthesis of gene clusters. Epigenetic regulation of gene transcription can be applied to wide range of fungi without any prior knowledge of the genome sequence (Cherblanc *et al*., 2012).

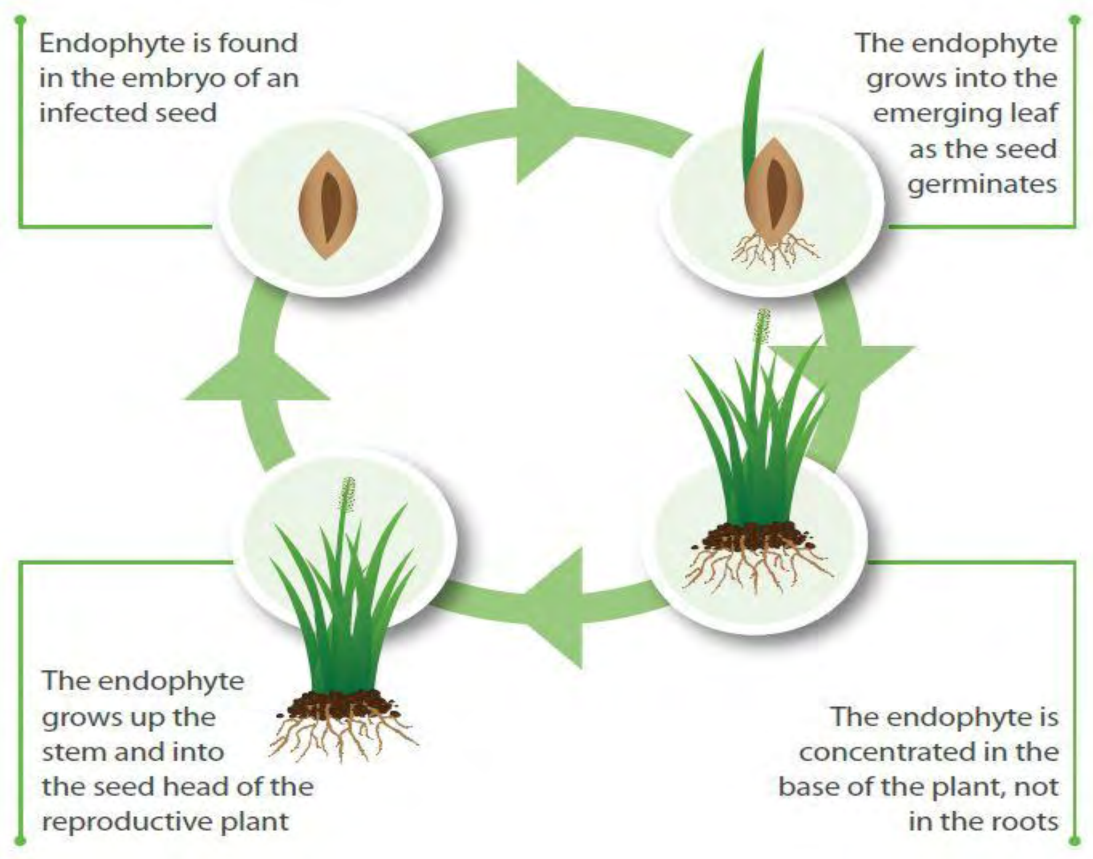

Figure-2.4: Host endophyte interaction (Kaul *et al., 2012*).

Therefore it is a relatively easy method to initiate the gene expression for secondary metabolite production (Rutledge and Challis, 2015).

DNA methylation and Histone modification are two major mechanisms to induce epigenetic modulation in fungal genome. AZA (5-azacytidine) acts as DNA methyltransferase inhibitor and SAHA (suberoylanilide hydroxamic acid) are reported as potent elicitors to fungal genome (Chernov *et al., 2013;* Fisher *et al., 2016*). AZA is a chemical analogue of the cytosine nucleoside (Momparler 2012). It tends to cause hypomethylation of the DNA by inhibiting DNA methyltransferase enzyme which catalyse the transfer of methyl group to the DNA. Also AZA incorporates into genetic material and leads to the dissembly of polyribosomes, defective methylation and acceptor function of t RNA which inhibits the production of proteins (Chapman and Brown, 2009). SAHA is a histone deacetylase inhibitor which by acting as a chelator for zinc ions present in active site of the histone deacetylase leads to the accumulation of the acetylated histones and acetylated protein further inhibiting the gene expression.

In a review by Pangeni *et al.*, 2014 has highlighted the potential of Resveratrol and recent advances in drug delivery. A wide range of pharmaceutical research has claimed Resveratrol as a potential candidate with the aim of improving bioavailability, increasing stability and minimizing toxicity related drug. The review provides detailed information about therapeutic activity of resveratrol. In other report by Kumar *et al.*, (2017) on Induction of cryptic and bioactive metabolites through natural dietary components in endophytic fungus colletotrichum gloeosporioides successfully establishes the importance of active dietary components which also interact with the epigenetic targets and can significantly induce the production of cryptic metabolites in the endophytic fungus and a. Another report by Ul-Hassan *et al.*, (2012) on Modulation of volatile organic compound formation in the mycodiesel-producing endophyte hypoxylon sp.CI-4. Thos report has figuratively highlighted the concept of using epigenetic modulation to enhance the production of volatile secondary metabolites in the fungus *Hypoxylon* sp. CI-4. Volatile Organic Compounds not previously observed as products of this organism appeared after exposure to the epigenetic modulators SAHA or AZA. The majority of newly appearing products were mono- and sesquiterpenoids.. Thus, it seems that simple chemical epigenetic methods can result in stable mutants having unique phenotypic characteristics suggesting that the CI4-B was not a random mutant and that the influence of SAHA on the fungus resulted in one or more permanent epigenetic modifications. In another research paper by Xinrui *et al.*, 2018 isolated triterpinoids and polyphenols. The methanol extracts of *Crataegus dahurica* plant extract were subjected to antioxidant assay and confirmed the presence of polyphenol content. The paper establishes the basis of the exploration of wild fruits for their polyphenol content.

Chapter – 3

AIM OF THE STUDY

- To develop an epigenetic variant of *Xylaria psidii* producing high Resveratrol content.
- To optimise the production of Resveratrol under shake flask condition.
- Enhancement of Resveratrol as compared to wild strain using epigenetic modulators and evaluating their *invitro* antioxidant content.

Chapter – 4

MATERIALS AND METHODS

4.1 Preparation of Potato Dextrose Agar (PDA) plates

39gm of PDA (Hi Media) was dispensed in 1000ml of lukewarm distilled water and pH was adjusted at 5.2. The media was then dispensed in 250ml of Erlenmeyer Flasks (Schott Duran) and autoclaved at 121ºC, 15psi for 15 minutes. Pre-sterilsed Petri plates (Tarsons) was dispensed with 25ml of PDA under sterile lab conditions in Laminar Air Flow chamber. The plates were allowed to solidify at the room temperature.

4.2 Sub-culturing of #22(P) VVLPM on PDA plates

#22(P) VVLPM cultures were procured from the laboratory repository maintained by Dr. Sanjai Saxena, Department of Biotechnology, Thapar Institute of Engineering and Technology. The isolated cultures were subcultured by point inoculating at the centre of the PDA plates and incubated at 26ºC ± 2ºC for 10 days. Morphological characters i.e. colony colour, texture, size, pigmentation were noted after every 24 hours of incubation.

4.3 Maintenance of Pure Culture of #22(P) VVLPM

A mycelia plug of 5mm was inoculated aseptically in PDA slants and vials supplemented with 10% of glycerol (w/v) and incubated at 26ºC ± 2ºC till the fungal growth was observed. Further the slants and vials were stored at 4ºC for future use.

4.4 Iidentification of endophytic fungi

4.4.1 Classical morphotaxonomy:

#22(P) VVLPM cultures were studied for microscopic properties. Different characteristics such as colony shape, colour, pigment production, diameter of culture were studied. For microscopic examination, a clean glass slide was taken and water drop was placed over it. By the help of fine needle, mycelial mass was taken and placed over the drop of water. It was teased properly using a fine tip needle and stained using Lacto phenol cotton blue. A cover slip of dimensions 18×10 mm was used to cover it while avoiding the formation of air bubbles. The slide was further mounted with DPX and observed under 10X, 40X and 100X using binocular microscope.

4.4.2 Molecular identification of #22(P) VVLPM

4.4.2.1 DNA isolation of endophytic fungi

Fungal cultures were grown over PDA for 4-5 days at 28˚C. Extraction buffer was prepared using 50mM Tris pH 8.0, 50mM EDTA, 3% SDS. In brief, 0.5-1g of mycelium was grounded to very fine powder by using liquid nitrogen in pestle and mortar. Add 660-750 µl of extraction buffer and crush again. Transferred the contents to 1.5 ml micro-centrifuge tube and add 10µl of ß- mercaptoethanol, 4µl of Proteinase K. Vortexed and incubated at 65°C in water bath for one hour (mixing after every 15 mins). Subsequently the reaction mix was spinned at 10,000 rpm for 15 min to remove all cell debris. Further, 6µl of RNase was added and incubated at 37°C for 30 min. Protein was precipitated by adding equal volume of phenol: chloroform (1:1) and centrifuged at 12,000 rpm for 15 min (repeat this step thrice). Aqueous phase was pooled and 20µl of 3M sodium acetate was added for DNA condensation. The tubes were then inverted and incubated at 4°C overnight. Further it was centrifuged at 12000 rpm for 15 min to obtain DNA pellet. The pellet was washed with 70 % cold ethanol. Centrifuged at 12000 rpm for 2 min. Decant off the supernatant and let it air dry. Finally, the pellet was dissolved in 20 µl of TE buffer. Agarose gel electrophoresis was done to perform the qualitative estimation of DNA.

4.4.2.2 Agarose gel electrophoresis:

Agarose gel (0.8%) was prepared in 1X TAE buffer and 0.5 µg/ml of EtBr (visualization dye) was added to it. This gel was poured in pre-set glass plate and comb was inserted to make wells. The gel was allowed to polymerize. After polymerization of gel, samples were mixed with 6X loading dye and were loaded in wells. Gel was allowed to run at 70 V for 1 h. Further, the gel was observed under UV trans-illuminator. For imaging of DNA, Bio – Rad Gel Documentation System was used. For the quantitative analysis of DNA, the absorbance of DNA was taken at 260 and 280 nm. 50 µg/ml of DNA sample is equal to 1O.D. The concentration of DNA was calculated by formula-

Concentration (µg/ml) = O.D. at 260 nm X 50 (µg/ml) X Dilution factor

4.4.2.3 PCR amplification of genomic DNA

Amplification of ITS1-5.8S-ITS2 rDNA sequence was carried out using universal primer pair i.e. ITS 1 and ITS 4 (White *et al.* 1990) synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (Verti 96 well Thermal cycler, Applied Biosystems). Amplification was performed in 25 µl of reaction mixture containing 2µl of extracted fungal DNA, 0.8 µM of each primer (ITS1 and ITS4), 2.5mM of dNTPs (Bangalore GeNei), 1.5 mM MgCl2 (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei). The conditions for Thermal cycler consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 1 min, 58ºC for 1.30 min, 72ºC for 1 min followed by final extension at 72ºC 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 Gel documentation System. The amplicon was sent for sequencing to GeNei, Bangalore.

4.5 Induction of epigenetic modulation using chemical elicitors

4.5.1 Preparation of AZA and SAHA PDA plates of *Xylaria psidii*

The wild type endophytic fungus *Xylaria pisidii* was subjected to epigenetic modification using 5-azacytidine (AZA) and suberoylanilide hydroxamic acid (SAHA) as chemical elicitors. The stock solutions of 1mg/ml of AZA & SAHA were prepared in DMSO. The PDA plates enriched with different concentration of AZA and SAHA ranging from 1 µM, 3 µM, 5 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM, 80 µM, 90 µM, 100 µM respectively were prepared and the plates were allowed to solidify at the room temperature. The mycelia plugs of *Xylaria psidii* was point inoculated at the centre of the AZA and SAHA enriched PDA plates respectively. The endophytic fungus was then incubated at 26ºC ±2ºC for 10 days.

4.5.2 Production of Culture

Mycelia plug of 5mm diameter was plugged out from the 10 days old culture of *Xylaria psdii* and was inoculated in culture bottles containing 30 ml of Potato Dextrose Broth (PDB) (Hi Media). The culture bottles were incubated at 120rpm at 26ºC ± 2ºC for 14 days for production of secondary metabolites (Rodrigues *et al.*, 2000; Santamari *et al., 2000;* Rosa *et al.*, 2003). After 15 days broth was separated from mycelia by filtration. Filtration was carried out aseptically using Whatman filter paper 4 of Merck Millipore (Rodrigues *et al., 2000*).

4.5.3 Culture filtrate extraction and biomass calculation

The spent broth of *Xylaria psidii* isolates were filtered using Whatman filter paper 4 and stored at -20 C till further use (Vicente and Calbello, 2001). Further the biomass of fungal spores was also noted for which the filter paper containing fungal spores was dried at 60ºC for overnight in hot air oven. Afterwards the weight was measured by keeping the filter paper on weighing balance and subtracting the prior calculated weight of the Whatman filter paper from it.

4.6. HPLC for affirmation of Resveratrol producing isolates

Different epigenetic variants were evaluated for the presence of secondary metabolite Resveratrol in their crude extracts. 50µl of cell free fungal extract was dissolved in methanol and injected into HPLC column to determine the concentration of fungal Resveratrol, different dilutions of standard Resveratrol (stock- 1mg/ml) ranging from 0.1-1 mg/ml were prepared in HPLC grade methanol. 20ul of each dilution was injected into C18 reverse phase discovery column with 4.6 mm Internal

Diameter x 150mm length. The data of the peak vs. concentration of the standard Resveratrol obtained were used to estimate the quantity of fungal Resveratrol in crude cell free extract.

4.7. Biological assays

4.7.1 Antioxidant assays

4.7.1.1 DPPH Scavenging assay

The free radical scavenging activity of the culture filtrate of AZA and SAHA epigenetic variants of #22(P) VVLPM was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals, according to the procedure described in the literature (Shi *et. al., 2012)* with minimal modifications.

Briefly, 20 µl of the culture filtrate was added to 230 µl of DPPH (100 µM in methanol) and mixed thoroughly. The mixture was incubated for 30 min at room temperature in dark. After incubation the absorbance was measured at 517 nm using BIOTEK® Powerwave 340 microtiter plate reader. Working DPPH used as positive control and methanol as negative control. Resveratrol (10-50 µg/ml) was used as standard. The test was performed in triplicate and the data was reported as mean ± SD. The percentage free radical scavenging activity of the fungal extract was calculated as:

$$\%FRS = \frac{Absorbance\ (Control) - Absorbance(Sample)}{Absorbance(Control)} \times 100$$

4.7.1.2 Trolox Equivalent Antioxidant assay (TEAC):

TEAC was analyzed by using the method described by Zulueta *et al.*, 1999. The assay utilizes the ability of an antioxidant to scavenge ABTS radical to determine the level of its activity. ABTS radical was generated by mixing 7 mM ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline 6-sulphonic acid) (TCI, Japan) dissolved in 0.1 M PBS of pH-7.4 and 2.45 mM potassium persulphate in 1:1 and incubated for 16 hrs at room temperature in dark. This ABTS mixture was diluted in PBS to an absorbance of 0.9-1.0 at 734nm using spectrophotometer (Hitachi U-2900, Japan). To carry out test 1ml of working ABTS solution was added to 10 µl of the extract and the reaction mixture was allowed to stand for 6 minutes. The absorbance was recorded at 734 nm. Phosphate buffer saline was used as blank and ABTS was used as control. Percentage free radical scavenging (%FRS) at different concentration was calculated using the formula-

$$\%FRS = \frac{Absorbance\ (Control) - Absorbance(Sample)}{Absorbance(Control)} \times 100$$

4.7.1.3 Ferric Reducing-Antioxidant Power (FRAP Assay)

Ferric reducing antioxidant power assay was performed according to the described method by Benzie and Szeto (1999) with slight modifications. FRAP working reagent was prepared with a mixture of 0.3M sodium acetate buffer, 10mM (TPTZ) and 20 mM Iron (III) chloride hexahydrate in ratio 10:1:1. 1ml of FRAP reagent was added to 10 µl of extract and mixed thoroughly. After the incubation at room temperature for 30 minutes the absorbance was measured at 595 nm. Ascorbic acid was used as a standard at concentration ranging from 10-100µg/ml and working solution of FRAP with deionized water was used as blank. A linear regression line was plotted between the concentrations of standard and their absorbance using which the concentration of sample was calculated. The ferric reducing antioxidant power was measured as µg ascorbic acid equivalent per mg of extract. The test was performed in triplicate and the data was reported as mean ± SD (Ramamoorthy *et al.*, 2007).

4.7.1.4 Total Phenolic Content

Total phenolic content was determined using Folin-Ciocalteu's (FC) reagent as described by (Kaur and Kapoor, 2002). Gallic acid (10 µg/ml - 100 µg/ml) was used as standard. To 100µl of 1mg/ml fungal extract 1.5ml of deionized water and 100 µl of FC reagent was added followed by incubation at room temperature (RT) for 10 minutes. Then 200 µl of Na_2CO_3 (6%w/v) was added and the reaction mix was allowed to stand at room temperature for 1 hour. Absorbance was noted at 760nm. A regression line was plotted between the concentrations of sample and further the concentration of sample was calculated. The test readings were in triplicates and reported as mean ± SD.

4.7.1.5 Total Flavonoid content

To the 200 µl of 1mg/ml of fungal extract 800 µl of deionized water was added and also 60 µl of $NaNO_3$ was added. The reaction mixture was incubated at RT for 5 minutes. $AlCl_3$ (10% w/v) was added and after incubation of 1 minute 400 µl of 1N NaOH was added to the reaction mixture and the volume was made up to 2ml. The absorbance was noted at 510 nm. Quercetin (50 µg- 250 µg/ml) was used as standard. A regression line was plotted between the concentrations of sample and further the concentration of sample was calculated. The test readings were in triplicates and reported as mean ± SD.

4.7.2 λ-DNA Nicking assay

Fungal extract of wild #22(P) VVLPM was evaluated for the ability to protect the λ- DNA. The reaction was performed in eppendrof tube containing 1µl of λ-DNA with fungal extract and also without fungal extract in 15µl of TE buffer and the volume was made up to 30 µl by adding Fenton's reagent (3µl of 2Mm $FeSO_4$, 3 µl of 30% H_2O_2 in Tris-buffer 10mM). The reaction mix was incubated for one hour at 37ºC. After incubation 1 µl of EDTA (pH - 8) was added to stop the reaction. The difference between both λ- DNA with and without fungal extract was evaluated on 1% agarose gel in Tris-acetate EDTA buffer. The gel was run at 70V for 1 hour and the bands were observed in XR+molecular imager Gel documentation system (Bio RAD,USA) (Ziogas *et al*., 2010).

Chapter – 5

RESULTS

5.1. Subculturing and Maintenance of fungal isolates

Endophytic cultures of #22(P) VVLPM were procured from the repository maintained by Dr.Sanjai Saxena, Professor, Department of Biotechnology at Thapar Institute of Engineering and Technology was inoculated on PDA plates (*Figure: 5.1)* and incubated at 26ºC ± 2ºC for 10 days.

Figure-5.1**:** Pure culture of the procured endophytic fungi

5.1.1. Preservation of culture isolates

Culture isolate was preserved on PDA slants and vials supplemented with 10% (w/v) glycerol and incubated at 26ºC ± 2ºC for the growth and stored at 4°C for maintenance of culture.

5.2. Culture production and extraction

For the production of secondary metabolite culture isolate was subjected to fermentation in PDB. The culture filtrates were filtered after 10 days of incubation using Whatman filter paper 4. It was found that the volume of spent broth has reduced with change in colour and characteristic fruity smell of culture filtrate was observed.

5.3. Identification of #22(P) VVLPM fungal isolate

5.3.1. Classical Morphotaxonomy of #22(P) VVLPM fungal isolate

The colonies were moderately growing (54 ± 2 mm), velvetenious white filamentous colonies with curly margins and radial crevices, floccose to downy colonies on different media after 15 days of incubation with 12 h photoperiod (*Figure 5.2*). Though fungus is cultivated in diverse media, the fungus was still in vegetative status i.e. did not produced any reproductive structure like stromata, conidia, conidiogenus cells or ascospores after 2–9 weeks of incubation (*Figure 5.2a-f*). Further, the isolate did not sporulate even after providing stress conditions like incubation under complete darkness and ultraviolet radiation, but it could be identified by their specific stromata (*Figure 5.2e and f*). Further identification using molecular technique revealed that the fungus belongs to the *Xylaria* sp.

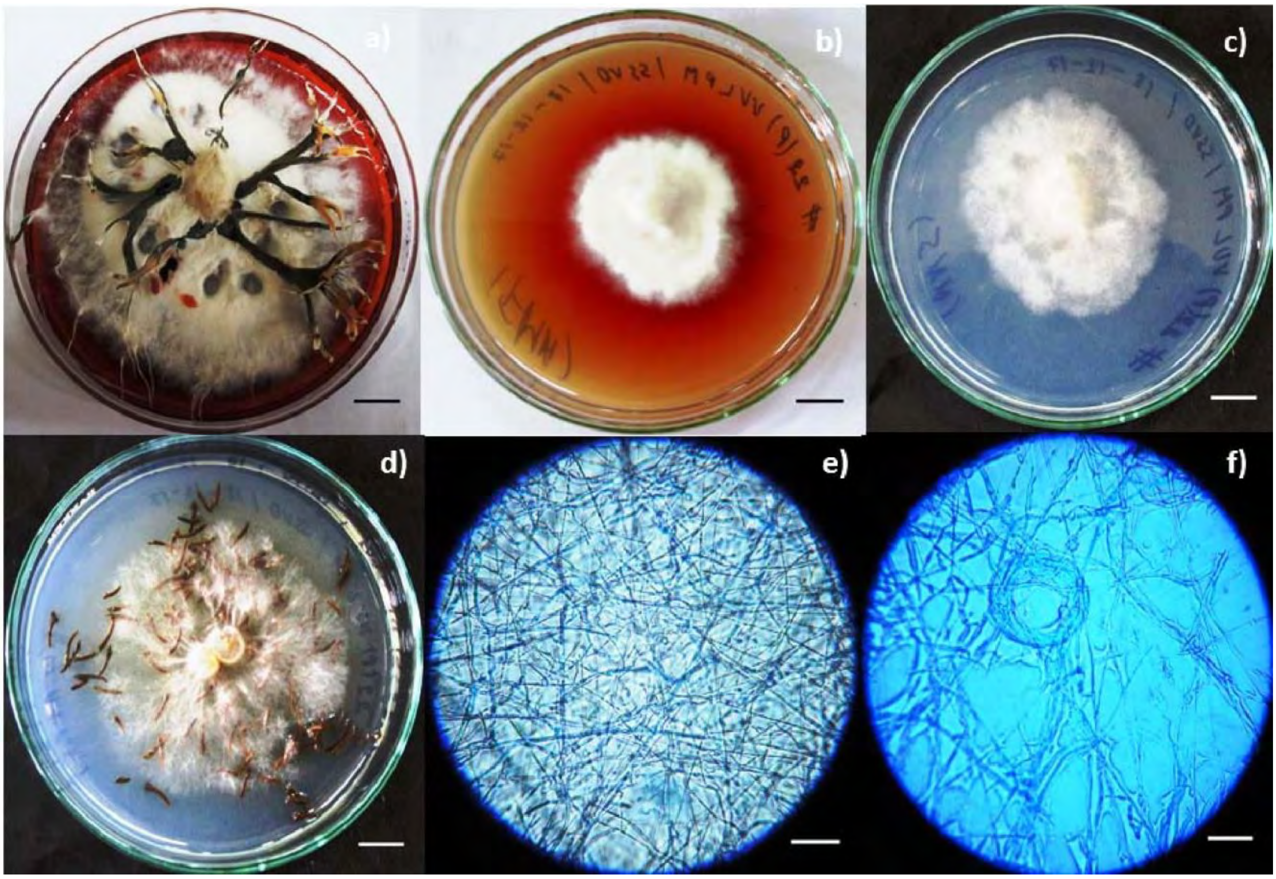

Figure-5.2: Colony morphology and Microscopic features of #22(P)VVLPM (*Xylaria*sp). a) PDA (pH 7) Red wine in color, b) Over FMA Media Reddish Brown in color, c-d) Over SNA and PLA Media white in color, e-f) Microscopic feature on SNA and PLA. Bars a-d) 10 mm; e – f) 10 µm.

5.3.2. Molecular identification of #22(P) VVLPM fungal isolate

5.3.2.1. DNA isolation of fungal Isolates

The size of fungal genomic DNA was approximately 10kb (*Figure: 5.3*). The ratio of absorbance at 260/280 nm was calculated as 1.72 determining the purity of DNA.

Figure-5.3: Lane 1-6 : Genomic DNA of #22(P)VVLPM resolved on 0.8% agarose gel.

5.3.2.2. PCR amplification of fungal isolate

The size of the PCR amplicon after resolving on 1.5% agarose gel was obtained in the range of 500-600 bp. The ITS-1 & ITS-4 primers were used for the amplification of the DNA (*Figure: 5.4*).

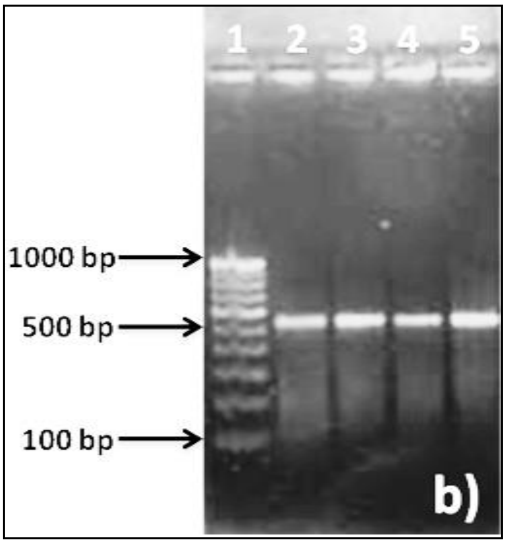

Figure-5.4: Lane 1: 1000bp ladder, Lane 2-5: PCR amplicon of #22(P)VVLMP.

5.3.2.2.1. Blast analysis

BLAST analysis of ITS sequence of #22(P) VVLPM exhibited its close homology with *Xylaria psidii*. However, to reconfirm a Maximum likelihood tree based upon Tamura and Nei model was prepared comprising of homologous sequences and #22 (P) VVLPM. In this phylogenetic tree #22(P) VVLPM clustered *Xylaria psidii* isolate SUT124 in clade I. Hence on the basis of morphological as well as phylogenetic analysis #22(P) VVLPM was assigned the name *Xylaria psidii*. The ITS sequence of #22(P) VVLPM has been submitted in Genbank with accession no.MH142837 (Table 5.1).

Table-5.1: Blast analysis of sequence of #22(P) VVLPM (accession no.MH142837)

S.No	Speices Name	Accession No.	Query Coverage	Sequence similarity
1.	*Xylaria psidii*, small subunit ribosomal RNA gene, internal transcribed spacer 1 and 5.8S ribosomal RNA gene	MH393292.1	92%	98%
2.	*Xylaria psidii*, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	KU291350.1	93%	97%
3.	*Xylaria psidii* isolate AT_L13_E2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	MF773655.1	91%	98%
4	*Xylaria psidii* isolate SUT124 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	DQ322158.1	87%	98%
5	*Sordariomycetes* sp. WF147 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	HQ130703.1	98%	91%
6	*Xylaria palmicola* isolate 604 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	GU322436.1	99%	91%
7	*Xylaria* sp. FPL-52(S) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	KF564638.1	89%	93%

8	*Xylaria* sp. 7 HMH-2010g 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	GU322435.1	99%	90%
9	*Xylaria* sp. BAB-4952 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, complete sequence	KR155042.1	99%	97%
10	*Xylaria psidii* strain 2508 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	FJ037730.1	97%	97%
11	*Xylaria* sp. BAB-5010 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	KR002893.1	94%	98%
12	*Xylaria* sp. ENT2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	KF493856.1	94%	98%
13	*Xylaria psidii* isolate LD small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene	MH393292.1	93%	97%
14	*Xylaria psidii* isolate LD small subunit ribosomal RNA gene, partial sequence; ITS 1	MH393292.1	93%	98%
15	*Xylaria psidii* internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2	KU291350.1	93%	97%

5.4. Subculturing of *Xylaria* isolates on AZA and SAHA PDA plates

The fungal isolates of *Xylaria psidii* inoculated on the PDA plates with different concentration of AZA and SAHA produced variant morphological characteristics of #22(P) VVLPM (*Figure-5.5)*. The colony diameter of AZA & SAHA variants of subcultured #22(P) VVLPM was also noted on 10th day of growth (*Figure: 5.6*).The colony diameter of wild strain of 22(P) VVLPM was 61mm. Among AZA variants the 10µM AZA treated fungal variant showed highest colony diameter of 67mm. While among SAHA variants the 5µM SAHA treated fungal variant showed the highest colony diameter of 54mm

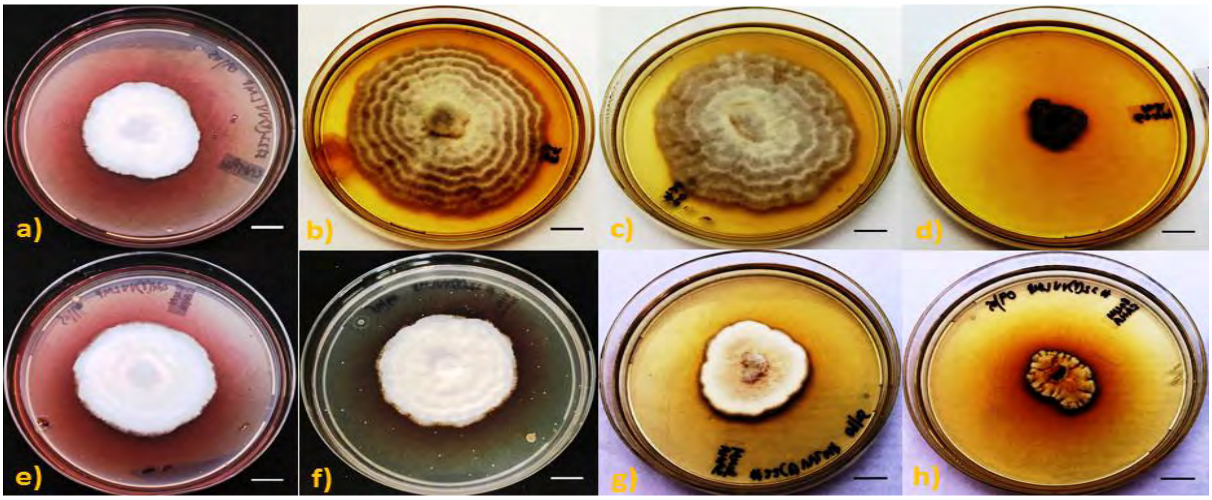

Figure-5.5: Epigenetic variants of #22(P)VVLPM a) Control (#22(P)VVLPM); b) AZA 10 µm on PDA medium; c) AZA 20 µm on PDA medium; d) AZA 60 µm on PDA medium; e) SAHA 1 µm on PDA medium; f) SAHA 10 µm on PDA medium; g) SAHA 30 µm on PDA medium; h) SAHA 60 µm on PDA medium(Bar : 10mm).

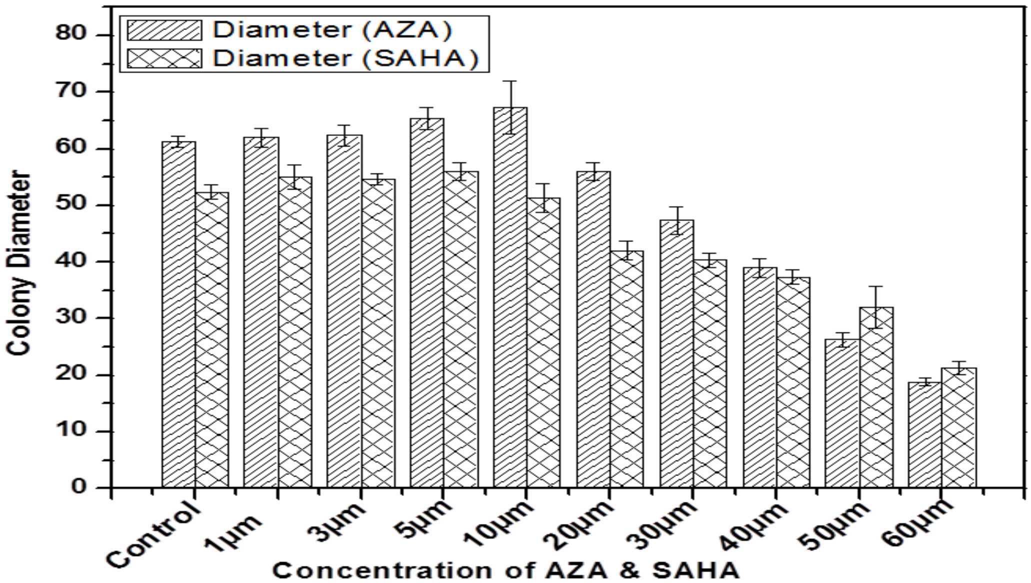

Figure-5.6: Diameter of #22(P)VVLPM on different concentrations of AZA and SAHA

5.4.1. Production and Extraction of culture filtrates

It was found that the volume of spend broth was reduced with the significant change in colour & characteristic smell of culture filtrates due to production of secondary metabolites. The culture filtrates were filtered after 10 days of incubation using Whatman filter paper 4. The biomass of culture filtrates were also noted (*Figure-5.7*). The biomass of wild strain was 0.65gm. Among AZA variants cell free extract of 3µM AZA treated fungal variant showed the highest biomass of 0.69gm and among SAHA variants 5µM SAHA treated fungal variant has highest biomass of 0.68gm.

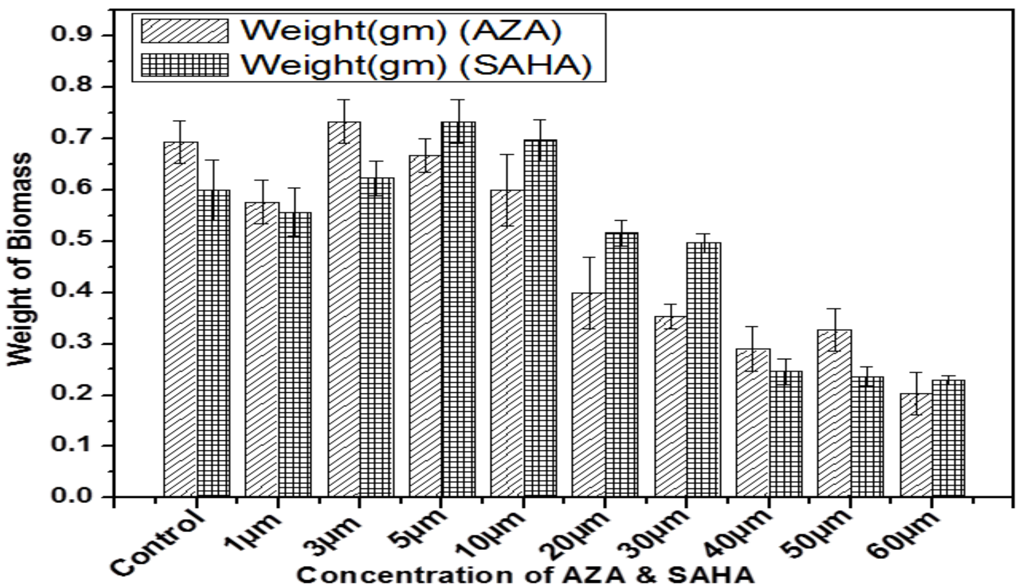

Figure-5.7: Weight of Biomass AZA and SAHA variants of #22(P) VVLPM

5.5. HPLC for affirmation of Resveratrol producing isolates

A symmetrical peak at RT 3.36 min on C18 reverse phase column similar to that of standard resveratrol was confirmed. Out of 26 epigenetic variants of #22(P) VVLPM which were obtained after treatment with different concentrations of AZA & SAHA it was found that 10µM of AZA and 5µM SAHA exhibited highest Resveratrol concentration of 48.94µg/ml and 52.32 µg/ml respectively as compared to control (35.43 µg/ml) and on increasing the concentration of AZA and SAHA the decrease in Resveratrol concentration was observed (Table- 5.2).

Table-5.2: Peak area of retention time of different standard concentration of Standard Resveratrol

Concentration (mg/ml)	Peak Area
1	288861.28
0.8	234551.81
0.6	195443.89
0.4	146776.54
0.2	105432.65
0.1	66476.86

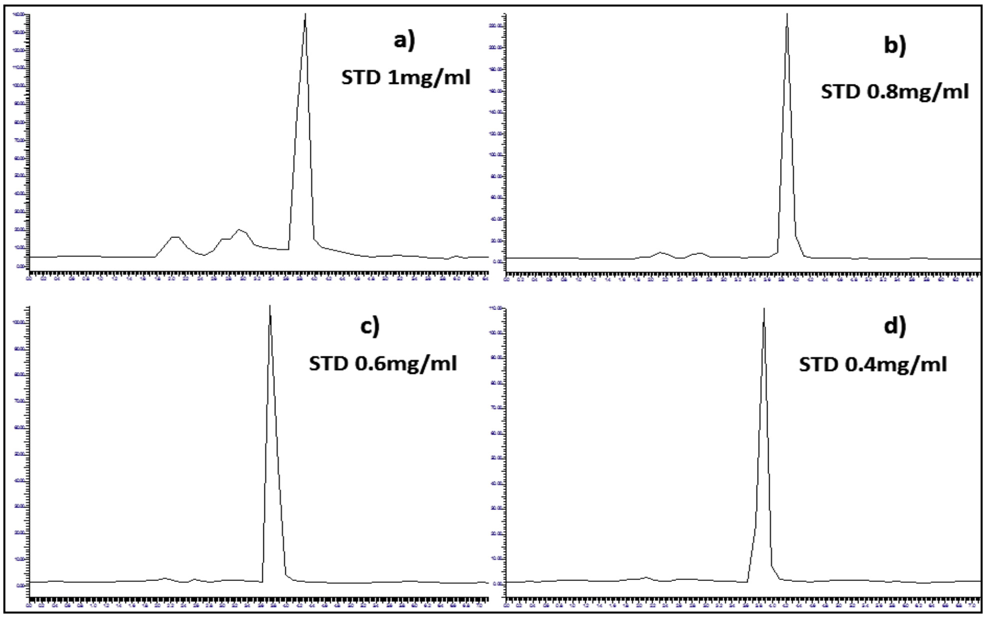

Figure-5.8: HPLC spectra of different concentration of Standard Resveratrol (a) 1mg/ml (b) 0.8mg/ml (c) 0.6 mg/ml (d) 0.4mg/ml

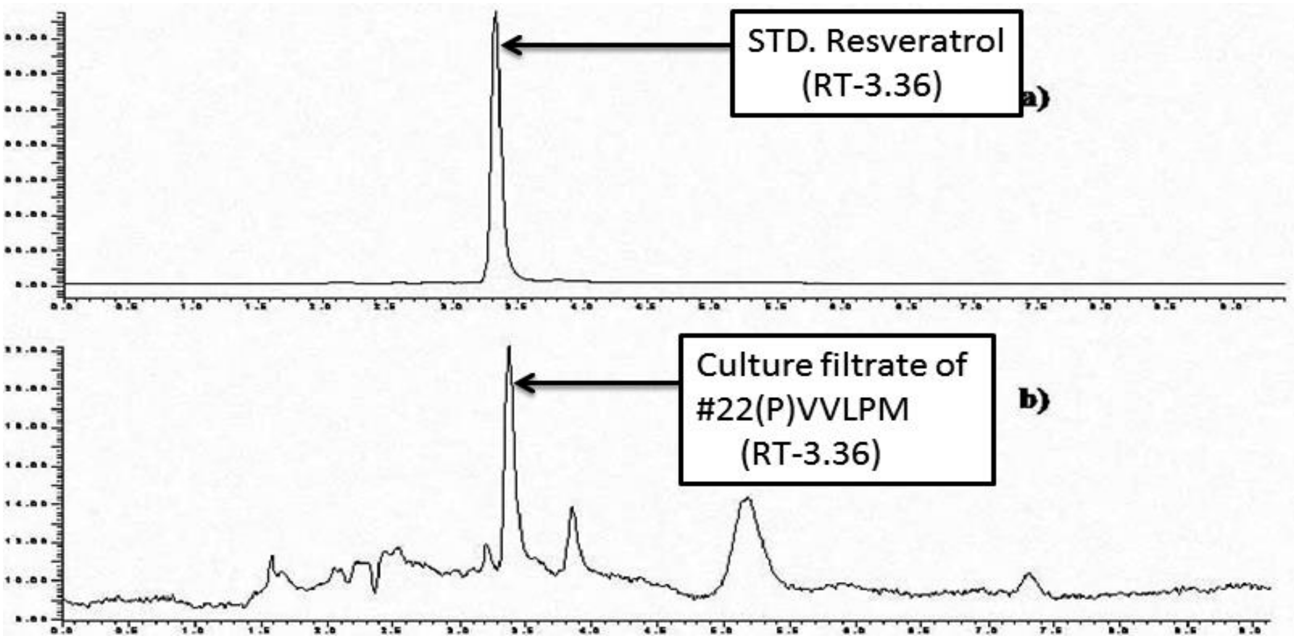

Figure-5.9: HPLC spectra of (a) Standard resveratrol, (b) crude residue of #22(P) VVLPM

Table-5.3: Peak area and concentration of Resveratrol of maximum resveratrol producing epigenetic variants of #22(P)VVLPM

	PEAK AREA (mAU)	Conc. Of RESV (µg/ml)	%increase or decrease
Control	134459.3	35.43	--
AZA 3	133459.2	35.01	-1.19%
AZA 5	139889.8	37.72	6.07%
AZA 10	**166477.8**	**48.94**	**27.60%**
SAHA 5	**174487.8**	**52.32**	**32.28%**
SAHA 10	147889.8	41.10	13.79%
SAHA 20	127864.8	32.65	-8.51%

(Note- the negative sign '-' indicates decrease in concentration of Resveratrol)

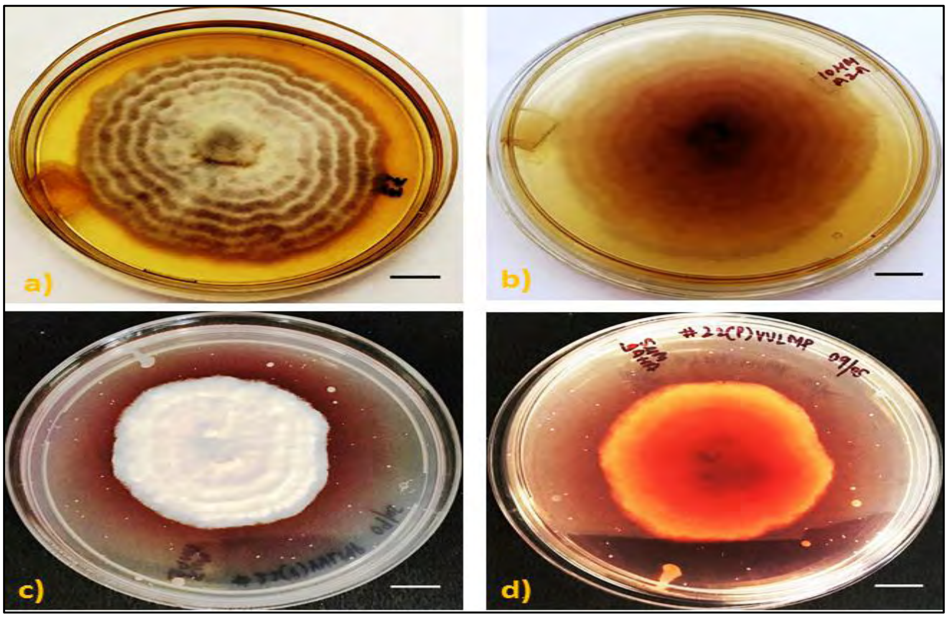

FIGURE- 5.10: Top resveratrol producing epigenetic variants of#22(P)VVLPM plate morphology; a) Front view of AZA 10 µm; b) Reverse side of AZA 10 µm; c) Front view of SAHA 5 µm; d)Reverse view of SAHA 5 µm (Bar : 10mm)

The colony characteristics of the top two RESV producing epigenetic variants like diameter, texture, and pigment production were analyzed (Table-5.4).

Table-5.4: Morphological characterstics of maximum reveratrol producing epigenetic variants.

	CONTROL 22(P)VVLMP	**10 AZA**	**5 SAHA**
DIAMETER ON PLATE	54.5 ± 0.14mm	56.5 ± 0.37mm	55 ± 0.61mm
COLONY CHARACTERSTIC	White velvety filamentous mycelia growth, produces red wine colour	White velvety filamentous colony with red wine colour. Pink pigment around the center of the growth.	White velvety filamentous colony with dark red wine colour. Pink pigment around the center of the growth.
DRY WEIGHT	0.58gm	0.39gm	0.52gm
CONCENTRATION OF RESV	35.43 µg/ml	48.94 µg/ml	52.32 µg/ml

5.6. Biological assays

5.6.1. Antioxidant assays

The culture filtrate of *Xylaria pisidii* were evaluated for their capacity of antioxidant activity using DPPH, ABTS, FRAP, TPC and TFC assay. The best activity and percentage free radical scavenging was observed in concentration 3µM, 5 µM, 10 µM and 20 µM.

5.6.1.1. DPPH Scavenging assay

Cell free extract of fungal variant treated with 5µM AZA and 10µM of SAHA showed the highest %free radical scavenging activity i.e. 29.17% and 31.53 %. The resveratrol was taken as standard. Also a significant increase in % free radical scavenging was observed as compared to wild strain whose % free radical scavenging activity was 12.23 %.

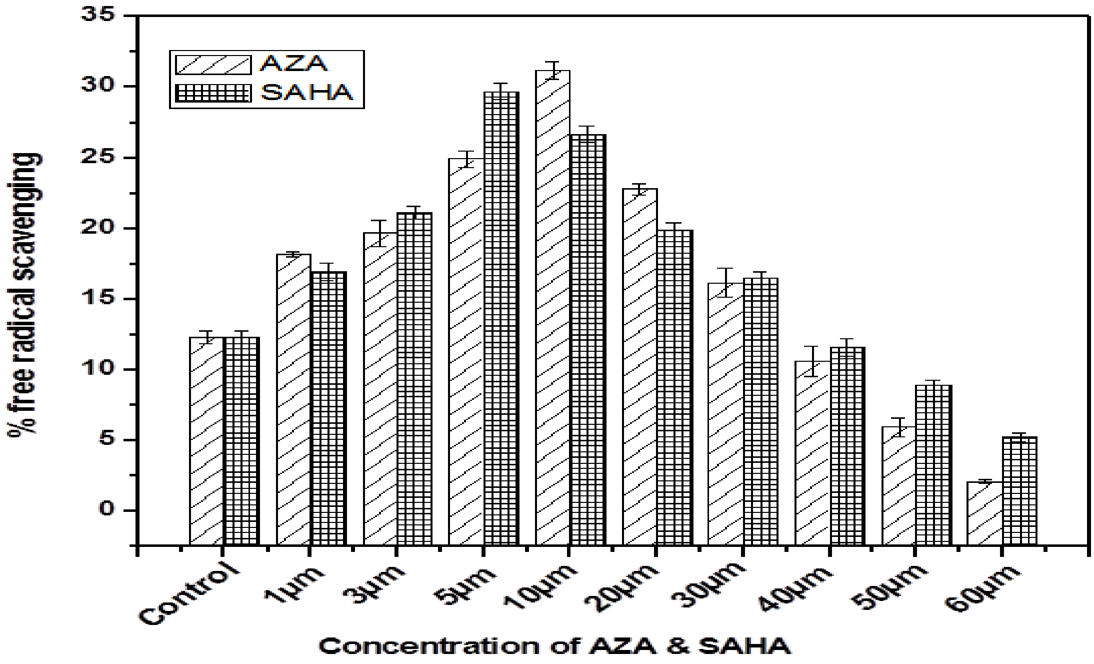

Figure- 5.11: DPPH scavenging assay

5.6.1.2. Trolox Equivalent Antioxidant Capacity assay (TEAC)

The highest % free radical scavenging activity was exhibited by cell free extract of fungus variant treated with 10 µM AZA was 46.15±0.40µg/ml and 5 µM SAHA treated cell free extract of fungus was 44.55±0.28 µg/ml epigenetic variants of #22(P)VVLPM was more as compared to control 34.04±0.47 µg/ml .

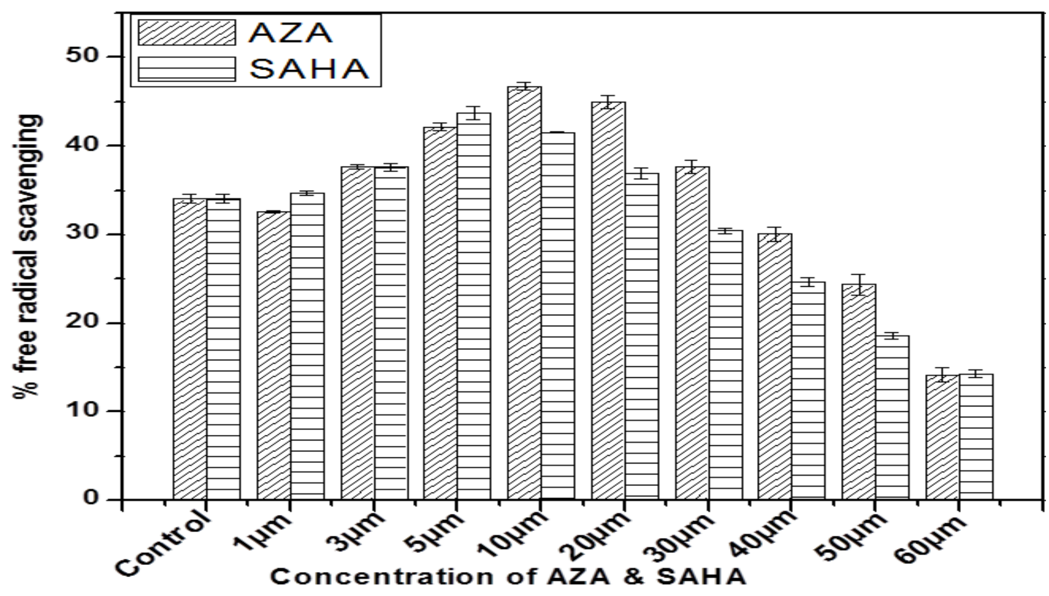

Figure- 5.12: Trolox Equivalent Antioxidant assay (TEAC)

5.6.1.3. Ferric reducing-antioxidant power (FRAP assay)

Ascorbic acid was used as standard. The concentration of samples was calculated as ascorbic acid equivalent per mg of extract. The highest equivalent concentration was evaluated in fungus extract treated with 5µM AZA, 10µM AZA, 20µM of AZA. The conc of 10µM AZA treated fungus extract was 38.25±0.49 µg/ml and 5 µM SAHA treated fungus extract was 33.91± 1.2 µg/ml (Figure-5.13).

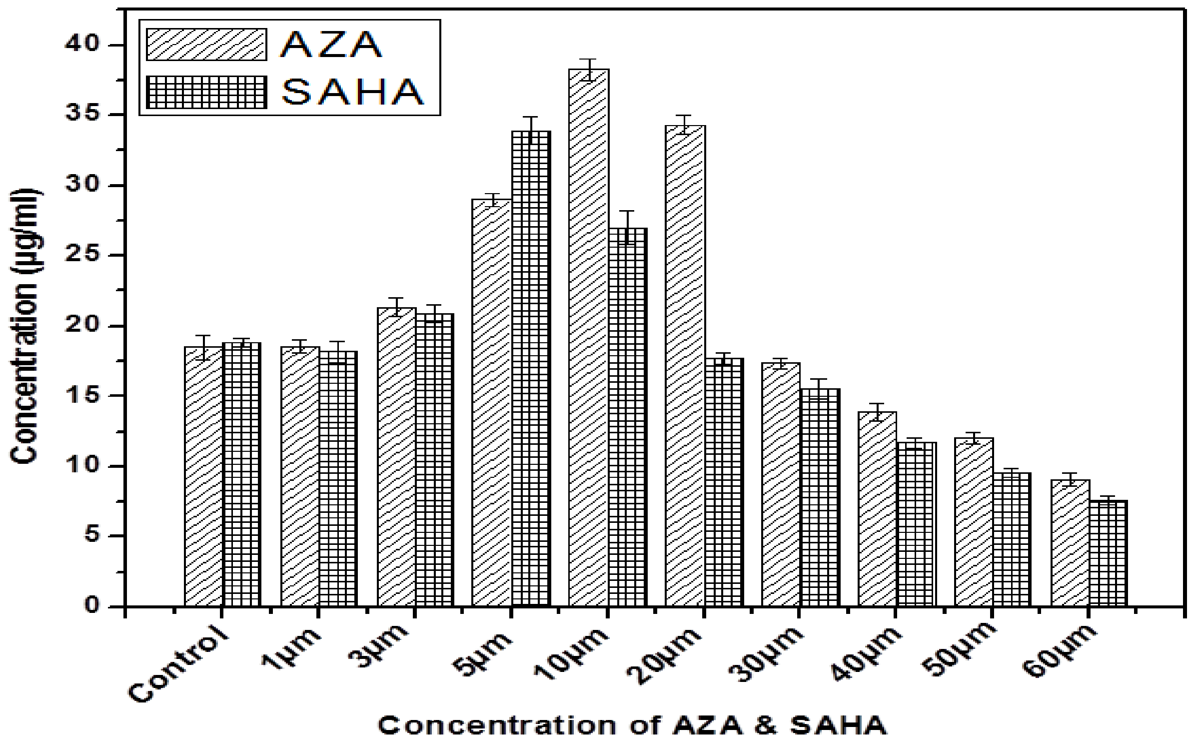

Figure-5.13: Ferric reducing-antioxidant power (FRAP) assay

5.6.1.4. Total Phenolic Content

The total phenolic content of the different concentration was calculated by taking gallic acid as standard. The concentration of phenolic content in sample was calculated as equivalent to gallic acid/mg of crude extract. The highest concentration was observed from 1 µM of AZA & SAHA to 20 µM of AZA & SAHA treated fungus extract followed by the decrease in the trend of concentration when the concentrations of AZA & SAHA increased in fungus culture The concentration of 10µM AZAtreated fungus extract was 48.37±0.76 and 5 µM SAHA treated fungus extract was 55± 0.21 (Figure-5.14).

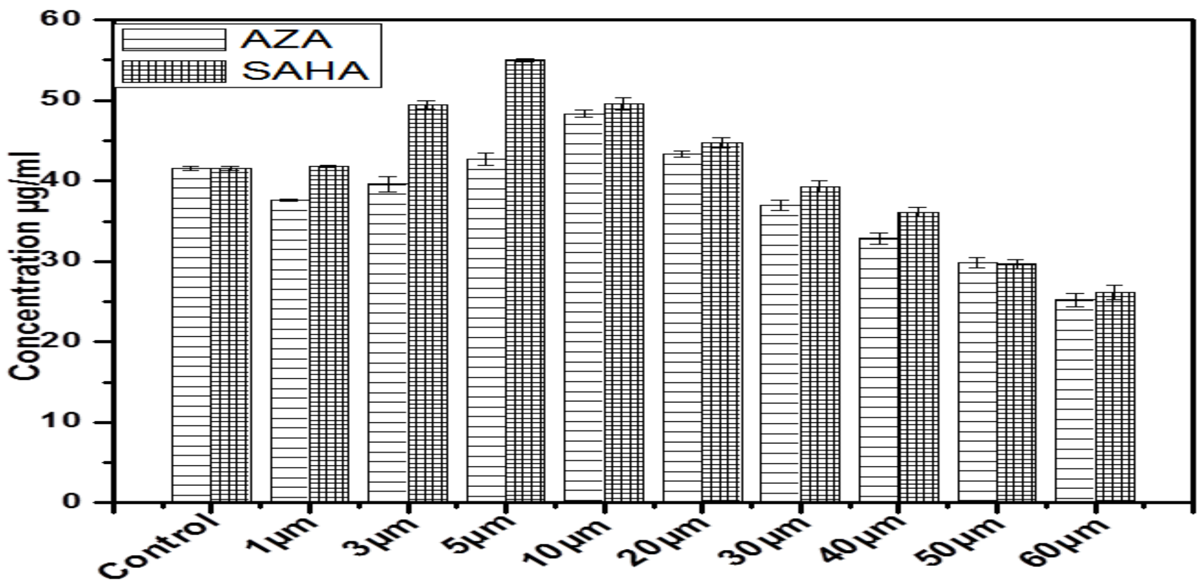

Figure-5.14: Total Phenolic Content of different epigenetic variants

5.6.1.5. Total Flavanoid Content

Total flavanoid content was calculated by taking Resveratrol as standard. The concentration of flavanoid content in sample was calculated as equivalent to resveratrol/mg of crude extract. The concentration as observed in 10 µM AZA treated fungus extract and 5 µM SAHA treated fungus extract was 46±0.65 and 49.2 ±0.41.

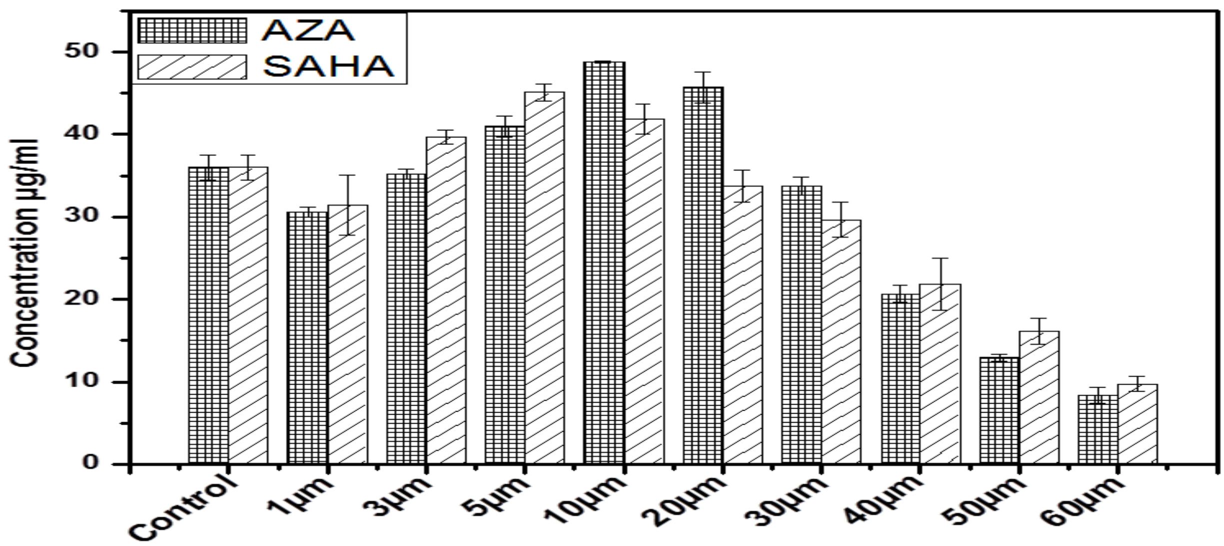

Figure-5.15: Total Flavanoid Content of different epigenetic variants

5.6.2. λ-DNA Nicking assay

The λ-DNA with fungal extract showed prominent bands on 1% agarose gel indicating that λ-DNA with the fungal extract of #22(P)VVLPM and cell free extract of #22(P) VVLPM treated with 5µM SAHA and 10 µM AZA have strong antioxidant properties whereas the λ-DNA without fungal extract was degraded by Fenton's reagent indicated as smear on the gel . Resveratrol was used as standard.

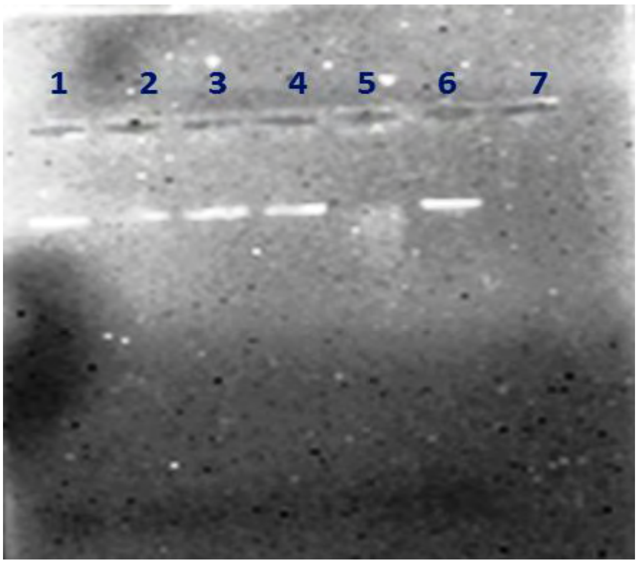

Figure-5.16: Lane 1-2: wild strain of #22(P)VVLPM + λ-DNA+ Fenton's Reagent, Lane 3: extract of fungus treated with 5 µM SAHA + λ-DNA+ Fenton's Reagent , Lane 4: extract of fungus treated with 10 µM AZA +λ-DNA + Fenton's reagent, Lane 5: λ-DNA without fungal extract, Lane 6: Resveratrol (1mg/ml) + λ-DNA+ Fenton's Reagent.

Chapter – 6

DISCUSSION

Endophytic fungi are the microbes which colonize inside plant tissue without causing apparent harm to the host. Besides upon colonization of host plant endophytes synthesize an array of secondary metabolite which may defend the host plant against survival and stress conditions and in turn the host plant supply nutrients and habitat for endophytic fungi. Therefore in addition to interaction of endophytes with the host plant, due to the course of co-evolution endophyte acquires the property of their host plants and produces analogue bioactive compounds out of which some have medicinal value (Strobel et al. 2003; Kusari et al. 2012; Ul-Hassan et al. 2012).

It is now proven that microorganisms have some cryptic genes (Silent gens) which remain unexpressed under normal lab condition but can be expressed when treated with *invitro* epigenetic modifiers like AZA and SAHA (Chemical elicitors) and leading to modulate the production of secondary metabolite which are very important for pharmaceutical, agrochemical as well as cosmeceutical industries (Abdulla et al. 2013; Hassan et al. 2012; Kumar et al. 2016).

Resveratrol is natural polyphenolic stilbene, firstly identified as major lead compound of the French paradox which is related to its antioxidant potential, it is also reported for its multifarious application in various diseases like neurodegenerative disorder, cardiovascular disease, and also have ability to promote the longevity of *Saccharomyces cerevisiae*. Due to these reasons resveratrol is now widely used as a nutraceutical and in cosmetics (Jimenez et al. 2007; Wang et al. 2013).

In the current research, a comparative study was performed for enhancement of resveratrol production via using different concentration of chemical elicitor which modulates the secondary metabolite production. *Xylaria* sp. # 22(P) VVLPM is a member of family *Xylariales,* though *Xylaria* sp. has been previously reported from *Vitis vinifera* (Mostert et al. 2000; Dwibedi et al. 2018). HPLC analysis played a crucial character for identification as well as quantification of resveratrol. Via HPLC analysis it is found that resveratrol concentration was maximum and enhanced in case of treatment with 5 µm SAHA and by 10 µm AZA followed by 10 µm SAHA and 5 µm AZA. In earlier reports on *Colletotrichum gloeosporioides* an endophytic fungus was also reported to have increase in cryptic compounds as well as their antimicrobial potential by using epigenetic modifiers grape extract and turmeric extracts (Sharma et al. 2017).

Endophytic fungi are renowned for their antimicrobial as well as antioxidant potential (Kahnduja and Bhardwaj 2003; Shrikanta et al. 2015). The resveratrol produced by endophytic fungus # 22(P) VVLPM in our study exhibited strong antioxidant potential. After treatment with different concentration of chemical elicitor like AZA and SAHA a significant increase in antioxidant potential was obtained. In case of DPPH increase in scavenging potential was found as compared to wild strain. Treatment with 5 µm SAHA and by 10 µm AZA was showing strong antioxidant potential among all epigenetic variants as compare to wild strain. In case of TEAC also same trend as in case of DPPH was obtained, whereas in case of FRAP, TPC and TFC the increment was observed in case of modification done using 3 µm AZA and SAHA , 5 µm AZA and SAHA, 10 µm AZA and SAHA only as compared to #22(P)VVLPM control. Kahnduja and Bhardwaj (2003) in their study found that RESV showed stronger antioxidant potential than catechin, myricetin and fisetin. Shrikanta et al. (2015) also observed antioxidant property which was attributed due to the presence of RESV in grapes, jack fruit and jamun.

Epigenetic modifiers such as HDAC inhibitors (SAHA) and DMNTs (AZA) inhibitors represents interesting chemical tool to express cryptic or silent gene which are unexpressed under standard laboratory condition.Earlier Successful attempt of epigenetic modulation have been made using 5- azacytidine in mycodiesel producing endophytic fungus *Hypoxylon* sp. (CI-4), producing a wide number of volatile organic compounds, including 1,8-cineole, 1-methyl-1,4-cyclohexadiene and cyclohexane, 1,2,4-tris(methylene), which was selected as a candidate for the modulation of VOC production (UL-Hassan et al. 2012). Chunshun et al. (2017)performed co-culturing experiment of endophytic fungi *Camporesiasambuci* (FT1061) and *Epicoccum sorghinum* (FT1062) and reported a new N-methoxypyridone analog, together with four known compounds. All these research strongly shouted that microbes have many genes which remain unexpressed under standard laboratory condition, and they need some epigenetic modifiers to activate these genes, leading to enhancement of novel secondary metabolite.

Chapter – 7

CONCLUSION

The treatment with different concentration of epigenetic modifiers such as HDAC inhibitors (SAHA) and DMNTs (AZA) inhibitors were found as a potent chemical tool for induction of cryptic gene in the endophytic fungus *Xylaria psidii* for enhancement of the resveratrol production as well as antioxidant potential. The present study shows positive correlation between resveratrol content and antioxidant potential.

Chapter – 8

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