Isolation, characterization and process optimization of resveratrol production from endophytic fungi

A

Thesis submitted

In the partial fulfilment of the requirements for the award of degree of

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IN

BIOTECHNOLOGY

Submitted By

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July 2019

CERTIFICATE

Certified that the thesis entitled "**Isolation, characterization and process optimization of resveratrol production from endophytic fungi**" submitted by Mr. Vagish Dwibedi, Reg. no. 901300012 in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a record of candidate's own independent and original research work carried out by himself under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "**Isolation, characterization and process optimization of resveratrol production from endophytic fungi**" in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy at Department of Biotechnology, Thapar Institute of engineering and Technology, Patiala is an authentic record of my own work during the period from January 2014 to June 2019, under the supervision of Prof. Sanjai Saxena, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab. This report has not been submitted for the award of any degree or certificate in this or any other university.

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Date: 26/7/2019

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VAGISH DWIBEDI

DEDICATED TO ...

MY PARENTS

(SATHISH KUMAR DWIBEDI & PREMA DEVI)

AND

MY GURUJEE

(PROFESSOR SANJAI SAXENA)

PUBLICATIONS

1. **Dwibedi V**, Saxena S (2018):"*Arcopilus aureus*, a Resveratrol-Producing Endophyte from Vitis vinifera". Applied Biochemistry and Biotechnology https://doi.org/10.1007/s12010-018-2755-x (IF= 2.2)
2. **Dwibedi V**, Saxena S (2019):"Diversity and phylogeny of resveratrol-producing culturable endophytic fungi from *Vitis* sp. in India" 3 Biotech https://doi.org/10.1007/s13205-019-1712-x (IF=1.80)
3. **Dwibedi V**, Kalia S and Saxena S (2019):**"**Isolation and enhancement of resveratrol production in *Xylaria psidii* by exploring the phenomenon of epigenetics: using DNA methyltransferases and histone deacetylase as epigenetic modifiers" Molecular Biology Reports (IF=2.11)
4. **Dwibedi V**, Saxena S (2019):"*In vitro* anti-oxidant, anti-fungal and anti-staphylococcal activity of Resveratrol producing endophytic fungi" Proceedings of the national academy of science, India Section B: Biological science https://doi.org/10.1007/s40011-019-01098-6 (IF=0.6)

Other Publications

5. Kumar S, Pagar AD, Ahmad F, **Dwibedi V**, Wani A, Wani A, Prasad V, Chhibber M, Saxena S, Singh I (2018):"Xanthine oxidase inhibitors from an endophytic fungus *Lasiodiplodia pseudotheobromae*" Bioorganic chemistry https://doi.org/10.1016/j.bioorg.2018.12..008 (IF=4.0)
6. Singh D, Babbar A, Jain V, Gupta D, Saxena S and **Dwibedi V** (2019): Synthesis, characterization, and bioactivity investigation of biomimetic biodegradable PLA scaffold fabricated by fused filament fabrication process. Journal of Brazilian society of Mechanical science and engineering https://doi.org/10.1007/s40430-019-1625-y (IF=1.80)

Publications (Revised Submitted)

7. **Dwibedi V**, Saxena S (2019): "Impact of precursor feeding, dietary supplementation, microbial and chemical elicitors on resveratrol producing endophyte *Arcopilus aureus* #12 VVLPM "World Journal of microbiology and biotechnology (IF=2.1)
8. **Dwibedi V**, Saxena S and Walia H (2019):"Comparative Study of the Resveratrol content, *in vitro* antioxidant and antidiabetic potential of Wine grapes widely produced in India" Journal of microbiology, biotechnology and food sciences (IF=1.5)

9. **Dwibedi V**, Sandhu J and Saxena S (2019): "First report of endophytic*Biscogniauxia* sp. from Indian grapevine, as a novel source of piceatannol"World Journal of microbiology and biotechnology (IF=2.7)
10. **Dwibedi V**, Rath S, Prakash R and Saxena S (2019): "Optimization of fermentation conditions through response surface methodology for enhancement of resveratrol production in *Arcopilus aureus* and its purification" Journal of Applied Microbiology (IF=2.7)

CONFERENCE PROCEEDINGS

ORAL PRESENTATION

1. **Dwibedi V** and Saxena S (2018): "*Isolation and enhancement of resveratrol production in Xylaria psidii by exploring the phenomenon of epigenetics: using DNA methyltransferases and histone deacetylase as epigenetic modifiers*" International symposium on Fungal biology: Advances, Applications and Conservations & 45th Annual meet of Mycological Society of India 19-21 November (2018), National fungal collection center Pune Maharashtra
2. **Dwibedi V** and Saxena S (2018): "Development of a bio-based packing material for post-harvest preservation of fruits and vegetables"DST- Lockheed Martin- Tata Trust _IIGP 2.0 (2018) (India Innovation Growth Programme 2.0) Innovation ID: IIGPUIBSUNB, Indian Institute of Technology Mumbai
3. **Dwibedi V** (2019):"Technological Challenge In Enhancing Shelf Life Of Post -Harvest Agri-produce By Innovative Bio-based Interventions"Design Thinking and IP workshop 13-16 march 2019 organised by DST- Lockheed Martin- Tata Trust _IIGP 2.0 (2018) (India Innovation Growth Programme 2.0) at Indian Institute of Technology Mumbai
4. **Dwibedi V** (2019):Development of a bio-based packing material for post-harvest preservation of fruits and vegetables"DST- Lockheed Martin- Tata Trust _IIGP 2.0 (2018) (India Innovation Growth Programme 2.0) Innovation ID: IIGPUIBSUNB, in Design thinking boot camp at Indian Institute of Management Ahmadabad

POSTER PRESENTATIONS

1. **Dwibedi V,** Kaur R, Walia H, Sodhi G and Saxena S* (2018): "Isolation of fungal endophytes to induce abiotic stress (extreme temp. and salinity) tolerance in wheat (*Triticum aestivum*)" International conference on food security food security challenges and opportunities, Department of Biotechnology TIET Patiala, Punjab

2. **Dwibedi V** and Saxena S (2018): "Endophytic fungi – A Unique bioresource for Resveratrol Production" International Conference on Drug Discovery: Biotech and Pharma at Cross Roads, Department of Biotechnology TIET Patiala, Punjab
3. **Dwibedi V** and Saxena S (2016): "Endophytic fungi a new source of industrially important compound – Resveratrol" National Conference on Emerging Trends in Fungal Biology and Plant Protection (ETFPP-2016), Centre of Advanced Study in Botany, Institute of Science, Banaras Hindu University, Varanasi

AWARDS

1. Best Poster Award (2018) at International Conference on Drug Discovery: Biotech and Pharma at Cross Roads, Department of Biotechnology TIET Patiala, Punjab
2. Winner in Down select competition- DST- Lockheed Martin- Tata Trust _IIGP 2.0 (2018) (India Innovation Growth Programme 2.0) Innovation ID: IIGPUIBSUNB (The Award carries a financial support up to 25 Lac INR.) "Development of a bio-based packing material for post-harvest preservation of fruits and vegetables"

WORKSHOPS/ TRAININGS

1. Three days' national workshop on "Computational Drug Design using molecular docking and virtual screening" sponsored by Schrodinger (26-29 April 2018) at TIET Patiala, Punjab
2. One day national workshop on "Intellectual Property Rights and Patenting" sponsored by Punjab Pollution boardatDepartment of Biotechnology TIET Patiala, Punjab
3. Seven days National workshop on National Workshop on (19-25 Feb 2015) "Isolation, Characterization and Conservation of Endophytes and Their Potential Applications in Agriculture and Allied Disciplines"at National fungal culture collection of India, Mycology and Plant Pathology group, Agharkar Research Institute, Pune.
4. 5 days' workshop on " Design Thinking and IP Workshop (12-16 march 2019)" sponsored by DST-Lockheed Martin- Tata Trust _IIGP 2.0 (2018) (India Innovation Growth Programme 2.0) at Indian Institute of Technology Mumbai
5. 7 days' workshop on " Bootcamp on Design Thinking (30-06 July 2019)" sponsored by DST-Lockheed Martin- Tata Trust _IIGP 2.0 (2018) (India Innovation Growth Programme 2.0) at Indian Institute of ManagementAhmadabad

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S.no	Symbol	
1.	%	percentage
2.	′	minutes
3.	≥	equals to or greater than
4.	°	degree
5.	°C	degree celsius
6.	µg	microgram
7.	µL	microliter
8.	µm	micrometer
9.	cm	centimetre
10.	g	gram
11.	h	hour
12.	L	litre
13.	M	molar
14.	mA	milliampere
15.	mg	milligram
16.	min	minute
17.	mL	millilitre
18.	mm	millimetre
19.	mM	millimolar
20.	mPa	megapascel
21.	nm	Nanometer
22.	psi	pounds per square inch
23.	rpm	revolutions per minute
24.	S	second
25.	U	unit (activity)
26.	V	Volume
27.	v/v	volume by volume
28.	w/v	weight by volume
29.	µg /mL	microgram per milligram
30.	µM	micromolar
31.	mg/mL	milligram per millilitre
32.	U/mL	units per millilitre

33.	ng/µl	nanograms per microliter
34.	bp	base pair
35.	α	alpha
36.	β	beta
37.	±	plus minus
38.	~	approximately
39.	mm^2	millimeter square
40.	mAU	milli absorbance unit
41.	V_m/V_f	volume of media to volume of flask
42.	psi	pounds per square inch
43.	$	dollar

LIST OF ABBREVIATIONS

S. No.	Abbreviations	Full form
1.	*4CL*	4 coumarate: coenzyme A ligase
2.	4-coumaroyl-CoA	4-Coumaroyl-coenzyme A
3.	ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
4.	ACN	Acetonitrile
5.	ADP	Adenosine diphosphate
6.	AMP	Adenosine monophosphate
7.	ANOVA	Analysis of variance
8.	ATCC	American type culture collection
9.	ATP	Adenosine triphosphate
10.	AZA	5-Azacytidine
11.	BBD	Box-Behnken Design
12.	BLAST	Basic Local Alignment Search Tool
13.	C18	Carbon 18
14.	CAGR	Compound annual growth rate
15.	CDA	Czapek Dox Agar
16.	CDB	Czapek Dox Broth
17.	CF	Colonization frequency
18.	CH_3OH	Methanol
19.	$CHCl_2$	Dichloromethane
20.	$CHCl_3$	Chloroform
21.	CHN	Carbon Hydrogen Nitrogen
22.	CMA	Corn Meal Agar
23.	CVDs	Cardiovascular disorders
24.	CZB	Czapekdox broth
25.	DAD	Diode array detector
26.	DCM	Dichloromethane
27.	DMSO	Dimethyl sulfoxide
28.	DNA	Deoxyribonucleic acid
29.	dNTP	Deoxynucleotide triphosphate
30.	DPPH	2,2-diphenyl-1-picrylhydrazyl
31.	*E.coli*	*Escherichia coli*
32.	EDTA	Ethylenediaminetetraacetic acid,

33.	ESI	Electron spray ionization
34.	FMA	Fusarium minimal agar
35.	FRAP	Ferric reducing antioxidant power
36.	FW	Fresh weight
37.	GLA	Grape leaf agar
38.	H_2O_2	Hydrogen peroxide
39.	H_2SO_4	Sulhpuric acid
40.	HDAC	Histone deacetylase
41.	HPLC	High Pressure Liquid Chromatography
42.	IC_{50}	Inhibitory concentration 50
43.	IR	Infra red
44.	ITS	Internal Transcribed Spacer
45.	JS	Jaccard's Index of Similarity
46.	LC-MS	Liquid chromatography-mass spectroscopy
47.	LOD	Limit of detection
48.	LOQ	Limit of quantification
49.	m/z	Mass to charge ratio
50.	MCL	Maximum Composite Likelihood
51.	MEA	Malt Extract Agar
52.	MEB	Malt Extract Broth
53.	MEGA	Molecular Evolutionary Genetics Analysis
54.	MHA	Muller Hinton agar
55.	MTCC	Microbial type culture collection
56.	NAD	Nicotinamide dinucleotide
57.	NADH	Nicotinamide dinucleotide (reduced)
58.	NADPH	Nicotinamide dinucleotide phosphate
59.	NCBI	National Center for Biotechnology Information
60.	NCTC	National Collection of Type Cultures
61.	NIH	National Institute of Health, USA
62.	NMR	Nuclear magnetic resonance
63.	OD	Optical Density
64.	OMA	Oat Meal Agar
65.	OVAT	One variable at time
66.	PBS	Phosphate buffer saline

67.	PCR	Polymerase chain reaction
68.	PDA	Potato Dextrose Agar
69.	PDB	Potato Dextrose Broth
70.	pH	Pouvoir hydrogen or Power of Hydrogen
71.	PTLC	Perparative thin layer chromatography
72.	RB	Richard's Broth
73.	RESV	Resveratrol
74.	R_f	Retention factor
75.	ROS	Reactive oxygen species
76.	RSM	Response surface methodology
77.	SAHA	Suberoylanilide hydroxamic acid
78.	*Sau*	*Staphylococcus aureus*
79.	SD	Standard deviation
80.	SDA	Sabouraud dextrose agar
81.	SNA	Synthetischer nahourstoffarmer agar
82.	*STS*	Stilbene synthase
83.	TAE	Tris acetate EDTA
84.	Taq	*Thermus aquaticus*
85.	TCA	Tri-chloro acetic acid
86.	TE buffer	Tris-EDTA buffer
87.	TEAC	Trolox equivalent antioxidant capacity
88.	TLC	Thin layer chromatography
89.	Tm	Melting temperature
90.	Tris	Tris (hydroxymethyl) aminomethane
91.	TSB	Tryptone Soya Broth
92.	USA	United States of America
93.	USFDA	United States Food and Drug Administrations
94.	UV	Ultraviolet
95.	WA	Water agar
96.	WHO	World Health Organization
97.	YEPB	Yeast Extract Peptone Dextrose Broth
98.	YEPDA	Yeast Extract Peptone Dextrose Agar
99.	YMG	Yeast and malt extract

EXECUTIVE SUMMARY

The present study focusses on the diversity of resveratrol producing fungal endophytes associated with different wine grape varieties grown in India. A total of 145 endophytic isolates were recovered of which 55 were from Pune region, 54 from Nashik while 26 were from Lucknow and 9 from Bengaluru. Only 30% isolates exhibited extracellular resveratrol production in the preliminary screening. However only 14 fungal endophytes exhibited significant resveratrol production in the range of 4.4g/L-89.1g/L in liquid cultures which was evaluated by HPLC analysis. Four potent resveratrol producing endophytes isolated were #12VVLPM; #4(P)VVLNM; #22(P) VVLPM and #19VVLPM. #12 VVLPM was identified to be *Arcopilus* aureus, while #22(P) VVLPM was identified to be *Xylaria psidii*. Both *Arcopilus aureus* and *Xylaria psidii* have so far not been reported to be resveratrol producers. The other two isolates viz. #4(P)VVLNM and #19VVLPM are generalists identified as *Fusarium solani* and Fusarium *equiseti*. Highest resveratrol production, 89.1 g/l was recorded in #12VVLPM identified as *Arcopilus aureus*.

The fungal resveratrol exhibited a strong anti-oxidant activity in all the tested assays.The endophytic isolate #12VVLPM exhibited the best activity similar to the standards in the entire anti-oxidant assay performed during the present course of investigations. Yet another important result was the anti-mycotic activity against different plant as well as human pathogenic fungi tested. The highest inhibition was observed against *B. cinerea* (57.8± 0.8%) followed by *Cercospora beticola* (ATCC24888) by extracts of #12VVLPM while the least inhibition was observed in #22(P) VVLPM and #4(P) VVLNM against same pathogen. Among the resveratrol producing endophytic isolates #12VVLPM exhibited the best inhibitory activity against all the cultures tested in the panel.

The fungal resveratrol was obtained in pure form ethnolic extract by eluting it in a column with mobile phase of MeOH: DCM :: 1.75:98.25. Fungal resveratrol (25 mg) was observed pure by TLC system 5% MeOH: DCM and concentrated by rotary evaporator. The homogeneity of the purified fungal resveratrol was confirmed by HPLC, which exhibited a single, symmetrical peak at R_t 3.36 min on C_{18} reverse phase column similar to that of standard resveratrol. For further confirmation of the presence of fungal resveratrol purified from the spent broth of *Arcopilus aureus*, ^{1}H-NMR, ^{13}C-NMR. MS and HR-MS, FTIR analysis was carried out which revealed the presence and purity of fungal resveratrol.

Optimization of different physic-chemical and nutritional parameters affecting resveratrol production by 'one-variable-at-a-time' approach resulted in the production 1.22 fold increases of (109.61[a] ± 0.7 µg/ml resveratrol from 89.1[a] ± 0.08 µg/ml from initial yield of resveratrol. The interactions of the most influential factors obtained by one-variable-at-a-time method were further optimized using a statistical approach, response surface methodology (RSM). This finally resulted in 1.49 fold increase production of resveratrol (133.5 µg/ml) yield of resveratrol. After optimization of various physiological and nutritional parameters using one variable at a time and RSM using shake flasks, the optimized conditions were put to test for epigenetic modification. This finally resulted in 1.85 fold increase yield of resveratrol i.e. 165.0 µg/ml. The present study provides prime information on a novel source for the fermentative production of the billion dollar drug resveratrol by the endophytic fungus *Arcopilus aureus*. Fungal resveratrol was found to be as efficient as that of standard resveratrol which paves new way for exploitation of these bio actively potent endophytic fungi for industrial bioprocess. This study a step forward to proving that endophytic fungi are able to produce billion dollar drugs which are highly demandable due to its multifarious application and paves a new way for replacement of conventional sources of these molecules by serving as alternative.

Chapter- 1

INTRODUCTION

1.0. INTRODUCTION

Resveratrol is a tri-hydroxy stilbene and is a member of naturally occurring polyphenols (Figure 1.1). Currently *trans*-resveratrol is a molecule of great commercial interest due to its multifactorial activities which are generally sought by pharmaceutical, cosmeceutical and nutraceutical industries. Resveratrol came into limelight when Renaud and De Lorgeril, (1992) related the possible health aid attributed to moderate and regular consumption of red wine (famously known as the French Paradox).

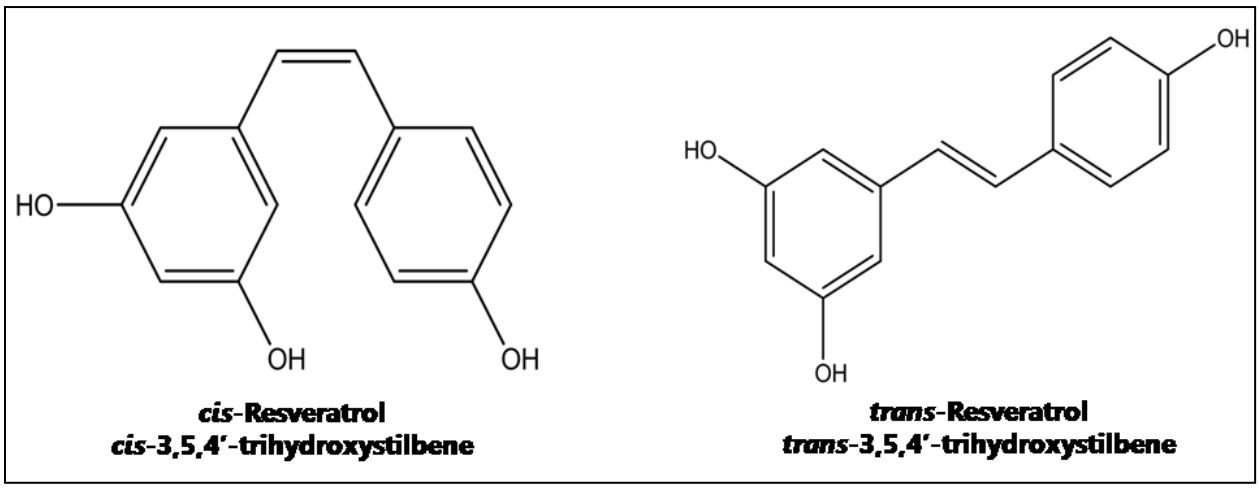


Figure 1.1: Structure of *cis*-resveratrol (left) and *trans*-resveratrol (right)

Ever since the concept of french paradox, *trans*- resveratrol has gained a great scientific attention, thereby leading to exploration of its biological activities. Today resveratrol has been associated to a plethora of beneficial health effects such as anti-oxidant, anti-inflammatory, prevention of cardiovascular diseases, diabetes, cancer apart from anti-ageing properties (Saxena and Srivastava, 2014; Duan et al., 2016; Kou et al., 2017; Turrini et al., 2018). Phytochemical studies have indicated resveratrol to be present in white hellebore (*Veratrum grandiflorum* O. Loes) as early as 1940's and later in 1963 from roots of *Polygonum cuspidatum*. *P. cuspidatum* has been used traditionally as an anti-platelet as well as anti-inflammatory agent in Chinese and Japanese herbal medicines. Since then resveratrol has been reported from 72 plant species till date, though not all of them are edible. It is present in high concentration in *Vitis vinifera* as a phytoalexin in response to fungal infections.

In plants resveratrol is primarily synthesized as a phytoalexin which is produced in response to fungal attack, UV irradiation or mechanical injury. As resveratrol (*trans*- resveratrol) has multiple

beneficial effects, it is being increasingly used as a constituent of health supplements and wellness products apart from being studied as a pharmacophore for drug development. Frost & Sullivan (2013) reported global supply market of resveratrol to be valued at $50.2 million with 90% into health and food supplement in the United State of America. The global resveratrol market which was valued at US $69 million in 2017 is projected to witness a CAGR of 9.1% in the forecast period of 2018-26.

Commercially, resveratrol is available in both pure forms in native as well as artificial combinations with polyphenols. *Trans*- resveratrol is overall stable compound but may become unstable when it comes in contact with light, and it is the only *trans*-isomer that has been consistently associated to multifarious health benefit in pharmacological as well as clinical studies while *cis*-resveratrol has limited success as an anti-platelet agent (Zhang et al., 2009). Resveratrol for nutritional and wellness supplements is predominantly resourced from the plant *P. cuspidatum* (syn. *Fallopia japonica*) root extracts. The leading producer of *trans*-resveratrol from root extracts is China using an extraction process. The level of *trans*-resveratrol purity in these extracts varies between 49%-99% (Zhang et al., 2006; Fan et al., 2010). Alternative sources are therefore being extensively explored to meet the rising demands. Researchers have attempted several methods for resveratrol production like chemical synthesis, semi-synthesis route using natural precursor, and *in vitro* cell culture based production (Tassoni et al., 2005; Barbulova et al., 2014). The drawbacks of these methods are that they required a large number of reaction steps which is challenging to pursue, and extraction of precursor for semi-synthesis of resveratrol is cost intensive proposition hence novel microorganisms are being screened as potential substitute for an environment friendly and relatively simple and economical method of resveratrol production (Tassoni et al., 2005; Beekwilder et al., 2006; Fan et al., 2010).

The resveratrol biosynthesis in plants is through the phenylpropanoid pathways, p-coumaric acid happens to be an essential intermediate which gets converted into p-coumaryl CoA through the enzyme 4 coumarate: coenzyme A ligase subsequent to which stilbene synthase or resveratrol synthase condenses 4 coumaryl co A with 3 molecules of malonyl co A to form resveratrol (Parage et al., 2012; Vannozzi et al., 2012). To meet the rising global demand of resveratrol, plant extraction is the classical method currently being used, however its major

drawback is low abundance in natural material which results due to seasonal, environmental or regional variations in the source. Yet another constraint is the complexity of the synthetic pathway and low total yield by chemical synthesis. Hence biotechnological approaches are explored as they offer the option of scalability using low cost or renewable resources which hold a great promise in commercial production of low volume, high value products.

Therefore, using recombinant DNA technology, researchers attempted mirror image of the resveratrol biosynthetic pathway of plants in microbes or developing plant tissue culture technology. Genetically modified yeast (*Saccharomyces cerevisiae*) and *E. coli* have been developed for production of *trans*-resveratrol (Beekwilder et al., 2006). However, there are issues related to yield when metabolically engineered micro-organisms are exploited vis a vis global demand of approximately 100 tons/year (Kiselev, 2011). Hence, scientists are exploring novel approach of mimicking the resveratrol biosynthetic genes in different microorganisms. Another auspicious approach is the exploration of the novel endophytes which may mimic the property of host plant under free fermenting conditions. It is already proven that due to close association and co-evolution of the endophytes with their host plant, they have potential of producing the same analogous compounds or phytochemicals (Tassoni et al., 2005).

Several endophytes possessing capacity to produce putative plants phytochemicals are being studied for their possible use as commercial strains for production via fermentation. Hence this technology could be cost-effective, environment friendly and commercially exploited for their large-scale production in future. Manipulation of fermentation conditions has been proved to be a promising strategy nowadays for obtaining novel compounds as well as enhancement of secondary metabolite production. Several types of research on fungal genome has already proved that inhibition of DNA methyltransferases and inhibition of histone deacetylase activity through an epigenetic modulator or gene disruption leads to the transcriptional activation of silent gene cluster which can improve the production of secondary metabolite in the free fermentative medium. Epigenetic regulation of gene transcription can be applied to a wide range of fungi without any prior knowledge of the genome sequence. Therefore, it is a relatively easy method for inducing the expression of the gene which is responsible for the production of

novel host secondary metabolite (Ul-Hassan et al., 2012; Li et al., 2017; Sharma et al., 2017; Deshmukh et al., 2018).

1.1. Exploration of endophytic fungi as resveratrol producers

The term Endophyte (Gr. endon, within; phyton, plant) was initially used by de Bary (de Bary 1866). Endophytes are a group of microorganisms comprising of fungi, actinomycetes and bacteria which reside within the intra- and intercellular plant tissues for all or a part of their life cycle. Fungal endophytes majorly comprise of mitosporic and meiosporic ascomycetes, coelomycetes and hyphomycetes which colonize the healthy living tissues of the host plant by latent infection in order to obtain shelter and nutrition (White and Torres, 2010; Aly et al., 2011; Kusari et al., 2012). These endophytes, in turn, produce an array of signal molecules to overcome the inherent defence mechanisms of the host plant which purportedly play a role in aiding the host plant in combating the different types of biotic and abiotic stresses (White and Torres, 2010; Aly et al., 2011). The signal molecules so produced by these endophytic microbes may possess novel activities which may be relevant to have potential applications in medicine, agriculture, and food industry. Many bioactive compounds possessing antimicrobial, anticancer, insecticidal and cytotoxic activities have been discovered from endophytic fungi. Some endophytic fungi develop the ability to produce putative plant phytochemicals, the very famous being *Taxomyces andreanae* from *Taxus brevifolia* producing Taxol in fermentation medium (Stierle et al., 1993). This generated ample interest in research groups to explore endophytic fungi to produce medicinal compounds which were being resourced from plants. Podophyllotoxin, is biosynthetically produced by *Podophyllum versipelle*, is highly admired as the precursor to be clinically used as an anti-cancer drug such as etoposide and teniposide. *Phialocephala fortinii* and *Trametes hirsuta* are the two endophytic fungal isolates which have been isolated from *Podophyllum peltatum* and *Podophyllum hexandrum,* respectively, which produce podophyllotoxin in the fermentation medium (Eyberger et al., 2006; Puri et al., 2006). Similarly, endophytic fungi for production of other plant products such as camptothecin (Puri et al., 2006; Kusari et al., 2012) and Vincristine (Aly et al., 2011) have also been identified and are being developed for the commercial bulk production of these plant-based medicinal compounds through fermentative route.

Thus endophytic fungi exhibit the biosynthetic capabilities of putative phytochemicals produced in their host plants where they exist asymptomatically (Moraga et al., 2019; Bacon and Hinton, 2019). This capacity to biosynthesize host phytochemicals may be attributed to horizontal gene transfer during the course of co-evolution with the plant driven by extreme environmental conditions (Gluck and Slot, 2018; Bacon and Hinton, 2019). Using molecular tools and analysis these horizontally transferred genes present in endophytic fungi can be tracked (Gluck and Slot, 2018; Feurtey and Stukenbrock, 2018). Thus, based on this presumption it was conclude that grape plant (*Vitis vinifera*) also holds potent endophytic fungi which are capable of produce resveratrol under free fermenting conditions.

Thus, in the current study, we systematically isolated and screened the endophytic fungi of *Vitis vinifera* for their potential to produce resveratrol with future implication to use it as the food and nutritional health supplement.

Chapter- 2

Present approach

Plants are abundant with microbial organisms, which include endophyte, epiphyte, mycorrhiza, pathogen and saprophytes. Endophytic fungi are phylogenetically diverse and ubiquitous group of microorganisms that colonize the internal tissue of a host plant without exhibiting any noticeable symptoms of their existence (Schulz et al., 1993; Bacon and White, 2000; Strobel and Daisy, 2003). An endophytic fungus differs from mycorrhizal fungi by the fact that endophytes are culturable on media while most fungi participating in mycorrhizal association are not culturable on synthetic natural media (they won't grow on petriplate) (Rozpądek et al., 2019; Sidhoum and Fortas, 2019). Another difference between mycorrhizal fungi and endophytes are that endophytes resides entirely within the plant tissue while mycorrhizal are restricted to only roots (Stone et al., 2004).

Endophytic fungi have ability to synthesize the analogous bioactive compounds that originated from their host plant (Strobel et al., 2004). It is tough to assume that production of these host secondary metabolite in plant does not proceed exclusively by endophytes but is rather the consequence of concomitant plant and fungal biosynthesis (Kusari et al., 2008). Further, it remains a rhetorical question that warrants the receiving partner to execute the analogous biosynthetic chemical reactions, as available in the donor. This intriguing query can only be answered after the biogenetic clusters of host plant and endophytes have been elucidated (Wani et al., 2015; Jia et al., 2016).

Fungal endophytes majorly comprise of mitosporic and meiosporic ascomycetes, coelomycetes and hyphomycetes which colonize the healthy living tissues of the host plant by latent infection to obtain shelter and nutrition (White and Torres, 2010; Aly et al., 2011; Kusari et al., 2012). These endophytes, in turn, produce an array of signal molecules to overcome the inherent defence mechanisms of the host plant which purportedly play a role in aiding the host plant in combating the different types of biotic and abiotic stresses (White and Torres, 2010; Aly et al., 2011). The signal molecules so produced by these endophytic microbes may possess novel activities which may be relevant to have potential applications in

medicine, agriculture, and food industry (Wani et al., 2015; Harman and Uphoff, 2019).

Many bioactive compounds possessing antimicrobial, anticancer, insecticidal and cytotoxic activities have been discovered from endophytic fungi. Some endophytic fungi develop the ability to produce a putative plant phytochemicals, the very famous being *Taxomyces andreanae* from *Taxus brevifolia* producing Taxol in fermentation medium (Stierle et al., 1993). Thus endophytic fungi are considered as fountain heads of putative host phytochemicals as well as novel bioactive compounds.

Hypothesis

As resveratrol has an increasing demand globally as a nutraceutical, cosmeceutical and also by the pharmaceutical industries, there is a need to bridge the demand and supply gap. Plant extraction cannot only meet the growing demand of resveratrol since approx. 1 mg of pure resveratrol is obtained by extracting 15 kg of roots of *Polygonum cuspidatum* where it is considered to be produced abundantly.

Till date, there is very scanty or limited data on exploration of endophytic fungi for their resveratrol producing potential. Hence, it is quite pertinent to isolate, screen resveratrol endophytic fungi and optimize the resveratrol production.

Hence the specific objectives of the proposed thesis are:

1. Isolation, screening and identification of resveratrol producing endophytic fungi.
2. Purification and characterization of resveratrol from selected endophytic fungus
3. Process optimization for maximum resveratrol production by the selected endophytic fungus

Chapter- 3

Review of literature

3.0. BACKGROUND

The medicinal properties of red wine grapes date back to 4500 years which is documented in the ayurvedic book, Charak Samhita's as "Darkchasva" (fermented juice of red wine) and was used as cardiotonic (Frémont et al., 1999; Paul et al., 1999). However, this aspect was rediscovered with the so-called 'French paradox' when it was observed that French people were at a low risk of death due to coronary heart disease despite their dietary habitat as they included red wine as an standing component of their diet. This generated an impetus to research on different components of red wine as well as wine grapes. However, it was later found that the cardioprotective effect of the red wine and wine grapes was attributed to a molecule, resveratrol (Saxena and Srivastava, 2014; Dwibedi and Saxena, 2018; Snopek et al., 2018).

Today the wellness and health supplement market is one of the fastest growing markets as evident from the unprecedented spike in the global sales in the past decade. The global nutrition and supplements market is expected to reach $249.4 billion by 2020 from $183.1 billion in 2015, at a CAGR of 6.4% during the forecast period (Global wellness monitor, October 2018). These wellness and nutritional products exploit a variety of natural products and bioactive molecules for maintaining a healthy life. *Trans*-resveratrol or *trans*-3, 4, 5-trihydroxystilbene has been found to be used in health supplements, wellness products due to its multiple therapeutic effects (Parker et al., 2005; Petrovski et al., 2011; Balea et al., 2018). Today, resveratrol is a constituent of health supplements and wellness products apart from being studied as a pharmacophore for drug development. As per the Frost and Sullivan report presented in 2013, the global resveratrol stock market was valued at $50.2 million with 90% going to the cosmetics and foods supplement market in the USA(Saxena and Srivastava, 2014; Dwibedi and Saxena, 2018). In 2017 the global resveratrol market was valued at 49 million US$ and by the end of 2025 it will reach 90 million US$, growing at a CAGR of 8.0% during 2018-2025 (Global Resveratrol Market Research Report, 2018).

resveratrol is a natural polyphenol, which has garnered much attention due to its countless profitable effects on human health which is arbitrate via various biosynthetic enzymatic pathways; cellular signaling as well as modification of gene expression. It has already been proved that resveratrol have multifarious beneficial application such as reducing or delaying the

progress of various neurological disorders like dementia (Alzheimer's) and Parkinson's disease; mitigate CVD's risk (cardiovascular diseases), reduce ischemic conditions, conquer with stress and delaying activity of ageing in unicellular microorganisms as well as in vertebrates (Su et al., 2013; Fei et al., 2018; Pan et al., 2018). Interest in resveratrol, a polyphenol often considered as the most important polyphenol in red wine as well as grapes exploded in 2003, when Sinclair and his co-researcher has found that resveratrol has ability to enhance the shelf lifespan of *Saccharomyces cerevisiae*. According to Sinclair's finding resveratrol could activate a gene *Sirtuin 1* which is also activated during calorie restriction in various species including monkey (Wood et al., 2004; Baur et al., 2006; Hubbard and Sinclair, 2014; Chao et al., 2017).

Resveratrol was identified as a phytochemical since 1940's when it was first isolated from a poisonous plant white hellebore (*Veratrum grandiflorum* O. Loes) as a resorcinol derivative (hence named resveratrol) and later from the medicinal plant *P. cuspidatum* (Saxena and Srivastava, 2014; Dwibedi and Saxena, 2018). Resveratrol also act as a phytoalexin in response to the attack or as a defense bioactive compound to intercept attack by the plant pathogenic fungus *Botrytis cinerea* in grapes (*Vitis vinifera*). The figure 3.1 illustrates the extent of research undertaken as number of citations on PubMed year wise.

Other natural stilbene which is derivative of resveratrol like piceatannol shows more bioavailability and bioactivity than resveratrol but is less abundant in natural sources(Wang et al., 2002; Bradamante et al., 2004; Wang et al., 2014). Stilbenes are synthesized via the shikimate pathway, where stilbene synthase (*STS*) catalyzes the formation of simple monomeric stilbene (i.e., resveratrol, pinosylvin or piceatannol). Based on encouraging therapeutic evidence, stilbene research has fueled interest in characterizing naturally occurring stilbene compounds for their pharmacological properties (Che et al., 2016; Braga et al., 2018). Stilbenes exist as stereoisomers *i.e.* in *cis* and *trans* form, depending upon where functional groups are attached concerning each other, on either side of the double bond. The naturally occurring stilbenes are generally present in *trans*form stereoisomers (Shen et al., 2009; Akinwumi et al., 2018). It is hypothesized as well as scientifically proved that *cis* and *trans* isomers of stilbenes are responsible for different biological and pharmacological activities (Reinisalo et al., 2015; Giacomini et al., 2016).

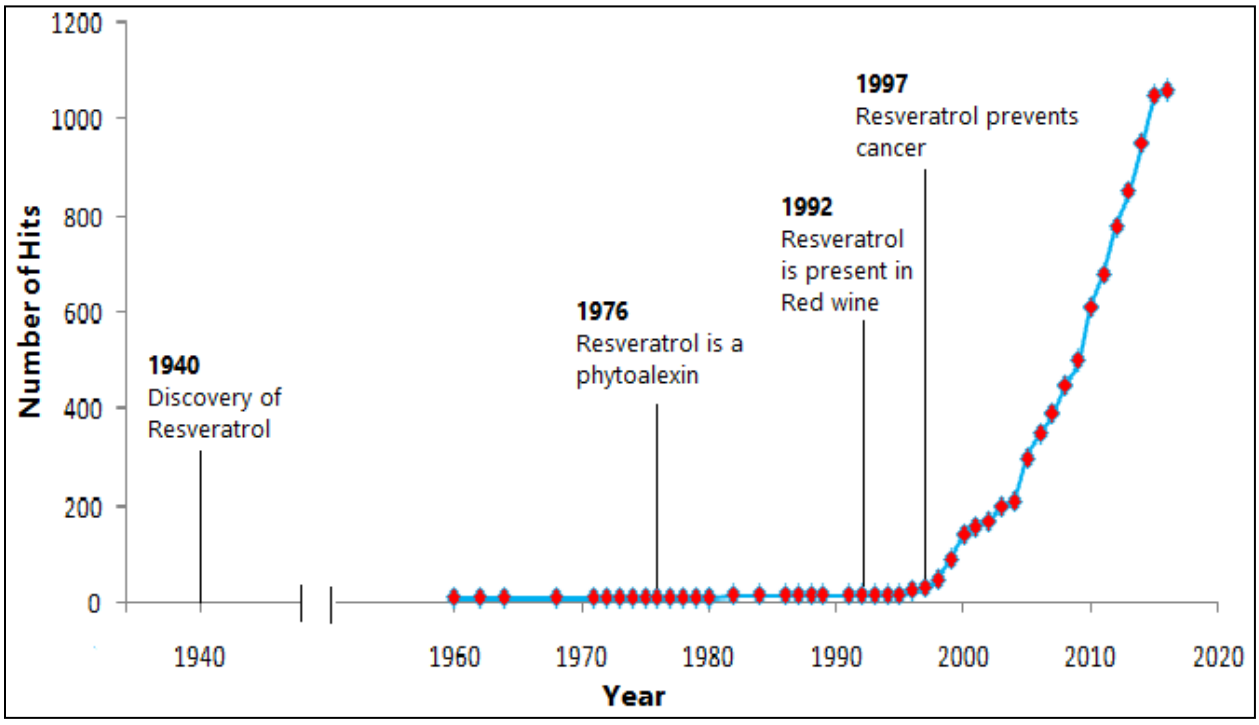


Figure 3.1. The number of resveratrol citations appearing on PubMed as a function of year (Source: Baur and Sinclair, 2006)

It is also scientifically proven that *trans* stilbene exhibits more potent activity compared to its *cis* isomer in antioxidant and anticancer assays. In one such study, it has been reported that *trans*-resveratrol to be ten times more potent in its ability to induce cell death in HL60 cell line as compared to *cis*-resveratrol (Kaur et al., 2007; Hong et al., 2009). The basic structural unit of the stilbenes comprises of two aromatic rings ligated by an ethylene bridge (C6-C2-C6), from this relatively simple structure stilbenes are generally classified into two categories, Monomer and Oligomer stilbenes.

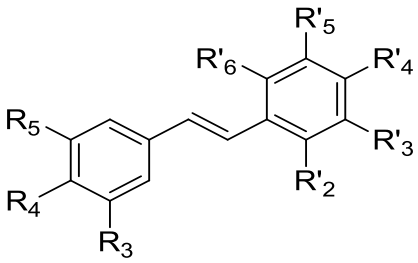


Figure 3.2. Chemical structure of stilbenes

Monomers are characterized by the number and position of hydroxyl groups, with sugar, methyl, methoxy and other residues and geometric (Stereo configuration) configuration, while oligomer stilbenes are characterized by a variety of skeletons from the oxidative condensation of

monomeric stilbenes, i.e. (dimers, trimers, tetramers) (Figure3.2, Figure 3.3) (Shen et al., 2009; Niesen et al., 2013; Tsai et al., 2017).

S.No	Stilbene	Source	Molecular formula	R_3	R_4	R_5	R_2'	R_3'	R_4'	R_5'	R_6'
1.	Pinosylvin	Scot pine, Red pine, *Genetum* sp.	$C_{14}H_{12}O_2$	OH	H	OH	H	H	H	H	H
2.	Resveratrol	*Fallopia* sp., *Polygonum sp.*, *Vitis* sp., *Arachis* sp.	$C_{14}H_{12}O_3$	OH	H	OH	H	H	OH	H	H
3.	Piceatannol	*Picea* sp., *Vitis* sp., *Vaccinium* sp., *Arachis* sp.	$C_{14}H_{12}O_4$	OH	H	OH	H	OH	OH	H	H
4.	Pterostilbene	*Vitis* sp., *Vaccinium* sp.,	$C_{16}H_{16}O_3$	OCH_3	H	OCH_3	H	H	OH	H	H
5.	Oxyresveratrol	*Artocarpus* sp., *Morus* sp.	$C_{14}H_{12}O_4$	OH	H	OH	OH	H	OH	H	H
6.	4-methoxy resveratrol	*Genetum* sp.	$C_{15}H_{14}O_3$	OH	H	OH	H	H	OCH_3	H	H
7.	Pinostilbene	*Genetum* sp., *Pinus* sp.	$C_{15}H_{14}O_3$	OCH_3	H	OH	H	H	OH	H	H
8.	Astringin	*Picea* sp.	$C_{20}H_{22}O_9$	OH	H	OGlu	H	OH	OH	H	H
9.	Isorhapontin	*Picea* sp.	$C_{21}H_{24}O_9$	OGlu	H	OH	H	H	OH	OCH_3	H
10.	Mulberroside A	*Morus* sp.	$C_{26}H_{32}O_{14}$	OGlu	H	OH	H	H	OGlu	H	OH
11.	Piceid	*Vitis* sp.	$C_{20}H_{22}O_8$	OH	H	OGlu	H	H	OH	H	H
12.	Gnetucleistol D	*Genetum* sp.	$C_{16}H_{16}O_4$	OH	H	OH	H	H	OH	H	OCH
13.	Gnetucleistol E	*Genetum* sp.	$C_{16}H_{16}O_4$	OH	H	OH	H	H	OCH_3	OCH_3	H
14.	Isorhapontigenin	*Genetum* sp., *Aiphanes* sp.	$C_{15}H_{14}O_4$	OH	H	OH	H	OCH_3	OH	H	H
15.	Rhapontigenin	*Vitis sp.*, *Genetum* sp.	$C_{15}H_{14}O_4$	H	OCH_3	OH	H	OH	H	OH	H
16.	Hydrangeic acid	*Hydrangea* sp.	$C_{15}H_{12}O_4$	H	H	OH	H	H	H	OH	COO

Figure 3.3. Some common stilbenes and their molecular formula

3.1. Biosynthesis of Resveratrol

In plants, resveratrol and its derivatives evolve from aromatic amino acid L-tyrosine and L-phenylalanine. Subsequently both aromatic amino acids follow the non-oxidative deamination reaction and get converted to phenylpropanoid (Figure 3.4). Resveratrol and other stilbenes are biosynthesized in plants via the phenylpropanoid pathway (Che et al., 2016; Braga et al., 2018; Milke et al. 2018).One of the essential intermediates of this biosynthetic pathway is p-coumaric acid which gets converted into p-coumaroyl-CoA by the enzyme 4 Coumarate: coenzyme A ligase (4CL) subsequent to which stilbene synthase or resveratrol synthase catalyses the condensation of 4 Coumaryl-CoA with 3 molecules of malonyl-CoA to form resveratrol (Li et al., 2016; Yang et al., 2016;Braga et al., 2018; Milke et al., 2018).

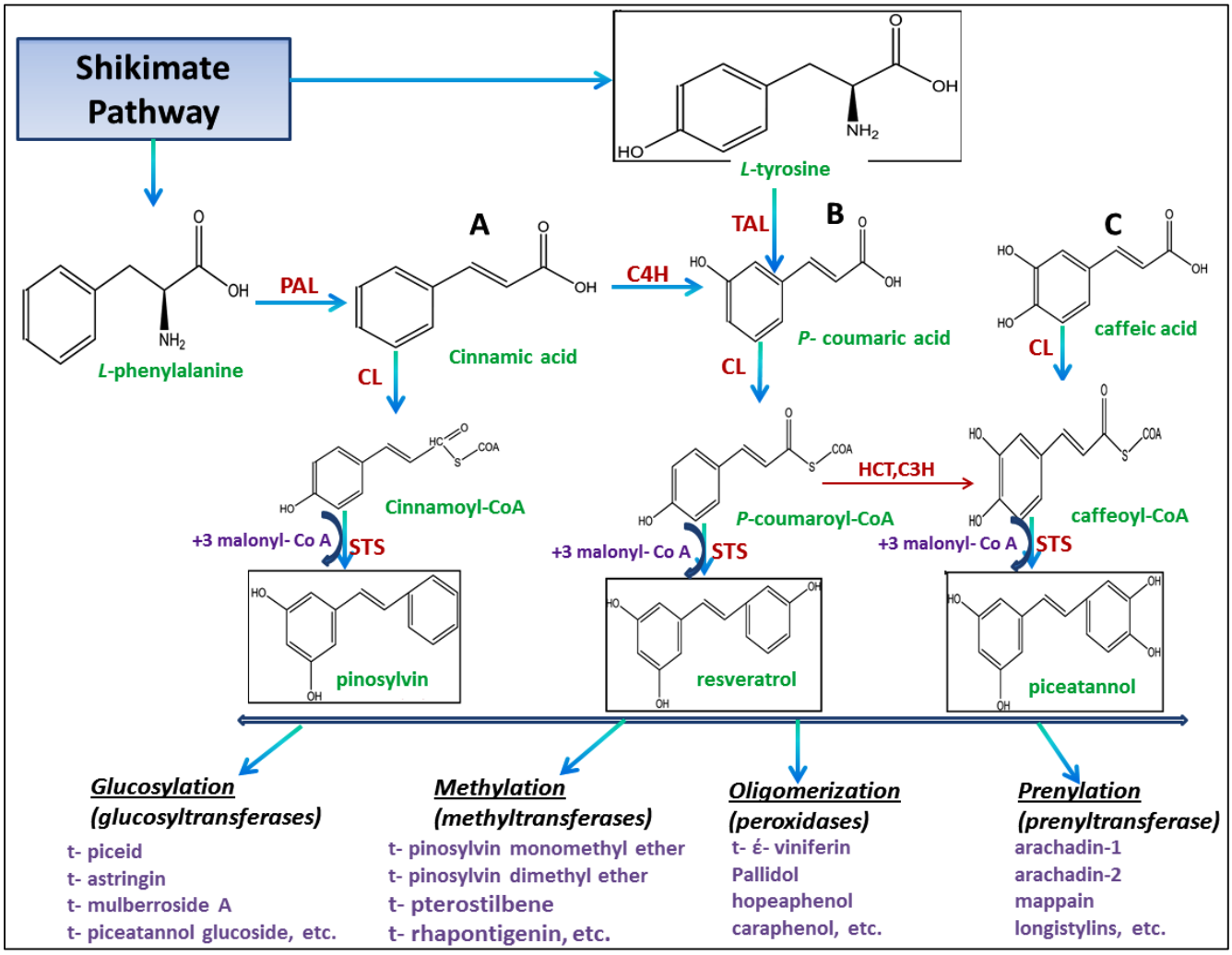


Figure 3.4. Biosynthetic pathway of resveratrol in plants

3.2. Medicinal properties of resveratrol

Resveratrol has been linked to a plethora of beneficial health effects such as anti-oxidant and anti-inflammatory activity, prevention of cardiovascular diseases, diabetes, cancer and has established itself as an anti-aging substance. An excellent safety profile of resveratrol further supports its use as an adjunct to pharmacological management of many inflammatory age-related chronic diseases. It was ascribed to lower prevalence of CVD's even though the dietary habits of French community due to red wine being an essential component of their diet. There is ample literature available on the therapeutic effects of Resveratrol (Duan et al., 2016; Turrini et al., 2018) (Figure 3.5).

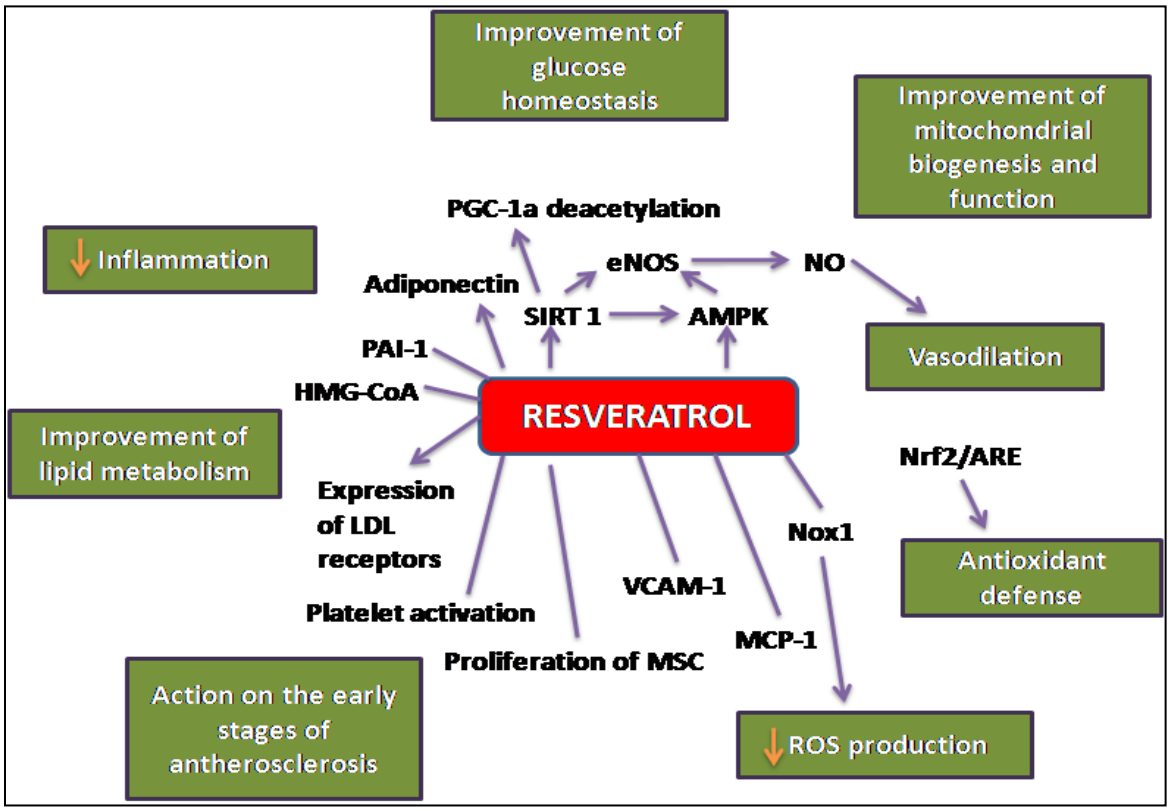


Figure 3.5. The multifarious health perspectives of resveratrol
(Source: Bonnefont-Rousselot, 2016)

3.2.1. Cardioprotective potential of resveratrol

Resveratrol, a phytoalexin found in grapes and wines, has been reported to exhibit a wide range of pharmacological properties and is believed to play a vital role in the prevention of human cardiovascular disease (Baur et al., 2006; Shi et al., 2012; Kukreja et al., 2014; Liu et al., 2016; Dwibedi and Saxena, 2018). Moderate drinking of red wine has long been known to reduce the risk of heart disease (Erdogan and Vang, 2016). It has been found that resveratrol inhibits LDL (low-density lipoprotein) oxidation which is the crucial event in the initiation of atherosclerosis (Kukreja et al., 2014; Erdogan and Vang, 2016; Chedea et al., 2017) (Figure 3.6). Rather than inhibiting the LDL, resveratrol also inhibits the platelets aggregation by which it protects from the cardiac attacks (Wang et al., 2002). Some studies on animal model clearly state that resveratrol can improve the cardiac function and survival when administered for the treatment of established strokes (Sung et al., 2014). These studies clearly indicate that resveratrol has ability to decrease the blood pressure and improved the cardiovascular functions. Resveratrol also blocks the biochemical parameters that embroil the existence of oxidative damage tissue

like catalase activities, sodium potassium activated ATP, glutathione, and superoxide dismutase activities (Mokni et al., 2013; Peuler, 2018). In another study on deoxycorticosterone acetate (DOCA)- salt hypersensitive model mouse it was found that one month of resveratrol administration exerted antifibrotic as well as anti-inflammatory potential which may play a role in attenuation of inauspicious cardiac changes which is associated with hypertension (Chan et al., 2011; Moes et al., 2018).

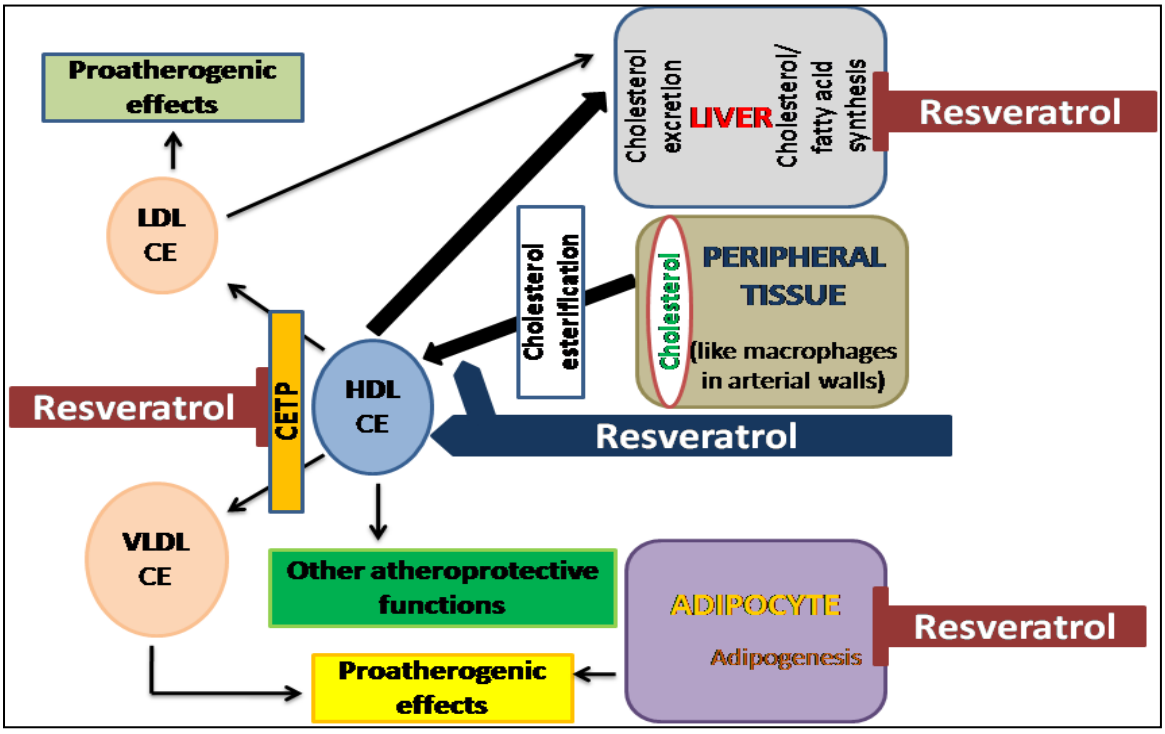


Figure 3.6. The cardioprotective potential of Resveratrol

(Source: Peuler, 2018)

The literature is available on preclinical studies exhibit the effect of resveratrol on cardiovascular disease (Leong et al., 2015; Muñoz et al., 2015; Zordoky et al., 2015) supported by the identification of multiple molecular targets like resveratrol, SIRT-1 (Sirtuin -1), AMPK (activated protein kinase), Nrf2 (nuclear factor 2) (Chong et al., 2012; Treviño-Saldaña et al., 2017). Mi-RNA play an important role in cardiac functions like cardio-protection, and heart pre-conditioning by resveratrol could result in regulation of these miRNA (Yu and Li, 2010; Leong et al., 2015; Muñoz et al., 2015). The most beneficial property of resveratrol is to stimulate the nitric oxide (NO) production by which it relaxed the endothelial mouse aortic ring constricted by phenylephrine and potassium chloride via NO production (Klinge et al., 2008; Yaziret al., 2018). All these

preclinical and clinical trials supported that resveratrol act as a cardioprotecting agent by plethora of pathways and mechanism which obstruct with the events responsible to stroke, atherosclerosis, myocardial ischemia and heart failure.

3.2.2. Chemo-preventive potential of resveratrol

Resveratrol possesses cancer chemo-preventive and cytostatic properties via initiation, promotion, and progression of carcinogenesis (Figure 3.7) (Chedea et al., 2017; Turrini et al., 2018). Figure 3.7 depicts how the resveratrol induces stress ligands in tumor cells. The stress proteins, *viz*. death receptor 5 (DR5) or the ligands for immune receptor NKG2D or ULBP's are upregulated in cancer cells on exposure to resveratrol. Therefore, the immune cells engage all these proteins which express the corresponding receptors (NKG2D) or ligand (TRAIL), which in-turn cause the death of the cancer cells. Resveratrol also inhibited growth and proliferation of Caco-2 cells through apoptosis. Human pancreatic cancer cell lines PANC-1 and AsPC-1 were also inhibited by resveratrol via apoptosis (Duan et al., 2016). Cancerous cell invades the healthy tissue with the help of matrix metalloproteinases enzyme; resveratrol was found to inhibit the matrix metalloproteinases activity (Avtanski and Poretsky, 2018; Chien et al., 2018). Tumors also develop new blood vessels by the angiogenesis process to fuel their rapid growth. Resveratrol can inhibit the angiogenesis process *in-vitro* (Nawaz et al., 2017) as well as *in-vivo* (Chen et al., 2013). Gao and Tollefsbol, (2018) have reported that resveratrol inhibits the MDA-MB-231 and MCF-7 human breast cancer cell lines by inducing apoptosis as well as modulating histone modifications and DNA methylation. Similarly, Signorelli et al., (2009) in their study found that resveratrol has ability to inhibit di hydro ceramide desaturase (DHCD) and subsequently induces the aggregation of di hydro ceramides verses ceramides thus advocate autophagy instead of apoptosis. Additionally resveratrol induces the expression and nuclear aggregation of Cyclooxygenase-2 (COX-2) without infecting COX-1, Ser-15 phosphorylation of p53 and aggregation of BcL-xS (Lin et al., 2011; Varoni et al., 2016). Tsai et al., (2018) also found that resveratrol also enhance miRNA, miR-663 expression in different human cell lines, these genes act as a tumor suppressor gene. Kumar et al. (2018) reported the novel mechanism GPE through MTA (metastasis-associated protein-1) down regulation in prostate cancer, their result showed that resveratrol inhibits the MTA 1 and MTA1-associated proteins compared to GPE, which

contains very little amount of resveratrol. Resveratrol has also been studied for its potential against colon cancer patients, and it is found that resveratrol has encouraging anticancer activity but due to low bioavailability, the ideal efficacy of resveratrol is restricted only in tumors those come into direct contact with resveratrol example, gastrointestinal cancer, skin cancer (Signorelli et al., 2009; Duan et al., 2016; Chien et al., 2018; Kumar et al., 2018; Tsai et al., 2018).

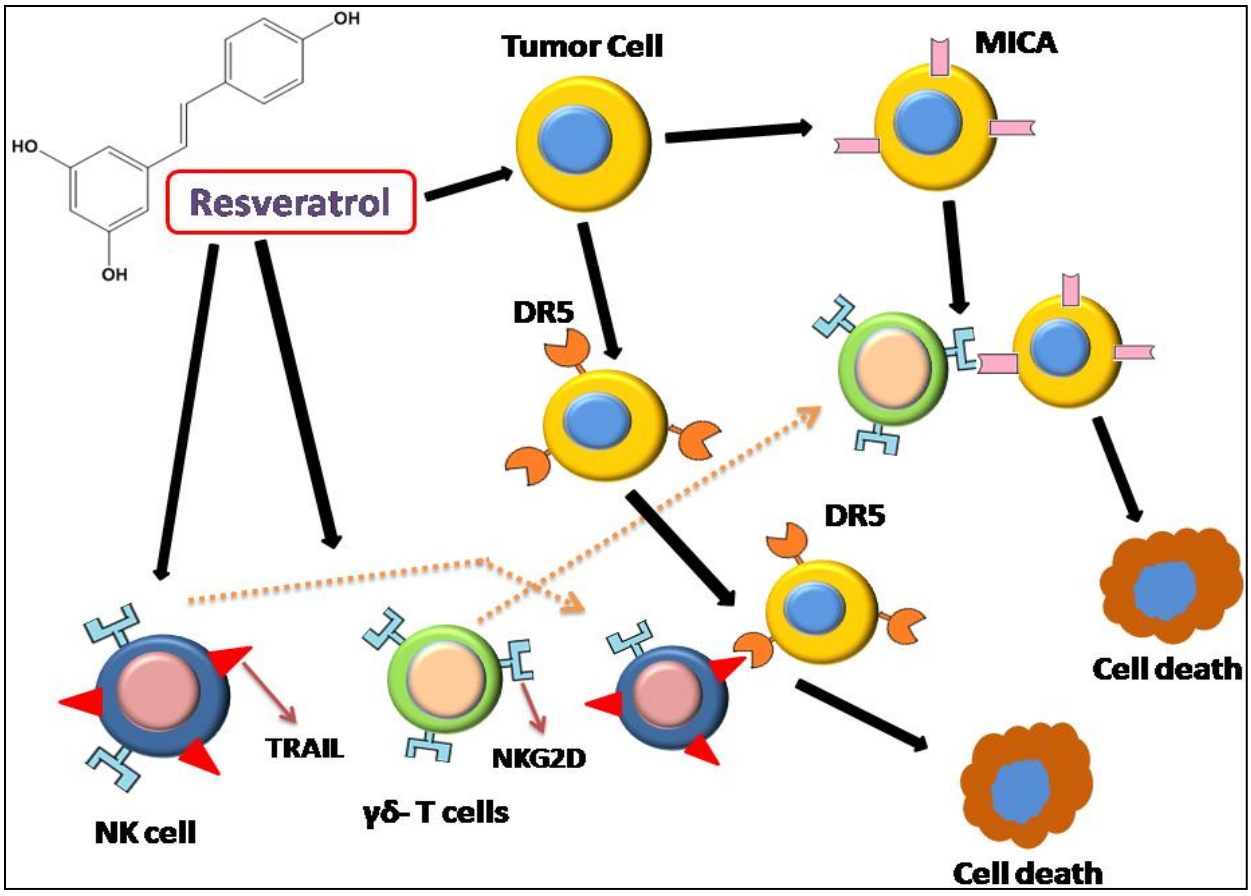


Figure 3.7. The chemo preventive potential of resveratrol
(Source: Ly and Dao, 2018)

3.2.3. Neuroprotective potential of resveratrol

Resveratrol has also been reported for its neuroprotective activity (Figure 3.8). It can delay the onset of neurodegradation due to the formation of β-amyloid plaque and oxidative stress. Alzheimer's dementia (AD) lead to the progressive loss in the memory but the etiology of the AD is entirely unknown, but the formation of β-amyloid plaques has been found to be highly neurotoxic which occurs in the AD (Sinha et al., 2002; Dasgupta et al., 2007; De La Lastra and Villegas, 2007; Shi et al., 2018).

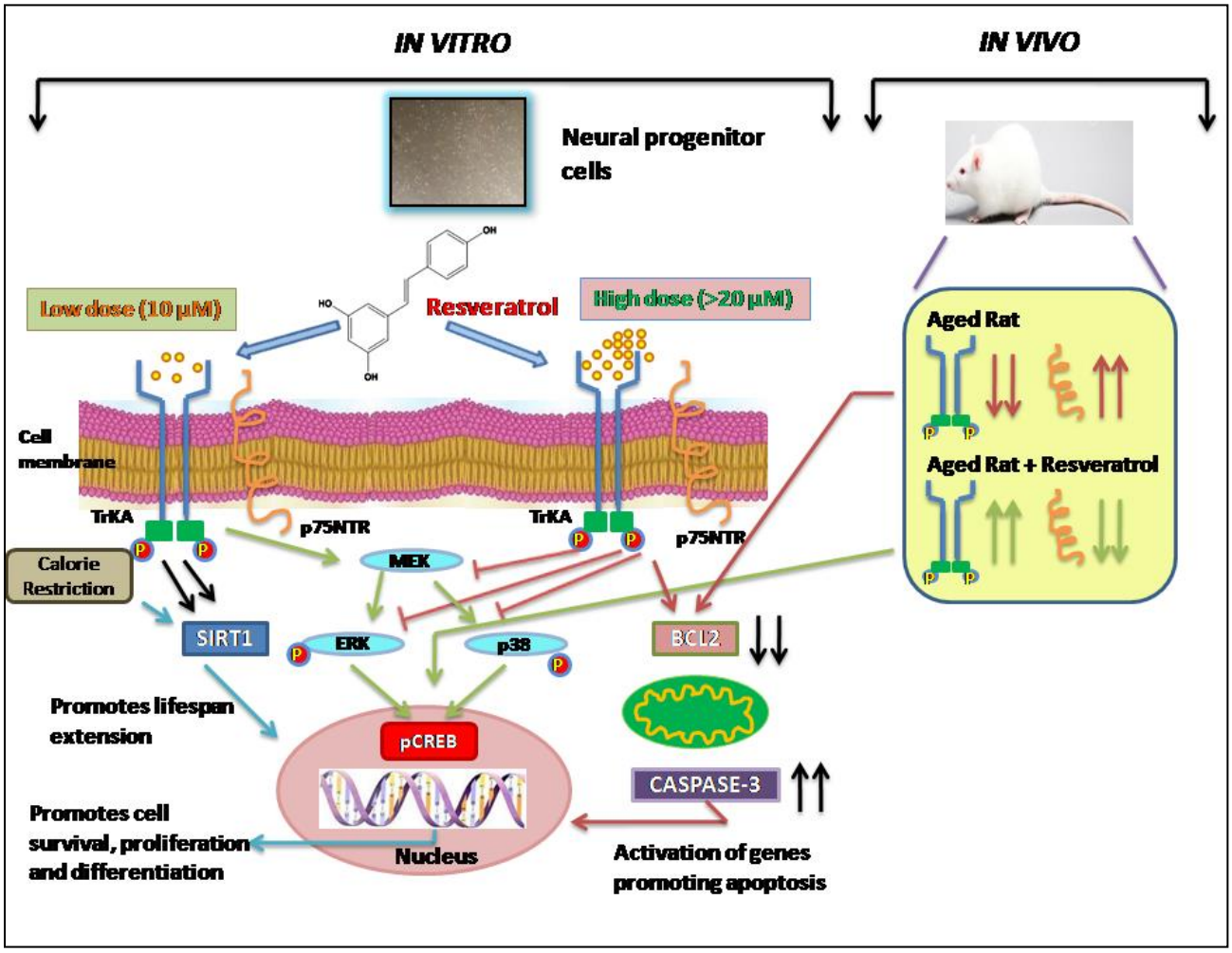


Figure 3.8. The picture depicts how resveratrol plays a role in neural progenitor cells proliferation and aged rat neurogenesis

(Source: Kumar et al., 2016)

Resveratrol has incredible ability to suppress *p53* gene activity which triggers an extensive death of brain cells (Pasinetti et al., 2015; Turner et al., 2015). Resveratrol also protects against microglia-dependent- toxicity in case of Alzheimer's by inhibiting the β signaling and there this activity also correlates with the activation of SIRT-1 protein (Ma et al., 2018). Lange and Li, (2018) reports the effect of resveratrol on age related mouse model (SAMP8 mice) and found that resveratrol enhances the mean life expectancy in model mouse (SAMP8). It is also found in some study that resveratrol and its derivatives increase the clearance of β-amyloid which is important feature of Alzheimer's Dementia (Bastianetto et al., 2015; Jia et al., 2017). Resveratrol also decreased the CSF MMP9 and increases the MDC (macrophage-derived chemokine), FGF-2 (fibroblast growth factor) and IL-4 (Interleukin-4) (Moussa et al., 2017). Resveratrol also induced

6-hydroxydopamine in Parkinson's mouse model. To overcome with chronic dysfunction, and oxidative stress and loss of dopaminergic neurons it decreases the level of COX-2, TNF-α mRNA and COX-2 protein expression (Bastianetto et al., 2015; Pasinetti et al., 2015; Turner et al., 2015; Moussa et al., 2017; Lange and Li, 2018).

3.2.4. Antidiabetic potential of resveratrol

Resveratrol has been found to improve metabolic health in people suffering from insulin resistance, in different animal models (de Matos et al., 2018; Ngo et al., 2018; Tastekin et al., 2018; Wong et al., 2018). The anti-hyperglycemic and anti-oxidant activity of resveratrol has been assessed by its modulatory effects on the activities of carbohydrate metabolizing enzymes in the kidney and the hepatic tissues of streptozotocin-nicotinamide-induced diabetic rats (Chaplin et al., 2018; Tastekin et al., 2018) (Figure 3.9).

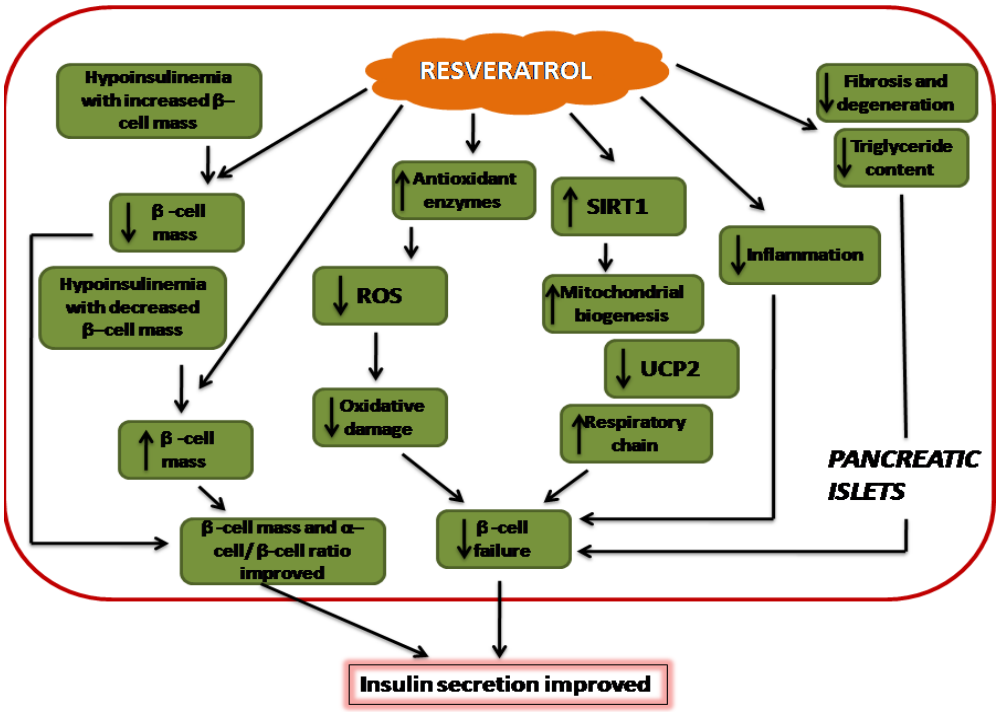


Figure 3.9. The role of resveratrol in improvement in insulin secretion in animals
(RSV – resveratrol, ROS – reactive oxygen species, UCP2 – uncoupling protein 2)
(Source: Szkudelski and Szkudelska, 2015)

It is also found that treatment of genetically modified mice (C57BL/KsJ-db/db mice) with resveratrol reduced the blood glucose level and hemoglobin A, which is similar in effect as compared to standard drug rosiglitazone (Crandall and Barzilai, 2013; Nanjan and Betz, 2014).

Similarly, resveratrol also reduces the gestational diabetes mellitus manifestation via triggering AMPAK supplementation of resveratrol and adequately improved glycemic control, insulin resistance as well as reproductive outcome of pregnant C57BL/KsJ-Lepdb/p mouse model (Movahed et al., 2013; Oyenihi et al., 2016). Resveratrol (250 mg/d) for 3 months was supplemented to 62 patients with type II diabetes in a clinical study last year was able to reduce the systolic pressure and hemoglobin A (Novelle et al., 2015; Oyenihi et al., 2016; Berman et al., 2017). These finding advocate that dietary resveratrol supplementation has promising antidiabetic potential.

3.2.5. Anti-ageing potential of resveratrol

Anti-ageing process attribute to both cosmetic maintenance of age through pertinent healthy appearance as well as steaming up with development of degenerative disease optimized in the cerebrum as well as in other tissues to extend life (Alarcon De La Lastra and Villegas, 2005; Baur et al., 2006; Labinskyy et al., 2006; Li et al., 2017). Resveratrol has been found to activate *SIRT1* and also exhibits the anti-oxidant effects (Figure 3.10). Resveratrol extends the lifespan in *Saccharomyces cerevisiae* (70% life span by activation of*SIRT-2*), *Caenorhabditis elegans* (by activating*SIRT-2*) (Baur et al., 2006; Burnett et al., 2011; Li et al., 2017) and *Drosophila melanogaster* (by activating *SIRT-2*), only if the genes of *SIRT2* are present in these organisms (Rascón et al., 2012; Bhullar and Hubbard, 2015; Kayashima et al., 2017).

It is also found that on an average 130mM of resveratrol can extend lifespan in honeybee by 38% (Baur et al., 2006; Burnett et al., 2011; Rascón et al., 2012). Scientists have also reported that resveratrol significantly decreased the food

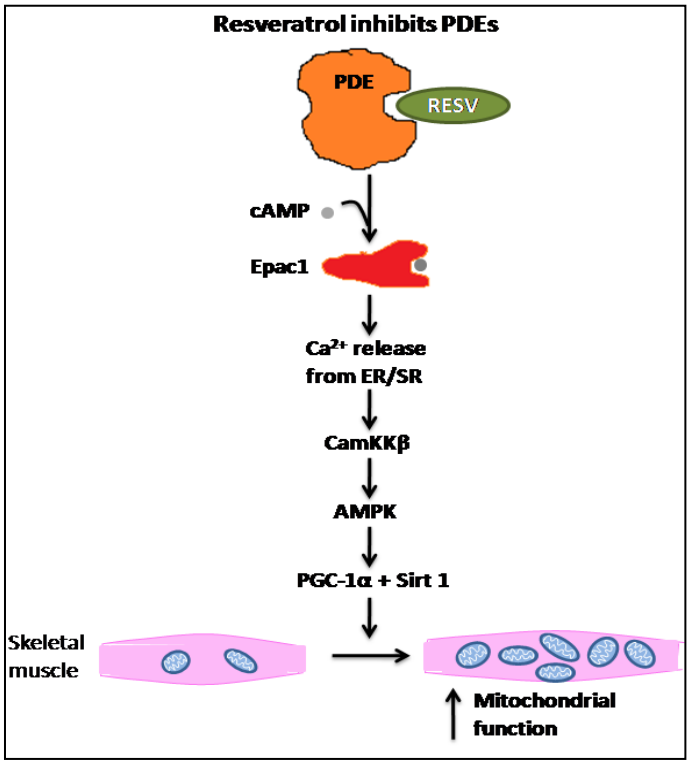


Figure 3.10. The antiaging potential of Resveratrol (Source: Park et al., 2012)

consumption in Honey bee as comparison to un-supplemented control. Activation of SIRT 1 protein undergoes modulation of number of proteins including FOXO family, peroxisome proliferator activated receptor co-activator-1α, NFkβ and protein kinase B (Ruderman et al., 2010; Chang et al., 2014).

Similarly, Pearson et al., (2008) found that long-term consumption of resveratrol in mice brings in transcriptional changes induced by calorie restriction and exhibited the reduction in sign of aging accumulated with improving insulin sensitivity, cardiovascular functions, neurodegeneration and apoptosis in vascular endothelium (Rascón et al., 2012; Bhullar and Hubbard, 2015; Kayashima et al., 2017; Li et al., 2017).

3.2.6. Antioxidant potential of resveratrol

Resveratrol is a billion-dollar molecule which is enormously being used as a medicinal molecule, as well as a pharmacophore for new drug development, with its multifactorial effects (Baur et al., 2006; Bastianetto et al., 2015; Bhullar and Hubbard, 2015). Much of this is attributed to its antioxidant potential as well as its propensity to avert and control oxidative stress (Figure 3.11). ROS (reactive oxygen species) comprises free radicals such as superoxide ions (O_2-), hydroxyl ions (OH-) as well as non-free radical species such as hydrogen peroxide (H_2O_2) (Sánchez-Moreno, 2002).

As ROS are continuously generated inside human body by physiological functions of the cell, they also damage the nucleic acids, fatty acids, and proteins. Resveratrol also has been used to prevent lipid oxidation in pharmaceutical products and enhance the nutritional quality and shelf life of pharmaceutical products (Gülçin, 2010; Salehiet al., 2018). Resveratrol exhibits a strong ABTES, DPPH, as well as hydroxyl ion scavenging potential as compared to Propyl gallate, Vitamin C, Vitamin E (Gülçin, 2010; Salehiet al., 2018). Resveratrol also has been found to possess potent inhibitory activity against TNF-α (tumor necrosis factor- α) and IL-1β (interleukin-1 β) (Estrov et al., 2003; Busch et al., 2012; Gu et al., 2018). As already displayed resveratrol is a dynamic antioxidant however its valuable effect is hindered by its low bioavailability (Sánchez-Moreno, 2002; Estrov et al., 2003; Gu et al., 2018). These outcomes strongly validate that resveratrol and its derivatives might be used as potential antioxidants in foods, cosmeceuticals and nutraceuticals.

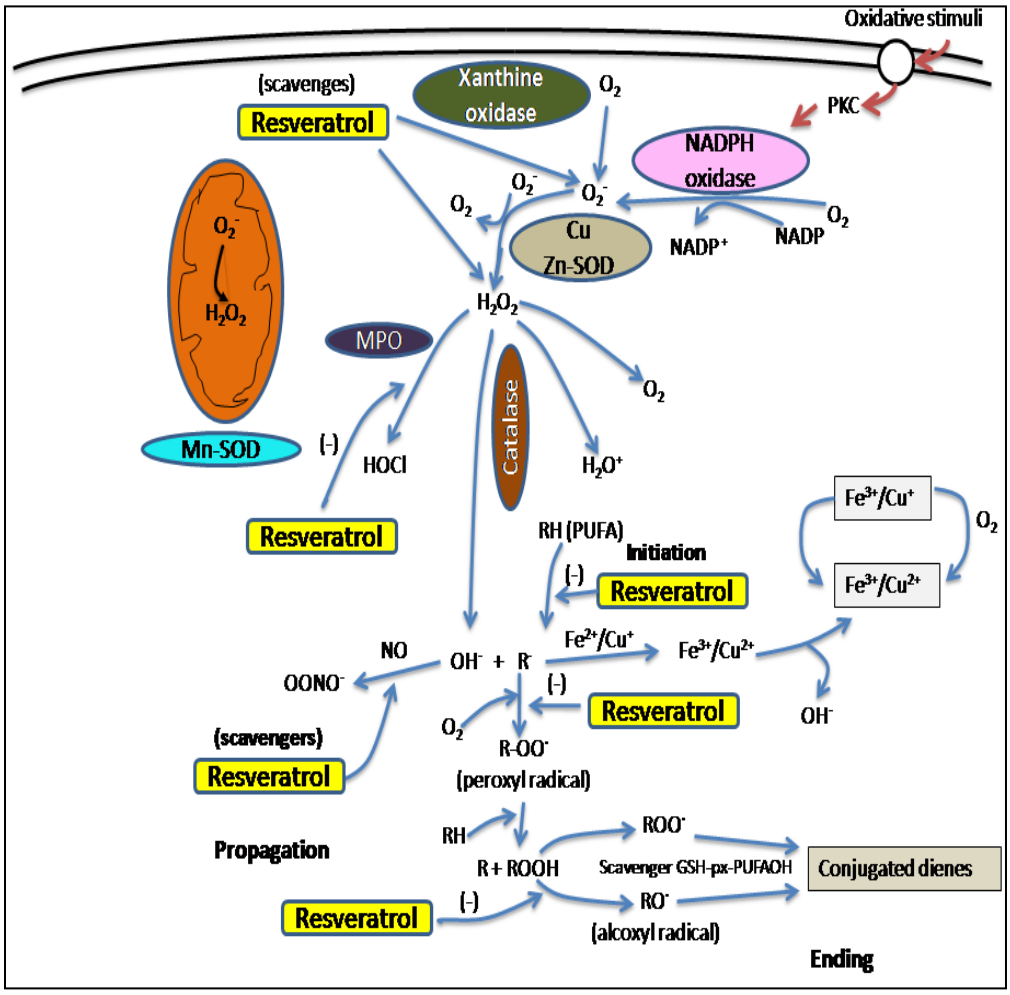


Figure 3.11. The mechanism of resveratrol as an antioxidant agent

(Source: De La Lastra and Villegas, 2007)

3.2.7. Antimicrobial potential of resveratrol

Resveratrol was initially known as vital phytoalexin produced by some berry plants against various stresses, U.V radiation and microbial infections to provide disease resistance (Figure 3.12) (Saxena and Srivastava, 2014; Dwibedi and Saxena, 2018; Snopek et al., 2018). Resveratrol inhibits *Helicobacter pylori* which is responsible for all type of gastric or intestinal ulcers and β cell lymphoma (Chan, 2002; Paulo et al., 2010; Ma et al., 2018). Resveratrol and its derivatives have been found to possess potential anti candidal activity (Houille et al., 2014; Ma et al., 2018). Resveratrol exhibits antiviral activity as it has been reported to inhibit HSV type 1 and 2, human cytomegalovirus, different influenza virus apart from synergistically boosting the efficacy of

some anti-HIV drugs (Saxena and Srivastava, 2014; Hwand and Lin, 2015; Ma et al., 2018; Xu et al., 2018).

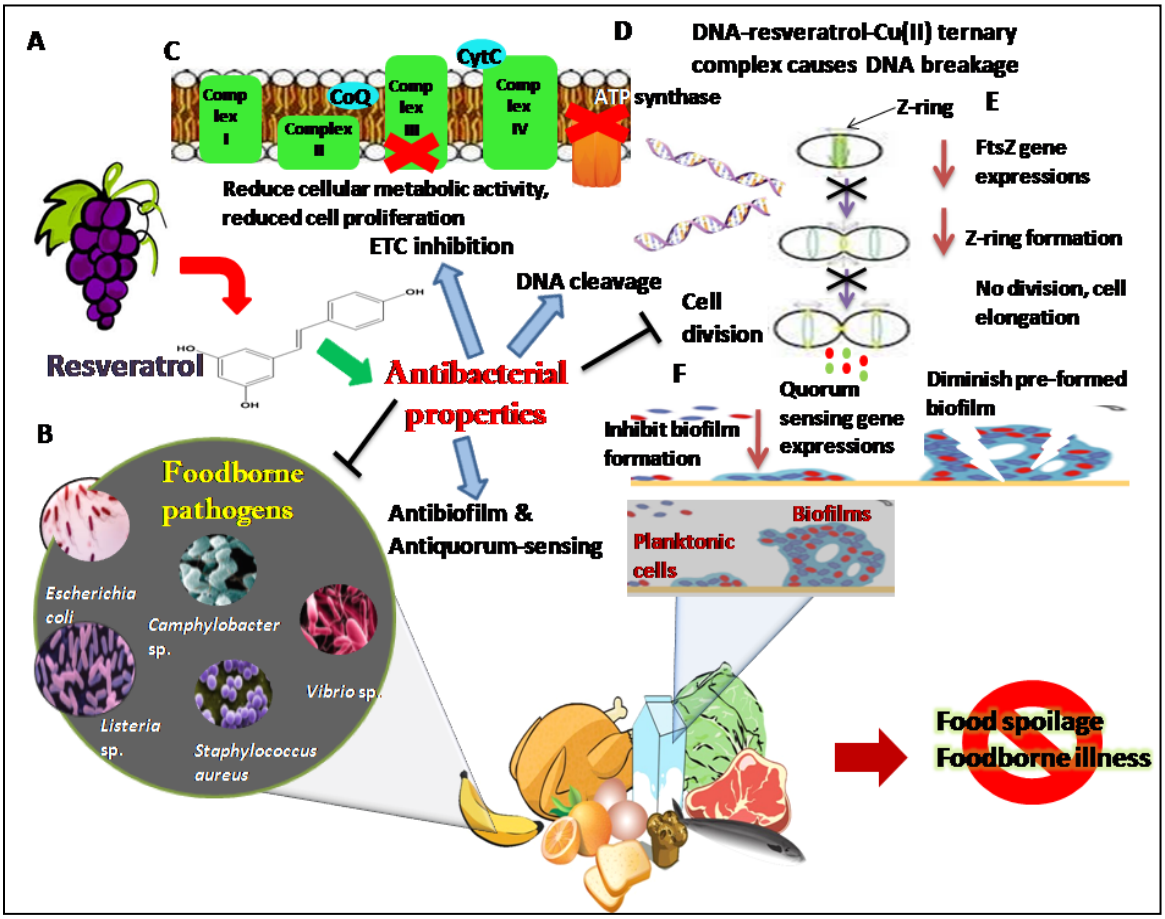


Figure 3.12. The overview of resveratrol as an antibacterial agent against food-borne pathogens (Source: Ma et al., 2018)

Thus, there are multifarious beneficial effects of resveratrol on humans which make it an attractive molecule to be incorporated as a nutritional or wellness supplement apart from making it a candidate molecule to be developed into a pharmacophore.

3.3. Resources of resveratrol for commercial production

As the demand of resveratrol is on rise due to its multifarious applications in pharmaceutical, cosmeceutical develop novel cost effective production methods to meet the global demand of resveratrol (Shi et al., 2012; Liu et al., 2016; Dwibedi and Saxena, 2018). Currently resveratrol is produced through extraction of the roots of *P. cuspidatum* (syn. *Fallopia japonica*).Large quantities of roots of *Polygonum cuspidatum* are extracted with solvents using liquid-liquid partition for the recovery of resveratrol (Sun and Zhang, 2003; Wang et al., 2013;Wei et al.,

2014;Mohammadet al., 2016). Chinese companies like Wuxi Gorunjie Technology Co. and Shanghai DND Pharm- Technology Co. produce trans-resveratrol from root extracts of *Polygonum* species (Figure 3.13). The major drawback of this current technology is massive annihilation of the plants for the recovery of roots for the extraction of resveratrol.

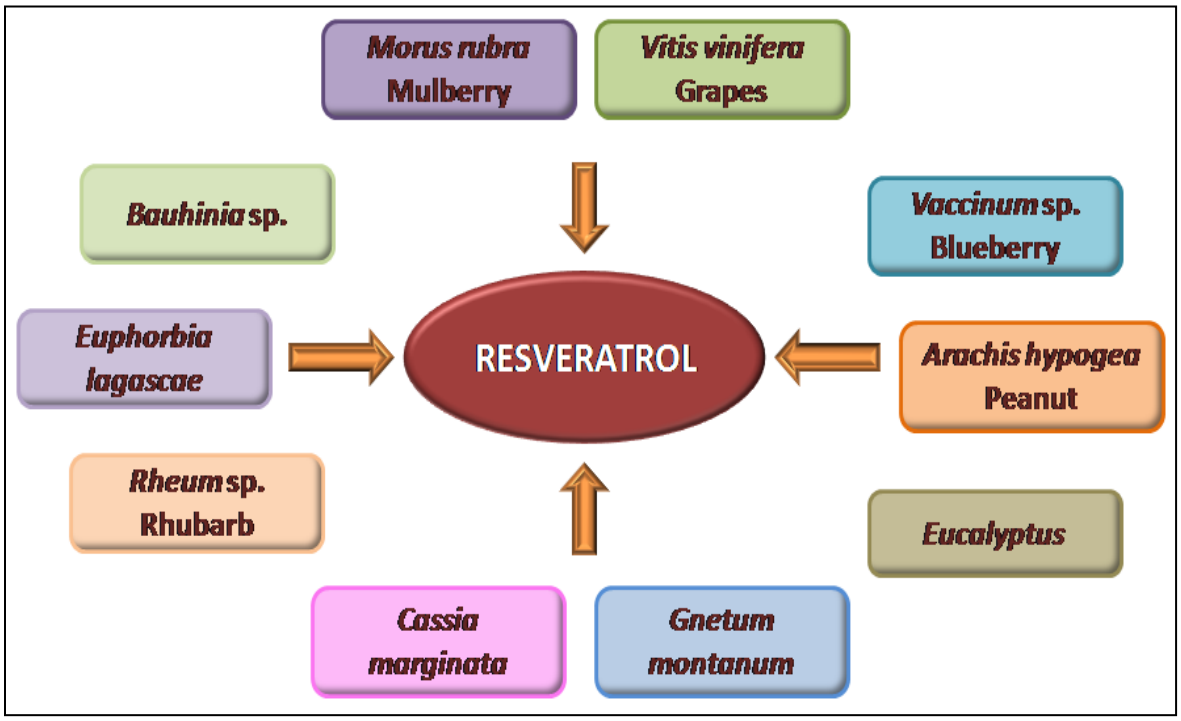


Figure 3.13. Sources of Resveratrol

(Source: Kukreja et al., 2014)

Resveratrol is found in variety of foods, the richest source is grapes which contains 1.5-7.8 mg/g fresh weight, peanuts contains 5.1 mg/g fresh weight, but all these fruits produce very low level of resveratrol (Burns et al., 2002; Donnez et al., 2009; Kiselev et al., 2011; Chu et al., 2018). There are various extraction techniques for extraction of resveratrol from plant and food materials, like enzymatic and alkaline extraction, solvent-solvent extraction, microwave assisted extraction, ultrasonic assisted extraction and supercritical fluid extraction (Sun and Zhang, 2003; Wang et al., 2013; Wei et al., 2014). Out of these conventional techniques solvent-solvent extraction techniques not only enhanced the yield, but also reduced the cost as well as time. However, to fulfill the global demand of resveratrol all these technologies need large amounts of raw material so it cannot be commercialized (Donnez et al., 2009; Kiselev et al., 2011).

3.3.1. Bioproduction of resveratrol reported from plants

Resveratrol correlates to a subgroup of secondary plant metabolites known as polyphenols with structure formula of C_6-C_2-C_6 and produced by the number of plant species including *Vitis vinifera*, *Morus rubra*, *Arachis hypogea*, and *Vaccinum* sp., etc. (Burns et al., 2002; Donnez et al., 2009; Kiselev et al., 2011; Chu et al., 2018) (Figure 3.13). They are derived via a class of phenylpropanoids and linked their biosynthesis with chalcone biosynthesis pathway. Phenylpropanoid pathway helps plants to combat different biotic and abiotic stress and STS enzyme play a significant role in the evolution of a vast array of stilbenes conferring a selective advantage in more than 70 plant species against microbial infection, injury, U.V radiation, abiotic and biotic stress, and some elicitors (Shen et al., 2009; Kiselev, 2011; Niesen et al., 2013; Tsai et al., 2017; Milke et al., 2018). Plant cell and tissue culture offer alternatives for the production of resveratrol. The production of trans-resveratrol in plant cell cultures, e.g., cell cultures of *Vitis* spp., *Arachis hypogea*, has been analyzed by several groups (Kiselev et al., 2011; Belchí-Navarro et al., 2012; Barbulova et al., 2014; Vuongetal., 2014) (Table 3.1). It was found that the untreated plant cell cultures accumulated less than 0.01% of resveratrol on dry weight basis (Tassoni et al., 2005; Shen et al., 2012). Different strategies such as pathogen attack, biotransformation using an exogenous supply of biosynthetic precursors or chemical elicitors and genetic manipulation are generally used for inducing higher resveratrol production.

However, challenge in this strategy is level of secondary metabolite production as during long-term cultivation it often becomes unstable and unpredictable. Further, the high rates of accumulation of mutations in plant cells necessitate some limitations in the subsequent commercial use of these cell cultures (Kiselev, 2011; Wilson and Roberts, 2012; Chu et al., 2018; Lange, 2018).

Table 3.1. Resveratrol reported from Plant and Food sources

Source of Resveratrol	**Family**	**Total stilbene content**	**Reference**
Arachis hypogaea	*Fabaceae*	0.002mg/g FW; 1.2 mg/g DW; 0.08 mg/g DW	Chung et al., 2003;Sobolev et al., 2011
Artocarpus lakoocha	*Moraceae*	85mg/g DW	Borah et al., 2017

Vitis vinifera	*Vitaceae*	13 µg/g DW; 0.5µg/g DW; 0.5µg/g DW; 1.5 mg/g DW; 0.004 mg/g FW; 0.121 mg/g DW	Timperio et al., 2012;Tavares et al.,2013;Lambert et al., 2013;Wang et al., 2016
Vaccinium myrtillus	*Ericaceae*	2µg/g	Rimando and Cody, 2005
Vaccinium corymbosum	*Ericaceae*	0.03µg/g FW	Rimando and Cody, 2005
Morus rubra	*Moraceae*	0.27 µg/mL	Ayinampudi et al., 2011
Morus albus	*Moraceae*	0.13 mg/g DW; 0.1 mg/g DW	Kim et al., 2010;Zhou et al., 2016
Bauhina racemosa	*Fabaceae*	NM	Aggarwal et al., 2004
Rheum acuminatum	*Polygonaceae*	0.5 mg/g DW	Rokaya et al., 2012
Cassisa margianata	*Fabaceae*	NM	Houghton et al., 1995
Genetum parvifolium	*Gnetaceae*	0.6mg/g DW	Deng et al., 2017
Eucalyptus sp.	*Myrtaceae*	NM	Aggarwal et al., 2004
Fallopia japonica	*Polygonaceae*	12 mg/g DW	Chen et al., 2013
Lycopersicon esculentum	*Solanaceae*	0.015 mg/g DW	Wang et al., 2016
Picea jezoensis	*Pinaceae*	0.04-0.1µg/g DW	Wang et al., 2016
Humulus lupulus	*Cannabaceae*	1mg/kg DW	Jerkovic et al., 2005
Robinia pesudoacacia	*Fabaceae*	0.017mg/g DW	Sergent et al., 2014
Saccharum sp.	*Poaceae*	12.3 µg/g DW	Boue et al., 2013
Pinus sylvestris	*Pinaceae*	NM	Aggarwal et al., 2004
Polygonum cuspidatum	*Polygonaceae*	3.8 mg/g DW; 0.06 mg/g DW; 0.15-1.8 mg/g DW	Vastano et al., 2000;Vrchotova et al., 2007;Benova et al., 2008
Veratrum grandiflorum	*Melanthiaceae*	NM	Kiselev et al., 2011

(Note: NM = not measured; DW = dry weight; FW = fresh weight)

3.3.2. Microbes as a source for resveratrol production

Currently, resveratrol biosynthesis pathway is known only in plants (Tavares et al., 2013; Che et al., 2016), and the key intermediates in order, are phenylalanine ammonia, cinnamic acid, and 4-coumarate Co-A. PAL (phenylalanine ammonia-lyase), CYP73A (cinnamic acid 4-hydroxylase), 4 CL (4-coumarate-CoA ligase), and stilbene synthase (STS) are the key enzyme in this pathway in order (Tavares et al., 2013; Che et al., 2016). Microorganisms have also been genetically manipulated to optimize the production of resveratrol through the fermentation route. Transgenic yeast and *E.coli* has been developed and is being tested for the production of trans-resveratrol (Beekwilder et al., 2006; Braga et al., 2018). The Danish Company, Fluxome first used

this isolate for the commercial production of resveratrol, which was designated as GRAS (Generally recognized as safe), in 2011 (Mei et al., 2015; Che et al., 2016; Braga et al., 2018). Similarly, metabolically engineered *E.coli* was able to convert 4-coumaric acid into *trans*-resveratrol producing a 100 mg/L yield (Zhao et al., 2012).Transformation of these genes in *E.coli* enhanced the resveratrol production as compared to transgenic yeast. A transformed metabolically engineered *E.coli* strain (JM109) which was fused with 4CL gene from *Arabidopsis thaliana* and *STS* gene from *Arachis hypogea*, was able to produce 100mg/L of resveratrol (Jeand et al., 2012;Braga et al., 2018). When the same strain of *E.coli* was transformed with 4CL from *Lithospermum erythrorhizon* and *STS* gene from *Arachis hypogaea, it* was able to produce 171 mg/L of resveratrol (Che et al., 2016; Yang et al., 2016; Braga et al., 2018; Milke et al., 2018) (Table 3.2).

However, the yield of resveratrol production by these metabolically engineered microorganisms is not very high to meet the global demands of 100 tons/year. Additionally, obtaining high purity resveratrol (98%) is difficult and very cost intensive (Zhao et al., 2012; Tavares et al., 2013; Mei et al., 2015).

Table 3.2. Metabolic engineered microorganism used for production of resveratrol

S.No.	Microorganisms	Introduced gene	Origin of gene	Stilbene content	References
YEASTS					
1.	*Yarrowia lipolytica*	*PAL/TAL,C4H, 4CL,STS*	*Rhodotorula glutinis (PAL/TAL), Streptomyces coelicolor (4CL), Vitis vinifera (STS)*	1.46 mg/L (RESV)	Li et al., 2016; Yang et al., 2016
2.	*Saccharomyces cerevisiae*	*PAL, C4H, 4CL, STS*	*Arabidopsis thaliana (PAL, C4H, 4CL), Rheum tataricum (STS)*	NM	Li et al., 2016; Yang et al., 2016
3.	*S. cerevisiae*	*TAL, 4CL::STS fusion protein*	*Rhodobacter sphaeroides (TAL), Arabidopsis thaliana (4CL), Vitis vinifera (STS)*	5.25 mg/L (RESV)	Jeandet et al., 2012; Delaunois et al., 2009

4.	*S. cerevisiae*	*TAL, 4CL::STS fusion protein, area*	*Rhodobacter sphaeroides (TAL), Arabidopsis thaliana (4CL), Vitis vinifera (STS), Escherichia coli (araE)*	3.1 mg/L and 1.27 mg/L (RESV)	Milke et al., 2018; Braga et al., 2018
5.	*S. cerevisiae*	*TAL, 4CL::STS fusion protein*	*Rhodobacter sphaeroides (TAL), Arabidopsis thaliana (4CL), Vitis vinifera (STS)*	14.4 mg/L (RESV)	Braga et al., 2018; Donnez et al., 2009
6.	*S. cerevisiae* with p-Coumaric acid	*PAL, CPR, C4H, 4CL, STS*	*Populus trichocarpa X Populus deltoides (PAL, CPR), Glycine max (C4H, 4CL), Vitis vinifera (STS)*	0.29 mg/L (RESV)	Delaunois et al., 2009; Yang et al., 2016
7.	*S. cerevisiae* with phenylalanine	*PAL, CPR, C4H, 4CL, STS*	*Populus trichocarpa X Populus deltoides (PAL, CPR), Glycine max (C4H, 4CL), Vitis vinifera (STS)*	0.31mg/L (RESV)	Che et al., 2016; Braga et al., 2018
8.	*S. cerevisiae*	*4CL, STS*	*Populus trichocarpa X Populus deltoides (4CL), Vitis vinifera (STS)*	1.45 mg/L (RESV)	Braga et al., 2018; Donnez et al., 2009
9.	*S. cerevisiae*	*4CL, STS*	*Nicotiana tabacum (4CL), Vitis vinifera (STS)*	5.8 mg/L (RESV)	Braga et al., 2018; Donnez et al., 2009
10.	*S. cerevisiae*	*PAL2, C4H, 4CL, STS*	*Arabidopsis thaliana (PAL, C4H, 4CL), Vitis vinifera (STS), Salmonella enterica (ACS)*	812 mg/L (RESV)	Li et al., 2016
BACTERIA					
11.	*Escherichia coli*	*4CL, STS*	*Streptomyces coelicolor (4CL), Vitis vinifera (STS)*	3.6 mg/L (RESV)	Che et al., 2016; Braga et al., 2018
12.	*Escherichia coli*	*4CL, STS*	*Arabidopsis thaliana (4CL), Arachis hypogea (STS)*	100 mg/L (RESV)	Che et al., 2016; Braga et al., 2018

13.	*Escherichia coli*	*4CL, STS*	*Nicotiana tabacum (4CL), Vitis vinifera (STS)*	16 mg/L (RESV)	Jeandet et al., 2012; Delaunoiset al., 2009
14.	*Escherichia coli*	*4CL, STS*	*Lithospermum erythrorhizon (4CL), Arachis hypogea (STS)*	171 mg/L (RESV)	Jeandet et al., 2012
15.	*Escherichia coli*	*TAL, 4CL, STS*	*Arabidopsis thaliana (4CL), Rhodobacter capsulatus (TAL),Rheum tataricum (STS)*	NM	Braga et al., 2018; Jeandet et al., 2012
16.	*Lactobacillus lactis*	*PAL, C4H, 4CL, STS*	*Arabidopsis thaliana (PAL, C4H, 4CL), Rheum tataricum (STS)*	NM	Milke et al., 2018; Braga et al., 2018
17.	*C. glutamicum* with Cerulenin	*4CL, STS*	*Petroselinum crispum (4CL),Arachis hypogea (STS)*	158 mg/L (RESV)	Milke et al., 2018 ;Braga et al., 2018
18.	*Escherichia coli*	*TAL, 4CL, STS*	*Rhodothorula glutinis (TAL), Streptomyces coelicolor A2 (4CL), Arachis hypogea (STS)*	22.6 mg/L (RESV)	Beekwilder et al., 2006
19.	*Escherichia coli* BW27784 with p-Coumaric acid	*4CL1, STS*	*Arabidopsis thaliana (4CL), Arachis hypogea (STS)*	1600 mg/L (RESV)	Lim et al., 2011
20.	*Escherichia coli* BW27784 with p-Coumaric acid	*4CL1, STS*	*Arabidopsis thaliana (4CL), Arachis hypogea (STS)*	2340 mg/L (RESV)	Wang et al., 2011; Bhan et al., 2013
21.	*Escherichia coli* with glucose	*TAL, STS, 4CL, YJiC*	*Streptomyces espanaensis (TAL), Arachis hypogea (STS), S. coelicolor (4CL), Bacillus licheniformis DSM 13 (YjiC)*	7.5 mg/L(RESV)	Choi et al., 2014

FUNGI

22.	*Aspergillus niger*	*PAL, C4H, 4CL, STS*	*Arabidopsis thaliana (PAL, C4H, 4CL), Rheum tataricum (STS)*	NM	Braga et al., 2018; Jeandet et al., 2012
23.	*Aspergillus oryzae*	*PAL, C4H, 4CL, STS*	*Arabidopsis thaliana (PAL, C4H, 4CL), Rheum tataricum (STS)*	NM	Braga et al., 2018 ; Jeandet et al., 2012

(Note: NM = not measured)

3.3.3. Endophytic fungi as a promising source for resveratrol production

The term Endophyte (Gr. endon, within; phyton, plant) was first coined by de Bary. Endophytes are a group of microorganisms comprising of bacteria, fungi, and actinomycetes which reside within the intra- and intercellular plant tissues for all or a part of their life cycle. Fungal endophytes majorly comprise of mitosporic and meiosporic ascomycetes, coelomycetes and hyphomycetes which colonize the healthy living tissues of the host plant by latent infection to obtain shelter and nutrition (White and Torres, 2010; Aly et al., 2011; Kusari et al., 2012). These endophytes, in turn, produce an array of signal molecules to overcome the inherent defence mechanisms of the host plant which purportedly play a role in aiding the host plant in combating the different types of biotic and abiotic stresses (White and Torres 2010; Aly et al. 2011). The signal molecules so produced by these endophytic microbes may possess novel activities which may be relevant and havepotential applications in medicine, agriculture, and food industry. Many bioactive compounds possessing antimicrobial, anticancer, insecticidal and cytotoxic activities have been discovered from endophytic fungi. Some endophytic fungi develop the ability to produce putative plant phytochemicals, the very famous being *Taxomyces andreanae* from *Taxus brevifolia* producing Taxol in fermentation medium (Stierle et al., 1993). This generated ample interest in research groups to explore endophytic fungi to produce medicinal compounds which were being resourced from plants. Podophyllotoxin, is biosynthetically produced by *Podophyllum versipelle*, is highly admired as the precursor to be clinically used as an anti-cancer drug such as etoposide and teniposide. *Phialocephala fortinii* and *Trametes hirsuta* are the two endophytic fungal isolates which have been isolated from

Podophyllum peltatum and *Podophyllum hexandrum*, respectively, which produce podophyllotoxin in the fermentation medium (Eyberger et al., 2006; Puri et al., 2006).

Similarly, endophytic fungi for production of other plant isolated drug such as camptothecin (Puri et al., 2006; Kusari et al., 2012) and vincristine (Aly et al., 2011) have also been identified and fermentation processes are being developed for the commercial bulk production of these plant-based medicinal compounds. As endophytic fungi exhibit the capability of producing the same functional compounds as their hosts and live asymptomatically in their hosts (Strobel and Daisy, 2003), they could be possibly explored as an alternative source for natural resveratrol production. This brought in another exciting observation that due to co-evolution with their host plants, the endophytic microbes acquired complete or partial biosynthetic capabilities of the host phytochemicals. This capacity to biosynthesize host phytochemicals by the endophytic microbes may be due to horizontal gene transfer who might have occurred during extreme environmental conditions (Strobel and Daisy, 2003). Using molecular tools, today these genes can be tracked down in the endophytic microbes.

Table 3.3. Endophytic fungi reported till date as a resveratrol producer

S.No.	Name of Fungi	Isolated Plant	Stilbene name	Resveratrol Content	References
1.	*Alternaria (MG1)*	*Vitis vinifera*	Resveratrol	6-123 µg/L	Shi et al., 2012
2.	*Alternaria* sp.	*Vitis vinifera*	Resveratrodehydes	NM	Wang et al., 2014
3.	*Aspergillus niger (C2J6)*	*Vitis vinifera*	Resveratrol	1.48 mg/L	Liu et al., 2016
4.	*Arcopilus aureus (#12VVLPM)*	*Vitis vinifera*	Resveratrol	89.1 mg/L	Dwibedi and Saxena, 2018

In plants, the phenylpropanoid pathway is well reported, and the enzymes involved in this pathway are used to construct genetically modified microorganisms to enhance the secondary metabolite in free fermentative condition. Shi et al., (2012); Liu et al., (2016) and Dwibedi and Saxena, (2018), reported that endophytic fungi isolated from grape could produce resveratrol (Table 3.3), and Li et al., (2016) and Yang et al., (2016) detected the activity of genes like *PAL*, *C4H*, *4CL*, and *STS* which are involved in the production of different type of stilbenes. Natural

drugs isolated from the plants and marine sources have always been the center of attraction in research. However, plants have long growth cycles, and some of them face extinction and extraction of resveratrol from a plant source is a prolonged process and involves the massive annihilation of the plants thereby disturbing the ecosystem. These drawbacks limit the industrial production of natural resveratrol. Endophytic fungi could be a better alternative to produce bioactive compounds in the free fermentative medium. There is minimal data on production of resveratrol from endophytic fungi; therefore, exploring endophytic fungi for this purpose is a new area.

3.3.3.1. Possibilities of developing a commercial strain from endophytic fungi

One of the promising strategies to produce putative phytochemicals cost effectively is to produce them via microbial fermentation which can be achieved either through recombinant DNA technology wherein the genes expressing the pathway of desired product are cloned and expressed in a suitable microorganism or by exploration of endophytes which have co-evolved with the host plant and possesses inherent biosynthetic capabilities of the host plant due to horizontal gene transfer (Strobel and Daisy, 2003; Aly et al., 2011; Kusari et al., 2012). The later process appears appealing since plant harbor diverse endophytes and possibilities exist to find or track the biosynthetic gene of interest which is responsible for the production of host metabolite or phytochemical which is much more comfortable and cost-effective proposition. This has generated a great deal of interest in endophytes possessing genes of the host biosynthetic machinery as these could be further manipulated for enhancing the desired compound as well as for exploration of novel/cryptic compounds by instigating the silent genes. The expression of silent or cryptic genes is generally induced by bringing in epigenetic modifications. Epigenetics primarily is related to changes in expression and regulation of genes which are not dependent on DNA sequences. Waddington, (1942) introduced the term epigenetics for the development of specific traits through the interaction of genes and its environmental factors. The activation of the silent genes can be achieved by different strategies such as chemical modification of the chromatin by addition of Histone deacetylase (HDAC) or DNA methyltransferases (DNMT) inhibitors (Waddington, 1942; Williams et al., 2008; Gerke and Braus, 2014; Duncan and Campbell, 2017; Xu et al., 2018).

Similarly, role of nutrients for the production of dietary metabolites from gut microbiota have been critically studied in context to development of diseases like irritable bowel syndrome (IBS); allergy, autoimmune diseases, diabetes, neurodegenerative diseases, and cancer (Bhat and Kapila, 2017), however minimal information exist on the role of nutrients in epigenetic modulation for induction of secondary metabolites production in microorganisms (Gerke and Braus, 2014;Sharma et al., 2017;Xu et al., 2018). Further using precursor in the fermentation medium can enhance or improve the production of the desired secondary metabolite in case the biosynthetic gene has been transferred from the host to the endophyte. Precursor feeding has been used as a strategy for production of secondary plant metabolites in plant cell culture as well as in genetically engineered microorganism which is mimicking the biosynthetic pathway of the plant. However, this strategy has been seldom used with wild or native microorganisms. In nature, fungi and bacteria live in complex communities, sharing similar niches, utilizing similar resources, and overcoming the same external cues for survival (Ul-Hassan et al., 2012; Cherblanc et al., 2013; Lin et al., 2013; Zutz et al., 2014). Thus the interaction between them in a growth medium could be mimicking their existence in the natural environment which would result in competition for nutrients, space leading to induction of silent biosynthetic pathways which may result in the production of novel compounds or enhance the production of desired secondary metabolite (Cherblanc et al., 2013; VanderMolen et al., 2014; Aghcheh and Kubicek, 2015; Netzker et al., 2015).

Chapter- 4

Materials and methods

4.0. MATERIALS AND METHODS

4.1. Study sites and plant sample collection

Mature, green, healthy samples of *Vitis vinifera i.e.*, leaves, stems were collected from Pune (12.07 °N, 77.03 °E), Nashik (20.000 °N, 73.78 °E), Bengaluru (12.9667 °N, 77.567 °E) and Lucknow (26.800 °N, 80.9000 °E) during the month of July-August 2016/2017 (Figure 4.1). Each plant sample was tagged and then placed in sterile zip bags and stored at 4 °C in the refrigerator till further use. The plant samples comprised of leaves and stem pieces from the vines of different varieties of *Vitis vinifera*. Ten samples each of leaves and stems were collected per variety from different locations and were stored in sterile zip pouches at 4°C by following the method of Huang et al., (2018).

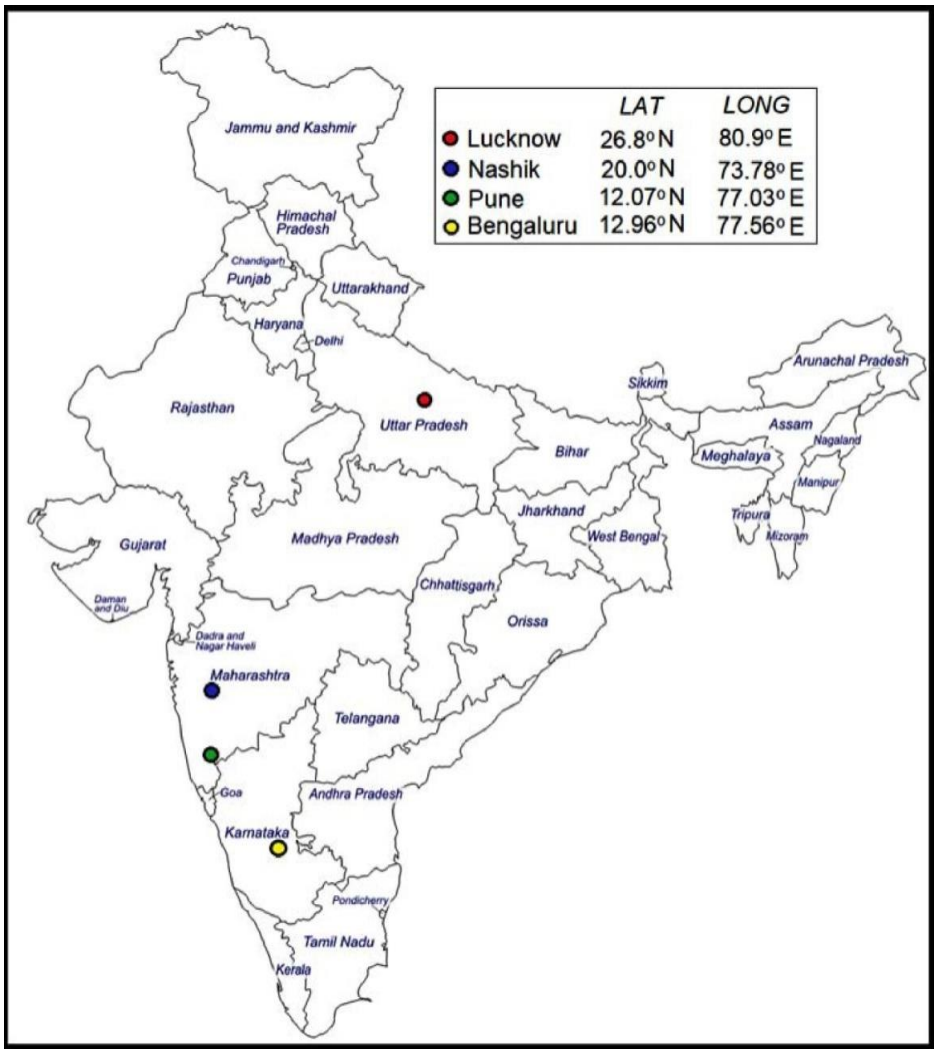


Figure 4.1. Map showing location of sampling sites used in present study

4.2. Isolation of Fungal endophytes and their preservation

To isolate the fungal endophytic fungi the leaf and stem samples were surface sterilized by dipping them in 0.1% solution of sodium hypochlorite for 1 min followed by 70% ethanol for 45s, followed by dipping in 30% ethanol for 30s and drying them aseptically. Finally, 3x3 mm segments from the sterilized leaves and 2 mm transverse sections of stems were prepared aseptically using sterile forceps and blade and were placed on potato dextrose agar supplemented with chloramphenicol (200mg/L) (Schulz et al., 1993; Arnold et al., 2003; Arnold et al., 2007). Under aseptic conditions the petriplate were then incubated at 26 ±1 °C for 10 days with 12 h light/dark photoperiod. Pure endophytic colonies after 10 days incubation was harvest from the edge of an advancing colony with help of sterile needle under optical microscope (Nikon) and transferred on freshly prepared potato dextrose agar (PDA) plates without chloramphenicol. Pure cultures of the endophytic fungi so obtained were preserved on PDA slants supplemented with 10% glycerol. To assess whether the sterilization method was effective in eliminating the surface fungi, imprint of treated fragments was made by pressing them against the surface of PDA plates that were incubated without plant segments. These plates were checked for the emergence of fungi from the imprints.

4.2.1. Morphotaxonomic studies of fungal endophytes isolated from Vitis vinifera

Preliminary identification of endophytic fungus was done using classical morphotaxonomic tools. Further their diversity was calculated by finding the colonization frequency, isolation rate, Sorenson's Index of Similarity, Jaccard's Index of Similarity, Simpson's diversity Index, Shannon-Weiner diversity index and establishing species evenness and species richness.

4.2.1.1. Morphotaxonomic studies

For morphotaxonomic studies, the endophytic fungi were grown over different media i.e. water agar (WA), czapek dox agar (CDA), grape leaf agar (GLA), potato dextrose agar (PDA), corn meal agar (CMA), fusarium minimal media (FMA), synthetic nutrient deficient agar (SNA) for 15-20 days at 26 ± 2 °C and 98% relative humidity (with 12 h of light/dark period). Growth rate of fungal colony, visual appearance, along with its microscopic features like hyphae characteristics, conidia formation and other cellular bodies were critically recognized under optical microscope (Nikon, Japan). All the microscopic observations were carried out using Nikon NIS element

software with minimum 30 observations per structure (Barnett and Hunter, 1972; Ellis and Ellis, 1985; Nagamani et al., 2006; Wang et al., 2016).

4.2.2. Fungal diversity studies

i. Calculation of colonization frequency and isolation rate

The percentage colonization frequency (%CF) and isolation rate (IR) was calculated by method adopted by Hata et al., (1995) and Gond et al., (2012). Samples were incubated and growth was examined daily for 6 weeks and colonization frequency was calculated by the following formula.

$$\text{Colonization frequency (\% CF)} = \frac{No.of\ individual\ fungi\ recorded}{total\ number\ of\ segments\ screened} \times 100$$

$$\text{Isolation rate (IR)} = \frac{No.of\ individual\ fungi\ recorded}{total\ number\ of\ segments\ screened}$$

The similarity of endophytic fungal assemblages of different location was compared using the following similarity indices.

ii. Sorenson's Index of Similarity (QS)

The Sorenson's Index of Similarity (QS) was calculated by the formula.

QS = 2a/ (2a+b+c)

Where, a= no. of common species in both endophytic populations;

b and c = no. of species specified to two different varieties or locations (Osono and Mori, 2004).

iii. Jaccard's Index of Similarity (JS)

Jaccard's index of similarity was calculated using the following formula:

JS = a/(a + b + c),

Where; a = no. of common species in both mycopopulation;

b and c = no. of species specified to two different varieties or locations (Verma et al., 2007).

iv. Simpson's Diversity Index (1-D)

The Simpson's Diversity Index (1-Dominance) (Simpson, 1951) was calculated by using the formula:

$$D = 1\left(\frac{\sum n\ (n-1)}{N\ (N-1)}\right)$$

Where, n= the total no. of organisms of a species,

N= the total no. of organisms of all species.

v. Shannon- Wiener Diversity Index

Shannon-Wiener Diversity Index was calculated using following formula (Shannon and Weaver, 1998; Verma et al., 2007; Bagchi and Banerjee, 2013)

$$H = -\sum_{i=1}^{s} Pi \ln Pi$$

Where, H = represents the diversity in a sample of species or kinds,

Pi = relative abundance of i^{th} species or kinds and measured by ni/N,

N = total no. of individuals of all kinds,

ni = number of individuals of i^{th} species,

ln = natrual log

vi. Species Evenness

The formula used for calculation of species evenness (Whittaker, 1972) is as under;

$$E = \frac{H'}{\ln S}$$

Where, H'= Shannon-Wiener index,

S = total number of species.

vii. Species Richness

Species richness was calculated by using following formula

$$\frac{S}{\sqrt{N}}$$

Where, S = the total no. of species,

N = total no. of isolates of all species.

4.3. Production of culture filtrates

Fermentative cultures of the endophytic fungi were produced by growing the fungi in a pre-sterilized potato dextrose broth (PDB, Hi-Media, Mumbai, India). Briefly, pre–sterilized PDB was inoculated with 5 mm mycelia plug of one-week old culture of the endophytic fungus and incubated at 26 ± 2 °C, 130 rpm for 10 days. After the incubation period is over, the fungal biomass was removed from the broth using filter paper followed by ultracentrifugation at 10,000 rpm (Hitachi RX II series, Japan) for 15 min at 26 °C temperature. The supernatant was collected

and passed through 0.22 µm NCM (nitrocellulose membrane) (GE Health care and life Sciences, Merck, Millipore, USA) to make it cell free (Sharma et al., 2017).

4.4. Liquid –liquid extraction

On the basis of phytochemical screening ethyl acetate (Merck, GR, USA) was used for recovering resveratrol from the lyophilized cell free culture filtrates of 145 endophytic fungal isolates, representing 145 morphotypes in a ratio of 3:1. The solvent phase so obtained were pooled and dry out over anhydrous sodium sulphate (Hi-Media, Mumbai, India) and subsequently concentrated *in vacuum* to obtain crude fraction. The crude fraction so obtained was reconstituted in methanol and was re-assayed for presence of resveratrol using previously described phytochemical tests (Shi et al., 2012; Wang et al., 2013). Crude fractions which exhibited positive results in the preliminary test were subjected to HPLC analysis for further confirmation as well as quantification.

4.5. Identification of polyphenols (resveratrol)

The culture filtrates of the endophytic fungi were subjected to three phytochemical tests viz., chromogenic reaction, acetic anhydride test and liebermann test to detect the presence of polyphenols (resveratrol).

4.5.1. Chromogenic reaction

Preliminary screening the fungal extract having polyphenols (resveratrol) was done by ferric chloride–potassium ferricyanide colour reaction. The chromogenic agent was prepared by using 0.1% $FeCl_3$: 0.1% K_3 [Fe (CN) $_6$] = 1:1 (v/v). Briefly, the reaction was started by the addition of 2 mL of sample followed by addition of 2 mL of MeOH and subsequently 3-4 drop of chromogenic agent was added. The change in colour was recorded. The presence of polyphenols (resveratrol) was characterized by blue colour. In this assay resveratrol served as positive control while un-inoculated PDB was used as negative control (Liu et al., 2016).

4.5.2. Acetic anhydride test

Present test was performed to confirm the presence of free hydroxyl group (–OH) which is present in the polyphenolic and stilbenoid compounds. The reaction was started by adding 100 µL of $C_4H_6O_3$ (acetic anhydride) to 100 µL of culture filtrate followed by addition of 500 µL of concentrated sulphuric acid. The formation of purple or pink precipitate confirmed the presence

of free hydroxyl group (–OH) group of the polyphenolic and stilbenoid compounds in the tested sample (Shi et al., 2012; Al-Jumaily et al., 2014).

4.5.3. Liebermann Test

In this test the culture filtrate was mixed with equal amount of sulphuric acid followed by addition of 2-3 drops of 1% sodium nitrate. Appearance of a blue-violet colour in the reaction mixture confirmed the presence of phenolic compound in the culture filtrate (Shi et al., 2012; Al-Jumaily et al., 2014). In this test, un-inoculated PDB was used as a negative control while resveratrol (1mg/ml; Sigma-Aldrich, USA) served as positive control. The blue- violet colour of the reaction mixture indicates the presence of a free para position in polyphenolic and stilbenoid compounds.

4.6. Estimation of resveratrol production through high performance liquid chromatography (HPLC)

The identification and concentration of resveratrol in crude bioactive fractions was determined via HPLC (PerkinElmer–200, USA) using C18 reverse phase discovery column (Sigma Aldrich, USA) attached with series -200 pump, UV-VIS wavelength detector and total chrome workstation software. The detector was set, and the peak areas were integrated automatically by the computer using the Total Chrom workstation HPLC analysis software programme. About, 20 µL of the sample of defined concentration was injected into the HPLC column and eluted using orthophosphoric acid (0.1%, pH- 2.3 Sigma Aldrich) and acetonitrile (Sigma Aldrich), 45%-55% ratio was used as mobile phase with a flow rate of 1 mL/min. The yield of fungal resveratrol was determined against a standard curve of standard resveratrol (0.1-1 mg/mL) under similar conditions. The peak area vs. concentration of the standard as well as number of peaks and peak height were used to estimate the concentration of polyphenols (resveratrol) (Shi et al., 2012; Wang et al., 2013; Liu et al 2016; Dwibedi and Saxena, 2018).

4.6.1. Preparation of sample

The stock solutions of standard resveratrol and fungal extracts were prepared (0.1-1 mg/mL) and 1 mg/mL of methanol respectively. The samples were sonicated for 15 min, followed by syringe filter through a cellulose membrane of 0.22 µm (GE Healthcare and Life Sciences, Merck, Millipore USA). The sample volume taken was 20 µL which was injected into the HPLC system.

The identification of trans-resveratrol in fungal extracts was evaluated by comparison of retention time and UV spectra with standard resveratrol. All the samples, standard resveratrol as well as fungal extracts were stored in dark and cool place to avoid the oxidation and isomerisation of trans-resveratrol to cis form.

4.6.2. HPLC method validation

i. Linearity of Accuracy

To establish the linearity of accuracy of methods a series of standard resveratrol solutions were prepared in the range of 0.1-1 mg/mL. For accuracy stock solutions of standard resveratrol (0.1, 0.2, 0.3 mg/mL) were prepared and then analyzed (n=10). Accuracy was assessed as a percentage accuracy and mean recovery (%).

ii. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined based on the standard solution of resveratrol used to build the calibration curve. LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively.

where, σ = standard deviation of regression equation intercepts (n = 10)

S = slope of the calibration curve

4.7. Identification of the top resveratrol producing endophytic fungi

For the identification of the top *resveratrol* producing isolates both the classical morphotaxonomic studies and molecular taxonomy tools were used.

4.7.1. Morphotaxonomy of top resveratrol producing endophytic fungi

For morphotaxonomic studies, the endophytic fungi were grown over different media i.e. water agar (WA), czapek dox agar (CDA), grape leaf agar (GLA), potato dextrose agar (PDA), corn meal agar (CMA), *Fusarium* minimal agar (FMA), synthetic nutrient deficient agar (SNA) for 15-20 days at 26 ± 2 °C and relative humidity (with 12 h of light/dark period). Growth rate of fungal colony, visual appearance, along with its microscopic features like hyphae characteristics, conidia formation and other cellular bodies were critically recognized under optical microscope (Nikon, Japan). All the microscopic observations were carried out using Nikon NIS element software with minimum 30 observations per structure (Ellis and Ellis, 1985; Barnett and Hunter, 1972; Nagamani et al., 2006; Wang et al., 2016).

4.7.2. ITS-rDNA based molecular taxonomy of top resveratrol producing endophytic fungi

For genomic DNA extraction, mycelia of 3-4 days old culture (approx. 0.1–0.2 g) was scrapped off with previously autoclaved inoculation loop and squeeze to very fine powder in a sterile mortar and pestle under liquid nitrogen. Further DNA extraction was done as per manufacturer's instructions (Wizard Genomic DNA purification kit, Promega, Madison, WI). The Internal Transcribed Spacer (ITS) region 1, 5.8S and 2 was amplified using ITS 1 and ITS 4 primers (White et al., 1990). The reaction mixture (25 µL) comprised of 1.5U of Taq DNA Polymerase in 10X Taq buffer containing 25mM $MgCl_2$ (Bangalore GeNei, Bengaluru, India), 2.5mM of dNTP, 1 µL of extracted genomic DNA, 10 mM of each primer. The conditions for the polymerase chain reaction were 96 ˚C for 5 min followed by 39 cycles of 95 ˚C for 45 s, 60 ˚C for 45 s, 72 ˚C for 45 s followed by final extension at 72 ˚C for 10 min (White et al., 1990). The ITS amplicons so obtained were examined using a 1.5% agarose gel under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software (Bio-Rad, Hercules, CA). The PCR products were purified with Wizard SV gel and PCR clean up system (Promega, Madison, WI) as per manufacturer's instructions. The obtained PCR products were sequenced at Eurofins (Bangalore, India).

The final sequence was then obtained by assembling the sequences using Sequencher ver.5 (www.genecodes.com) and submitted to the GenBank. The sequence similarity search of the ITS sequences was performed using the BLAST algorithm tool of NCBI. The selected sequences of reference taxa obtained using BLAST were aligned with the ITS sequence of the endophytic fungi using the Clustal W option in MEGA 6. The aligned sequences were then trimmed to make alignment uniform and the aligned files were saved in FASTA and MEGA format. The evolutionary relationship was inferred by employing Maximum Likelihood method using Tamura and Nei Model (2011). Thousand bootstraps were used to assess the evolutionary history. Gaps were considered as missing data.

4.8. Screening for STS gene in resveratrol producing endophytic fungi

The resveratrol producing endophytic fungi were screened for the presence of STS (Stilbene synthase) gene by using two different sets of Primers, (STS-F: ATGGCTTCAGTTGAGGAAAT, STS-R: TTAATTTGTAACCATAGG) and (STS-F: GCAGGTGGAACTGTCCTTCGAAC, STS-R:

CTAAATTGAGTTTTGCTTCAACTGC) (Xu et al., 2011; Rühmann et al., 2013). Briefly, the 25 µL of the reaction mix comprised of, 1 µL (30 ng) of isolated genomic DNA sample together with, 10 µM of specific primer, 2.5mM of dNTP, 1.5U of Taq DNA Polymerase in 10X Taq buffer added 25 mM $MgCl_2$ (Bangalore GeNei), 10mM each primer. The PCR reaction conditions consisted of initial denaturation at 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s followed by final extension at 72 °C for 5 min (White et al., 1990). The amplicons were examined using a 1.5% agarose gel under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

4.9. In-vitro Antioxidant activity

4.9.1. DPPH Scavenging Assay

The DPPH free radical scavenging property of the crude compound was determined according the Ho et al. (2012). Briefly, to 1 mL of DPPH (100 µM in methanol) 50 µL of the fungal extract (10-50 µg/mL) was mixed and incubated in dark at room temperature for 30 min. Further, the absorbance was recorded at 517 nm using Biotek throughput reader, Powerwave 340.Methanol was used as the negative control. Resveratrol (10-50 µg/mL) was used as standard and working DPPH used as the control. Free radical scavenging activity was expressed as percentage. The percentage of scavenging activity was expressed as calculated as:

$$\text{Free radical scavenging } (\%) = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100$$

IC_{50} (µg/mL) of the sample was evaluated by using linear regression. The test was performed in triplicates and data were represented as mean ± SD.

4.9.2. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay was performed according to Re et al. (1999). The $ABTS^+$ radical was generated by mixing of 7 mM ABTS (in 0.1M PBS, pH=7.4) and 2.45 mM potassium persulphate in equal volume and the mixture was allowed to stand in dark at room temperature for 16 h to produce a solution of dark green color. $ABTS^+$ radicals working solution was prepared by diluting the above solution with phosphate buffer saline to an absorbance of 0.9 to 1.0 at 734 nm. For carrying out the assay, 10 µL of extract (50-300 µg/mL) was added with 1.0 mL of diluted $ABTS^+$ solution. The reaction mixture was incubated for 6 min and decrease in O.D was recorded at 734

nm using spectrophotometer (Hitachi U-2900, Japan). Phosphate buffer saline was used as blank and working $ABTS^{+}$ solution was taken as control. Trolox was used as the standard (50-300 µg/mL) and radical scavenging capacity was expressed as µg of Trolox equivalent/mg of sample. The percentage of scavenging activity was expressed as calculated as:

$$\text{Free radical scavenging } (\%) = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100$$

IC_{50} (µg/mL) of the sample was evaluated by using linear regression. The test was performed in triplicates and data were represented as mean ± SD.

4.9.3. Metal ion Chelating scavenging assay

The ferrous ion chelating potential of the fungal extracts was evaluated according to Danagoudar et al. (2017). In this assay ferrozine can quantitatively chelate Fe^{2+} ions and form a red-brown coloured complex. Measurement of color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions. Briefly, the reaction mixture comprised of 1 mL of different concentrations of the fungal extracts (0.2-1.0 mg/mL) and 500 µL of 2mM of $FeCl_3$. The reaction was started by the addition of 200 µL of 5mM ferrozine. After vigorously shaking the reaction mixture was left standing at 30 °C for 10 min and the decrease in the colour intensity was measured at 562 nm against a reagent blank. Resveratrol was used as standard. The percentage of scavenging activity was expressed as calculated as:

$$\text{Free radical scavenging } (\%) = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100$$

The test was performed in triplicates and data were represented as mean ± SD

4.9.4. Nitric oxide radical scavenging assay

Nitric oxide radical inhibition was evaluated using Griess-llosvory reaction (Patel et al., 2010). The reaction mixture (3 mL) consisted of 2 mL of 10 mM sodium nitroprusside, 0.5 mL saline phosphate buffer and 0.5 mL of standard solution or fungal extract (ethyl acetate fraction) (100–500 µg/mL) which was incubated at 25 °C for 2:30 h. After incubation, 500 µL of the reaction mixture was added with 1000 µL sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and permit to stand for 5 min for the completion of the reaction of diazotisation. After this, further 1 mL of the N-(1-Naphthyl) ethylene diamine dihydrochloride was mixed and was permitted to stand for 30 min at room temperature. The concentration of nitrite was assayed at 546 nm and

was calculated with the control absorbance of the standard nitrite solution. Buffer was used as a blank and resveratrol was taken as the standard solution. The percentage inhibition was calculated using the formula:

$$\text{Free radical scavenging } (\%) = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100$$

The test was performed in triplicates and data were represented as mean ± SD.

4.9.5. Hydrogen peroxide radical scavenging assay

The ability of fungal extract (ethyl acetate fraction) to scavenge hydrogen peroxide was calculated by method of Ruch et al. (1989), with minor modifications. A solution of 43 mM of hydrogen peroxide was prepared in 1 M phosphate buffer (pH 7.4). Various concentration of fungal extract (0.2 to 1.0 mg/mL) was added to in 600 µL (43 mM) of hydrogen peroxide solution. The intensity of hydrogen peroxide at 230 nm was determined after 10 min of incubation against a blank solution containing phosphate buffer without hydrogen peroxide. Resveratrol and Ascorbic acid were used as standards. The percentage of scavenging activity was expressed as calculated as:

$$\text{Free radical scavenging } (\%) = \frac{OD_{control} - OD_{test}}{OD_{control}} \times 100$$

The test was performed in triplicates and data were represented as mean ± SD.

4.10. λ-DNA Nicking assay

Oxidative λ- DNA damage protection ability of fungal ethyl acetate extract was assessed by the method of Danagoudar et al. (2017). Briefly, the reaction was performed in an eppendorf tube containing 1 µL of λ- DNA (0.5µg) with and without fungal extract (ethyl acetate fraction) (2 µL of 300 µg/mL) in 15 µL TE buffer and was incubated with Fenton reagent (3 µL of 2 mM $FeSO_4$, 3 µL of 30% H_2O_2 in Tris buffer 10 mM) in a final reaction volume of 30 µL for one hour at 37 °C. After that 1 µL of 0.5 mM EDTA (pH 8.0) was added to stop the reaction. TE buffer was also used in control. The relative difference between oxidized and native DNA was analysed on 1% agarose gel prepared in Tris-acetate-EDTA buffer (pH-8.5). The gel was run at 70 V for 1 hour at room temperature and after that band intensity was documented using XR+ molecular imager Gel documentation system (Bio RAD, USA). All the tests were performed in triplicates and their mean ± SD was calculated.

4.11. In vitro Antifungal activity

Fungicidal activity of the culture extract of resveratrol producing fungi were assessed against a panel of plant pathogenic fungi comprising of *Botrytis cinerea* (MTCC 359), *Collectotricum gloeosporioides* (MTCC 9623), *Cercospora beticola* (ATCC 24888) and *Rhizoctonia solani* (MTCC 4634) by dual culture method. From 7 days old culture of resveratrol producing endophytic fungi and plant pathogen fungi, 6 mm mycelial agar plugs were prepared aseptically. These were then placed on a SDA plate (90 mm dia) at 5 cm from each other. Subsequently, the plates were incubated at 28 °C for 15 days. The growth of the endophytic fungi and pathogen was observed regularly, and radial growth was measured by the mean colony diameter till 15th day of inoculation. The percentage of inhibition was calculated by following formula (Ghildiyal et al., 2008);

$$\frac{R1 - R2}{R1} \times 100$$

Where, R1=radial growth of pathogen;

R2=radial growth of pathogen inoculated with endophytic fungi. All the tests were performed in triplicates and their mean ± SD was calculated

4.12. In-vitro Antistaphylococcal activity

The extracts obtained were evaluated fortheir antistaphylococcal potential against a battery of *Staphylococcus aureus* cultures (NCTC 6571, MTCC 96, MTCC 737, Sau G9, and Sau 902 (Sau cultures were isolated from burns and pus, respectively)) using agar well diffusion assay. Briefly, the plates were swabbed with 18–24 h old 0.5 McFarland adjusted cultures. 5 mm wells were punched in Muller Hinton Agar (MHA) plates using a sterile cork borer. Subsequently, 30 µL of the fungal extract (1 mg/mL) was dispensed in each well and allowed to diffuse at 37 °C for 20 minutes. Similar concentration of Streptomycin (Hi Media) served as a positive control. The plates were incubated overnight at 37 °C. Antistaphylococcal activity was determined by measuring the zone of inhibition formation around the well (Ma et al., 2018). All the tests were performed in triplicates and their mean ± SD was calculated.

4.13. Purification and characterization of fungal resveratrol

The potent bioactive crude fraction of selected endophytic fungus (#12VVLPM) was further subjected to purification by thin layer chromatography (TLC) and column chromatography. Further, the purified fraction was characterized by employing various analytical techniques viz. HPLC, LC-MS, HR-MS, ^{1}HNMR and ^{13}CNMR.

4.13.1. Thin layer chromatography

The crude fraction of the potent endophytic fungus (*Arcopilus aureus*) was further fractionalization by subjecting to PTLC (preparative thin layer chromatography). The glass thin layer chromatography plate of width of 0.5 mm was construct by overlaying silica gel G (Sigma Aldrich; 381276) onto 20 x 15 x 5 mm fresh glass plates (Merck Millipore; RP-8F254S). After that PTLC was activated by incubating at 100 °C for 3 h prior to use. Simultaneously the test sample was spotted on to activated PTLC glass plate just 1 cm above the edge of plate with the help of capillary tube. Concurrently, the PTLC chamber was soaked with different polar and nonpolar solvent systems for 30 min. The PTLC plate along with test sample was then placed inside the saturated PTLC chamber and allowed to run for one hour till the solvent front reached the desired level. After the solvent reached the desired level PTLC plate was taken out and allowed dry at room temperature. The chromatogram was appearing by visualizing the PTLC plate under UV light (Thermo Fisher Scientific; UVGL-58). Resveratrol (1 mg/mL) was used as standard for the comparison of retention factor (R_f) value. R_f value of each band was obtained as ratio of distance moved by solute to that of solvent (Babu et al 2005; Park and Boo, 2013; Wang et al., 2013)

$$\text{Retention Factor } (R_f) = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

4.13.2. Purification of fungal resveratrol by column chromatography

The extract containing fungal resveratrol was initially evaluated with the standard resveratrol through TLC optimization technique. The selected solvent system for the best separation of bioactive fraction formed the basis to decide the mobile system for silica-based column chromatography. Briefly, prior to packing the silica column, the silica gel (60-120 mesh, Merck Millipore) was dried in an oven for one hour at 100 °C. Approximately, 30 g of dried silica was

suspended in methanolic extract and packed in a glass column (30 x 3 cm, Borosil, India) fitted with a G_0 filter. The packed column could be equilibrated with DCM for 1 h. Around 1 g of the bioactive residue absorbed on dried silica was loaded onto the pre-packed column. The gradient elution was carried out using a gradient of DCM- Me0H (100:0 → 0:100). Different fractions were obtained and observed by TLC using optimized solvent system and R_f value was determined (Wang et al., 2013; Soural et al., 2015; Kumar et al., 2018).

4.14. Structure elucidation of pure compound

4.14.1. LC- MS analysis

The purified fungal resveratrol (Stock- 1 mg/mL) in methanol (Merck, HPLC grade) was analyzed in LC–MS system (Waters, Micromass Q–TOF micro using Waters Alliance 2795 separation module). The confirmation was done based on molecular ion of standard and fungal resveratrol by comparing their molecular ions and fragmentation pattern (Dincheva et al., 2011; Ramirez-Lopez and DeWitt, 2014; Kumar et al., 2018).

4.14.2. Nuclear magnetic resonance spectroscopy (NMR)

Approximately, 5 mg of the pure compound dissolved in 0.5 mL of deuterated methanol (Sigma Aldrich) was used for recording the NMR spectra. NMR spectra were recorded at 400 MHz on a JOEL II-400 NMR spectrometer (400.131 MHz proton and 100.525 MHz carbon frequencies) at 18 °C. Proton and carbon 90 ° pulse widths were 10.9 sec and 8.37 sec µs respectively (Liu et al., 2016; Kumar et al., 2018).

4.15. Process optimization for maximum Resveratrol production

4.15.1. One variable at a time approach for maximizing resveratrol production

This is a conventional method for any optimization study for the enhanced production of any biological product. In the present study, this approach was followed wherein the different physiological parameters such as temperature, pH, agitation rate, inoculum age, incubation period and nutritional parameters such as carbon source, nitrogen source were evaluated and optimized. All experiments were carried out in triplicates and their mean and standard deviation was calculated (Shi et al., 2012; Saran et al., 2015).

4.15.1.1. Optimization of basic parameters

i. Estimation of fungal biomass (Growth kinetics)

To determine the fungal biomass produced by *Arcopilus aureus*, fungus was grown in potato dextrose broth (PDB) liquid medium in 250 mL of Erlenmeyer flask and incubated at 30 ºC. After 0, 2, 4, 6, 8, 10, 12, 14, and 16 days of incubation, fungal mycelium were harvested and fresh weight (FW) was recorded. The day on which maximum resveratrol was produced was selected for further optimization. All the tests were performed in triplicates and their mean ± SD was calculated (Shi et al., 2012; Saran et al., 2015; Zhou et al., 2018).

ii. Selection of best production medium for maximizing resveratrol production

To begin with, maximum resveratrol production from the selected organism was examined in different production medium reported in the literature. For this, different production media were tested by inoculating the fungus in CZD YEB Broth, CZD Broth, PDB Broth, YMG Broth, Grape Leaf Extract Broth, Richards Broth, SDB Broth, CMB Broth, Ashthana Hawker Medium, Wickerham Medium, M4 Medium and Tryptone Soy Broth. The medium showing maximum resveratrol production was selected for further optimization. All the tests were performed in triplicates and their mean ± SD was calculated (Shi et al., 2012; Jeandet et al., 2014; Saran et al., 2015).

iii. Optimization of medium-to-flask volume ratio (V_m/V_f ratio)

The effect of medium-to-flask volume ratio in the range of 0.12-0.7 was tested on mycelial growth and resveratrol production. The fungus *Arcopilus aureus* was grown in 250 mL of Erlenmeyer flask along with different volume of potato dextrose medium analogues to each volumetric ratio. Mycelium was harvested from fermentation media after 8 days. The weight of mycelium was calculated, and resveratrol production was also analyzed by the help of HPLC from culture filtrate (Shi et al., 2012; Saran et al., 2015; Zhou et al., 2018). All the tests were performed in triplicates and their mean ± SD was calculated.

4.15.1.2. Optimization of Physiological parameters

i. Estimation of optimum pH, Temperature and Agitation

The resveratrol producing fungus was grown in 50 mL of PDB medium in 250 mL of Erlenmeyer flask at different pH range 3-10 (3, 4, 5, 6, 7, 8, 9 and 10) and temperature range 12-39 ^{o}C (12, 15, 18, 21, 24, 27, 30 33, 36 and 39 ^{o}C). The pH of medium was adjusted by using 1N HCl/NaOH.

Agitation rate indirectly measures the dissolved oxygen concentration and it's an important factor for production of secondary metabolite. Hence, the production of resveratrol from the selected fungus was examined at different agitation rate i.e. static, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 rpm in a New Brunswick incubator shaker (model AG-27; Multitron) under optimized conditions obtained so far.

After 8 days of incubation the weight of mycelium was calculated and resveratrol production was also analyzed by the help of HPLC from culture filtrate (Shi et al., 2012; Goutam et al., 2014; Zhou et al., 2018). All the tests were performed in triplicates and their mean ± SD was calculated.

4.15.1.3. Optimization of Nutritional parameters

i. Estimation of optimum carbon source and concentration

In order to study the effect of carbon source on resveratrol production, different sugars were evaluated in the optimized fungal medium. The carbon sources tested were Glucose, Fructose, Galactose, Sucrose, Glycerol, Maltose, Lactose and Starch. Further the media was supplemented with different concentrations of the selected carbon source in the range of 0.25-5% (0.25, 0.50, 1.0, 2.0, 3.0, 4.0, and 5.0%) for their effect on resveratrol production. After 8 days of incubation the weight of mycelium was calculated and resveratrol production was also analyzed by the help of HPLC from culture filtrate in order to optimize the concentration of the selected carbon source (Shi et al., 2012; Garyali et al., 2014; Goutam et al., 2014; Zhou et al., 2018). All the tests were performed in triplicates and their mean ± SD was calculated.

ii. Estimation of optimum nitrogen source and concentration

In order to study the effect of nitrogen source on resveratrol production, different nitrogen sources were evaluated in the optimized fungal medium. The nitrogen sources tested were ammonium chloride (NH_4Cl), yeast extract, corn steep liquor, peptone, tryptone, urea (NH_2CONH_2), beef extract and ammonium sulphate (NH_4SO_4). Further the media was supplemented with different concentrations of the selected nitrogen source in the range of 0.25-5% (0.25, 0.50, 1.0, 2.0, 3.0, 4.0, and 5.0 %) for determining the best concentration for maximizing resveratrol production. After 8 days of incubation the weight of mycelium was calculated and resveratrol production was also analyzed by the help of HPLC from culture filtrate

(Shi et al., 2012; Garyali et al., 2014; Qiao et al., 2017; Zhou et al., 2018). All the tests were performed in triplicates and their mean ± SD was calculated.

In "One- variable-at-a-time" (OVAT) approach, physiological and nutritional parameters were optimized in a sequential manner by incorporating one optimized condition before optimizing the second parameter. This procedure is conventionally used and is normally preferred for optimization procedures. However, this procedure is time consuming and also has a limitation of non-interaction of different physiological and nutritional parameters. It is possible that the interactions of these parameters may further result in an increase in resveratrol production.

4.15.2. Process Optimization for fungal resveratrol production by Response surface methodology

Various physiological and nutritional parameters play an important role in fermentative production of any bioactive compounds (Shi et al., 2012; Saran et al., 2015). The classical approach for process optimization of bioactive compounds is generally by OVAT approach and the other method which is being used to optimize the parameters is based on the statistical approach known as "Response surface methodology (RSM)" (Shi et al., 2012; Saran et al., 2015; Tian et al., 2017). In the present study fungus *Arcopilus aureus*, showed maximum resveratrol production, was selected for further process optimization of medium components as well as growth conditions for maximization of resveratrol production.

4.15.2.1. Experiment design using Response Surface Methodology (RSM)

Response surface methodology is a statistical procedure which is used to obtain the optimized conditions from all possible combinations of significant physiological and nutritional factors. In the present investigation, Box-Behnken Design (BBD) falling under RSM was used to obtain maximum resveratrol production from the selected endophytic fungus.

i. Box-Behnken Design (BBD)

Once the variables having substantial influence on resveratrol production, i.e. response, were identified, BBD was used to optimize the levels of these variables as per the procedure described by Myers et al., (2016). Here, BBD was performed as it incorporates replication of medial point (000). The ranges of these variables were decided according to the ranges determined by one-variable-at-a-time approach.

The statistical software package 'Design-Expert® 6.0 Stat-Ease, Inc., (Minneapolis, USA) was used to analyze the experimental design. A set of 54 experiments was generated. The design matrix with different variables was set at three levels (-1, 0, +1) (Table 4.1). All the variables optimized by one-variable-at-a-time were taken at a central coded value treated as zero. The least and highest ranges of variables were examined. All the experiments were carried out in 250 mL of Erlenmeyer flask containing 50 mL of PDB medium at 30ºC with initial pH of 7.0. After 8 days of incubation the weight of mycelium was calculated and resveratrol production was also analyzed by the help of HPLC from culture filtrate (Shi et al., 2012; Garyali et al., 2014; Qiao et al., 2017; Zhou et al., 2018). All the tests were performed in triplicates and their mean ± SD was calculated.

The optimized conditions of the parameters obtained as the response (dependent variable) was represented as a second order polynomial equation to express the effect of different variables.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where, Y=represents response; X_i and X_j=independent variables which affect the response; β_0 defines regression coefficient for the intercept, β_i for linear, β_{ij} for cross product terms and β_{ii} for quadratic.

Table 4.1. Experimental range and levels of the six most influential variables used in RSM

Factors	Variables	Actual	Coded	Actual (0 level) *	Coded	Actual
A	**RPM**	90	-1	115	+1	140
B	**Temperature (ºC)**	20	-1	30	+1	40
C	**Incubation days**	5	-1	8.5	+1	12
D	**pH**	5	-1	7	+1	9
E	**Glucose (%)**	0.5	-1	1.25	+1	2
F	**Peptone (%)**	0.25	-1	0.625	+1	1

4.15.2.2. Statistical analysis and modelling

The data on resveratrol production was subjected to Analysis of Variance (ANOVA) appropriate to design the experiments. The mathematical relationship of the independent variable and the response (resveratrol production) was calculated by the second order polynomial equation. This

equation is used to evaluate the linear, quadratic and interactive effects of independent variables on the selected response. The response for each run were subjected to multiple non-linear regressions using the software 'DESIGN EXPERT' to obtain the coefficients of the second order polynomial equation included in the final models. The f-test was used to evaluate the significance of the models. Proper care was taken to eliminate the problems of aliases while fitting the models.

Values close to 1 indicate a good model. Three dimensional plots were generated to present the amount of resveratrol produced under optimized conditions. The concentric circles in three-dimensional plot represent the region of maximum resveratrol production.

4.15.2.3. Validation of response surface model

In order to determine the accuracy of the model, the concentration of six factors (rpm, temperature, pH, incubation days, glucose and peptone), which have a major influence on resveratrol production as obtained by response surface methodology were randomly selected within the design space.

4.15.3. Analytical methods

After 8 days of incubation, fungal biomass was separated with the help of Whatman filter paper no. 4 (GE Healthcare Life Sciences, USA), followed by ultracentrifugation at 8,000 ×g (Hitachi RX II series, Japan) for 10 min at 30ºC to make cell free filtrate. The cell free culture was then passed through 0.22 µm nitrocellulose membrane (GE Healthcare Life Sciences, USA) (Yang et al., 2016). After that culture filtrate was extracted with three volume of ethyl acetate. The ethyl acetate fraction was carefully pooled out and evaporated under rotary evaporator at 35ºC. Concentration of resveratrol in ethyl acetate fraction of the selected potent culture isolate was determined using HPLC (Perkin Elmer, USA) attached with 200 series pump, UV-VIS wavelength detector and total chrome workstation software. Briefly, 20 µL of ethyl acetate fraction (crude) was dissolved in HPLC grade methanol and injected into the C_{18} reverse phase column. Isocratic mode of elution of orthophosphoric acid (0.1%, pH- 2.3, Sigma Aldrich; V800287) and acetonitrile (Sigma Aldrich; 34851), 45%-55% ratio was used as mobile phase with a flow rate of 1 mL/min. To calculate the concentration of fungal resveratrol, various dilutions of standard resveratrol (0.1 - 1.0 mg/mL) were prepared in HPLC grade methanol. 20 µL of each dilution of

standard resveratrol was injected into C_{18} (5 µm, 25 cm X 4.6 mm) reverse phase discovery column (Sigma Aldrich; 58298), column oven temperature (34 °C). The data of the peak area vs. concentration of the standard resveratrol obtained were used to estimate the quantity of fungal resveratrol in crude ethyl acetate (EA) fraction (Shi et al., 2012; Wang et al., 2013; Jeandet et al., 2014; Saran et al 2015; Liu et al 2016; Dwibedi and Saxena, 2018).

4.16. Development of epigenetic variants

The wild type endophytic fungus *Arcopilus aureus* was used for epigenetic modulation using 5-azacytidine (AZA) and suberoylanilide hydroxamic acid (SAHA) as modulating compounds. All the stock solutions were prepared in DMSO. The endophytic fungus was inoculated on PDA enriched with different concentration of AZA and SAHA (1-100 µm (1, 5, 10, 20, 30, 50, 70 µm)) for 10 days. Several epigenetic variants resulted from different concentrations of AZA and SAHA and the most notable culture i.e. maximum resveratrol producer was selected for future study. Further the AZA and SAHA variants in combination was also carried out and the resveratrol concentration was also determined (Table 4.2).

The 5 mm fungal mycelial disc of 8 day old endophytic fungus obtained was inoculated. The flasks were incubated in incubator shaker at 26 ± 2 ˚C, 120 rpm for 8 days. After the completion of incubation, fungal mycelia was separated from spent broth with the help of Whatman filter paper 4 followed by ultracentrifugation at 12,000 rpm for 15 min and then separated through 0.2 µm NCM (nitrocellulose membrane) to get cell free filtrate (Gonzalez et al., 2003; Ul-Hassan et al., 2012; Kumar et al., 2016). The filtrates of the epigenetically modified cultures were then quantified for the presence of RESV using HPLC.

4.16.1. Stability study of epigenetic variant

The epigenetic variants were sub-cultured up to 5 generations on AZA and SAHA enriched PDA plates in which the maximum RESV concentration appeared during HPLC analysis same as that of the first epigenetic variant. Henceforth, the stability of the variants was also evaluated using HPLC (Gonzalez et al., 2003; Ul-Hassan et al., 2012; Kumar et al., 2016).

Table 4.2. The different combinations of AZA and SAHA

Conc. → / Combinations ↘	AZA 1μM	AZA 5μM	AZA 10μM	AZA 20μM	AZA 30μM	AZA 50μM	AZA 70μM
SAHA 1μM	AZA 1μM + SAHA 1μM	AZA 5μM + SAHA 1μM	AZA 10μM + SAHA 1μM	AZA 20μM + SAHA 1μM	AZA 30μM + SAHA 1μM	AZA 50μM + SAHA 1μM	AZA 70μM + SAHA 1μM
SAHA 5μM	AZA 1μM + SAHA 5μM	AZA 5μM + SAHA 5μM	AZA 10μM + SAHA 5μM	AZA 20μM + SAHA 5μM	AZA 30μM + SAHA 5μM	AZA 50μM + SAHA 5μM	AZA 70μM + SAHA 5μM
SAHA 10μM	AZA 1μM + SAHA 10μM	AZA 5μM + SAHA 10μM	AZA 10μM + SAHA 10μM	AZA 20μM + SAHA 10μM	AZA 30μM + SAHA 10μM	AZA 50μM + SAHA 10μM	AZA 70μM + SAHA 10μM
SAHA 20μM	AZA 1μM + SAHA 20μM	AZA 5μM + SAHA 20μM	AZA 10μM + SAHA 20μM	AZA 20μM + SAHA 20μM	AZA 30μM + SAHA 20μM	AZA 50μM + SAHA 20μM	AZA 70μM + SAHA 20μM
SAHA 30μM	AZA 1μM + SAHA 30μM	AZA 5μM + SAHA 30μM	AZA 10μM + SAHA 30μM	AZA 20μM + SAHA 30μM	AZA 30μM + SAHA 30μM	AZA 50μM + SAHA 30μM	AZA 70μM + SAHA 30μM
SAHA 50μM	AZA 1μM + SAHA 50μM	AZA 5μM + SAHA 50μM	AZA 10μM + SAHA 50μM	AZA 20μM + SAHA 50μM	AZA 30μM + SAHA 50μM	AZA 50μM + SAHA 50μM	AZA 70μM + SAHA 50μM
SAHA 70μM	AZA 1μM + SAHA 70μM	AZA 5μM + SAHA 70μM	AZA 10μM + SAHA 70μM	AZA 20μM + SAHA 70μM	AZA 30μM + SAHA 70μM	AZA 50μM + SAHA 70μM	AZA 70μM + SAHA 70μM

4.17. Statistical analysis

All the assays were performed in triplicates and the data was represented in terms of mean ± standard deviation. The results were analyzed by ANOVA followed by Tukey's post hoc test ($p<0.05$). Statistical analysis was performed using Graph Pad Prism 7 software and $p< 0.05$ was considered significant.

Chapter- 5

Results

5.0. RESULTS

5.1. Isolation of Endophytic fungi

A total of 145 isolates were isolated from 525 segments of leaf and stem from all the plant samples collected. Among 145 isolates 55 isolates (19 Merlot, 4 Wild, 12 Pinot Noir, 10 Shiraz and 10 Muscat) were recovered from Pune, Maharashtra; 54 isolates (16 Merlot, 6 Wild, 6 Pinot Noir, 20 Shiraz and 6 Muscat) from Nashik, Maharashtra; followed by 27 isolates (5 Merlot, 12 Wild, 4 Pinot Noir, 3 Shiraz and 3 Muscat) from Alambagh, Lucknow and 9 isolates (4 Merlot and 5 Wild) from Bengaluru, Karnataka (Figure 5.3, Table 5.1).

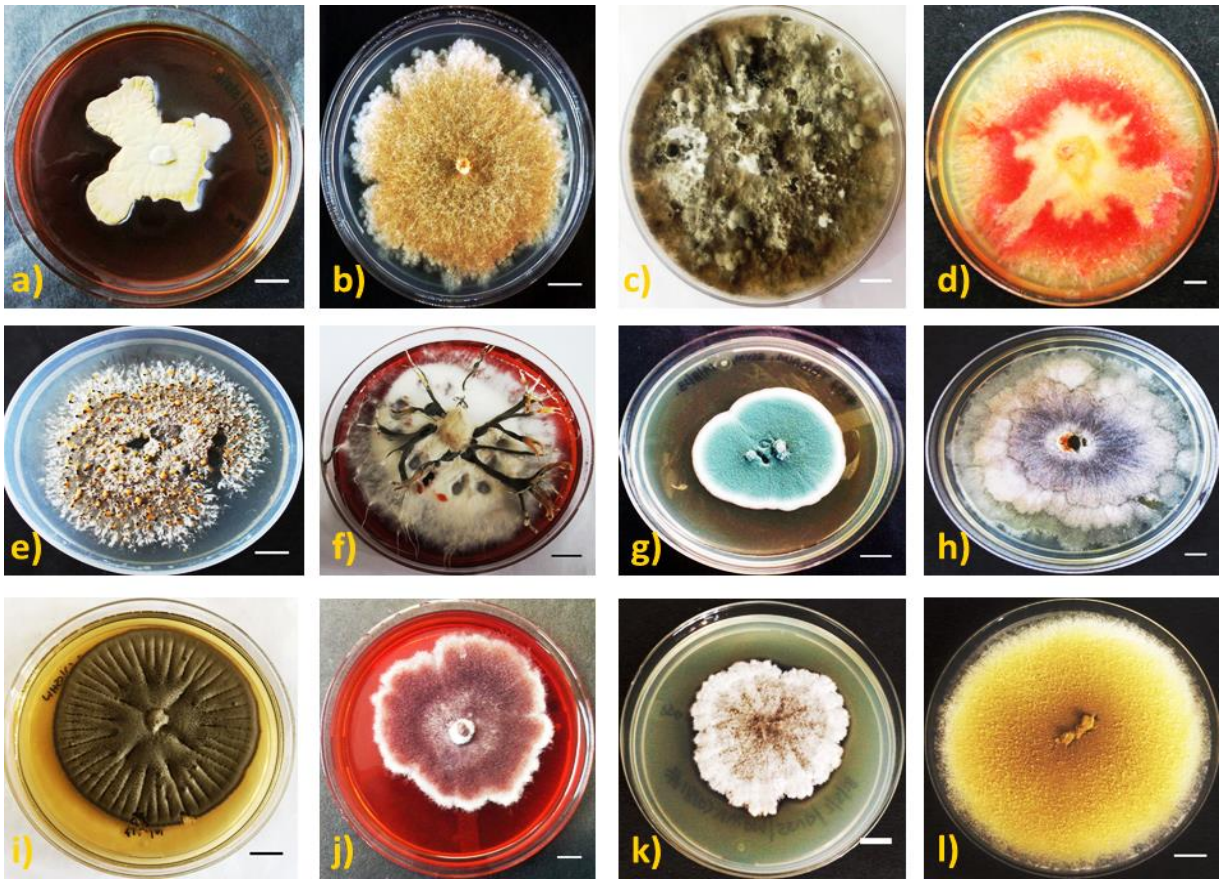


Figure 5.1. Plate culture of some of characteristic endophytic fungi from different parts and location of *Vitis vinifera*. (a) *Arcopilus aureus*, (b) *Fusarium incarnatum*,(c) *Nigrospora* sp., (d-j) *Fusarium solani*, (e) *Colletotrichum falcatum* (f) *Xylaria psidii*, (g) *Penicillium* sp., (h) Unidentified, (i) *Aspergillus* sp.,(k) Unidentified, (l) *Botryosphaeria* sp. (Bar: 10 mm)

Figure 5.1 and Figure 5.2 depicts some of the endophytic fungi isolated during the study and the morphotaxomony of some of the isolates, respectively.

5.1.1. Fungal Diversity Studies

A total of 145 isolates belonging to eighteen different fungal genera were isolated from 525 segments of leaf and stem in totality (Figure 5.3). Collectively, among all the endophyte

isolates recovered from *Vitis vinifera* in present study, there were 58.2% Hyphomycetes, 19.3% Ascomycetes, 21.0% Coelomycetes and 1.7% Basidiomycetes (Figure 5.4).

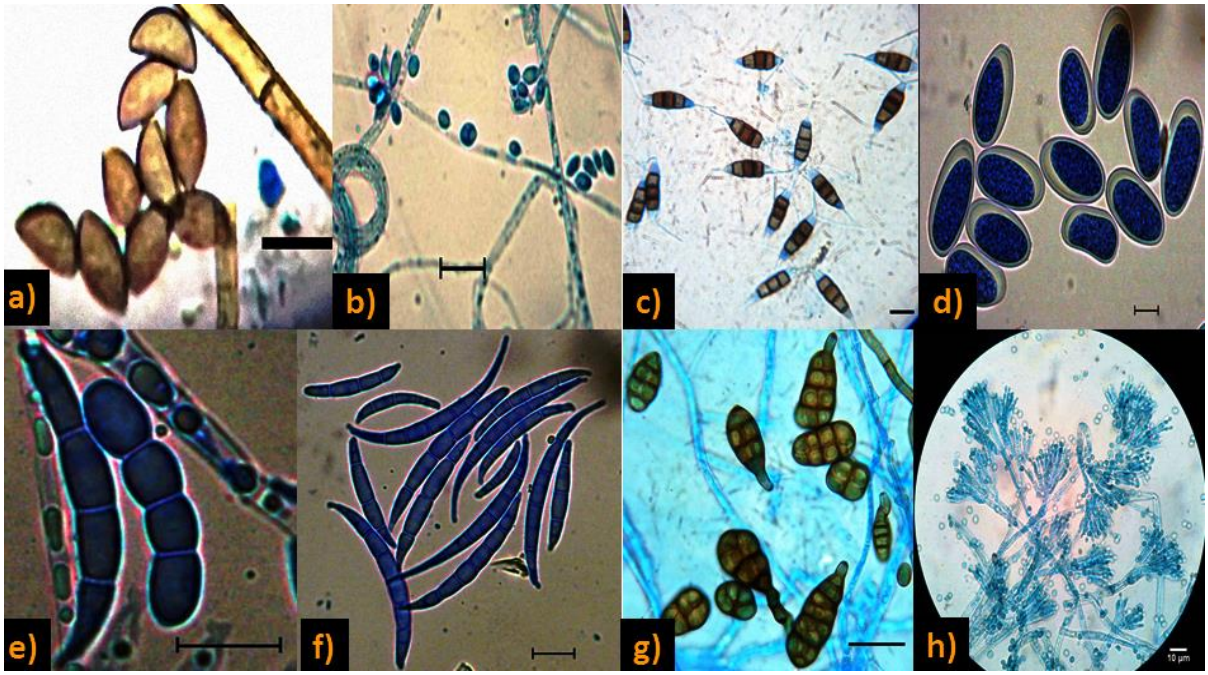


Figure 5.2. Microscopic features of endophytic fungi isolated during the study. (a) Lunate, reniform shaped ascospores of *Arcopilus aureus,* (b) mature conidia of *Quambalaria sp.,* (c) Conidia of *Pestalotiopsis* sp., (d) mature conidia of *Lasiodiplodia pseudotheobromae,* (e-f) macro and microconidia of *Fusarium* sp., (g) Conidia of *Alternaria* sp., (h) stipulate, mature and phialides of *Penicillium* sp. (Bar: 10 µm)

The maximum endophytic fungi were isolated from Pune, Maharashtra (37.9%) and lowest were recovered from Bengaluru, Karnataka (6.2%). Among the different *Vitis vinifera* varieties the maximum endophytic fungi were present in *Vitis vinifera* (Merlot, 13.79%) isolated from Nashik, Maharashtra and least were isolated from *Vitis vinifera* (Shiraz, 2.07%) and *Vitis vinifera* (Muscat, 2.07%) from Alambagh, Lucknow.

Nashik (Maharashtra) exhibited highest colonization frequency (36%) while Bengaluru, Karnataka exhibited least (18%) colonization frequency. Whereas, among different varieties *Vitis vinifera* (Shiraz, 66.7%) from Nashik, Maharashtra exhibited the highest colonization frequency followed by *Vitis vinifera* (Merlot, 53.3%) from Nashik, Maharashtra and the least colonization frequency was that of *Vitis vinifera* (Wild, 10%) from Pune, Maharashtra (Table 5.1). The dominant fungi observed were *Aspergillus*, *Fusarium*, *Alternaria* and *Botryosphaeria*. *Xylaria, Quambalaria* and *Arcopilus* were also isolated which have not been reported earlier from Indian grape wines (*Vitis vinifera)*.

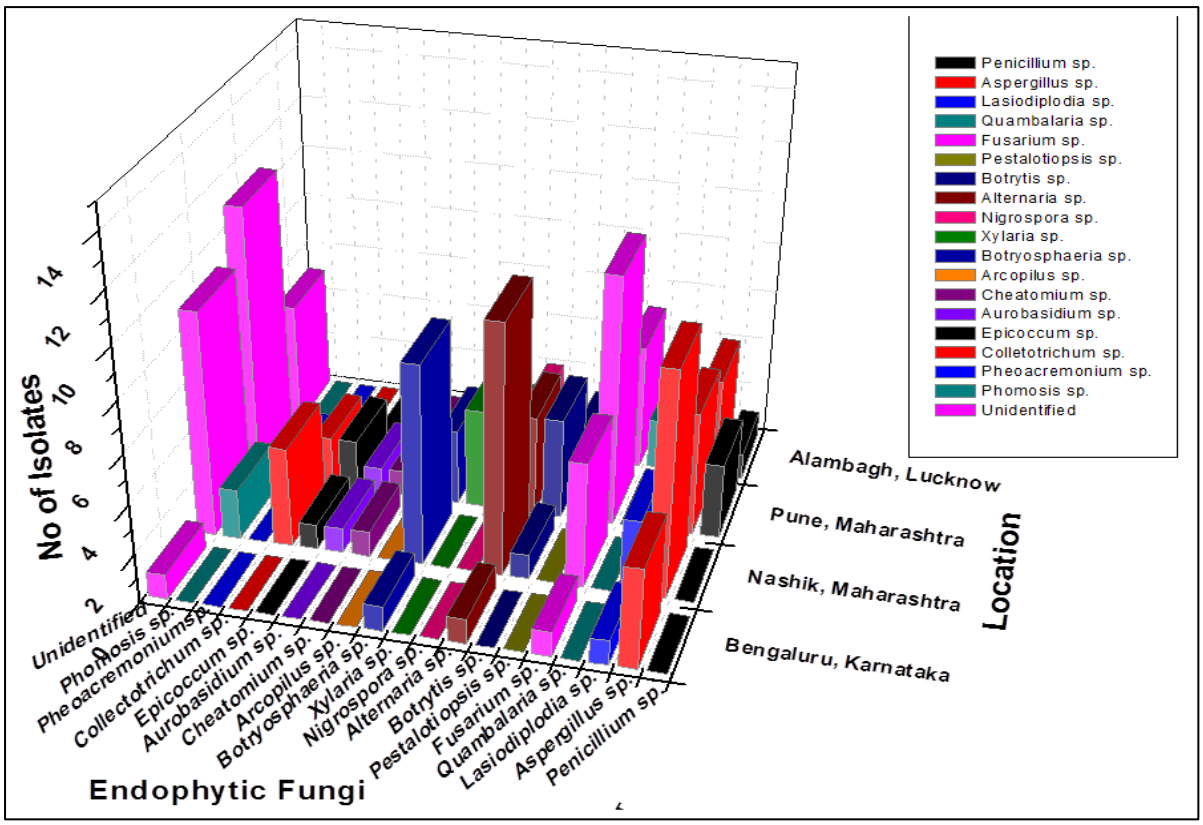


Figure 5.3. The relative recovery of different endophytic groups from the different location in India.

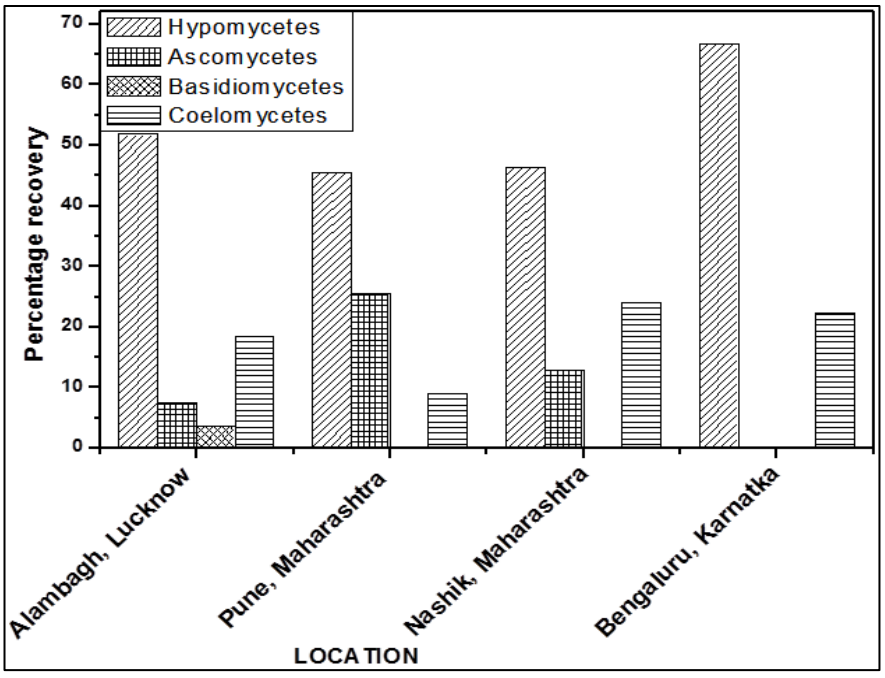


Figure 5.4. Percentage recovery of endophytic fungi isolated from different locations in India.

Table 5.1. Colonization frequency of different fungal endophytes of *Vitis vinifera* according to variety and location(collected from different parts of India)

Location	Plant sample	No. of segments	No. of isolates	Culture code	Morphological identification	%CF	
						Variety wise	Location wise
Alambagh, Lucknow	*Vitis vinifera* (Merlot)	25	5	#3VVLLK	*Penicillium* sp.	20	21.6
				#6(b)VVLLK	Unidentified		
				#9VVLLK	*Aspergillus* sp.		
				#15VVLLK	*Aspergillus* sp.		
				#1VVSTL	*Fusarium* sp.		
	Vitis vinifera (Wild)	25	12	#1(b)VVSTL	*Alternaria* sp.	48	
				#3VVSTL	*Fusarium*sp.		
				#37VVSTL	*Fusarium* sp.		
				#1VVRLLK	Unidentified		
				#1VVRSTL	*Lasiodiplodia* sp.		
				#4VVLLK	Unidentified		
				#50(d)VVLLK	*Fusarium* sp.		
				#8VVLLK	*Pestalotiopsis* sp.		
				#8VVGSTL	Unidentified		
				#21VVGSTL	*Aspergillus* sp.		
				#9VVGLLK	Unidentified		
				#9VVRLLK	*Botrytis sp.*		
	Vitis vinifera (Pinot)	25	4	#4VVSTL	*Nigrospora* sp.	16	
				#8VVSTL	*Pestalotiopsis sp.*		
				#5(b)VVLLK	*Aspergillus* sp.		
				#13VVRLLK	*Fusarium* sp.		
	Vitis vinifera (Shiraz)	25	3	#20VVRLLK	Unidentified	12	
				#9VVSTL	*Xylaria* sp.		
				#2VVSTL	*Nigrospora* sp.		
	Vitis vinifera (Muscat)	25	3	#7VVSTL	*Pestalotiopsis sp.*	12	
				#24VVSTL	*Pestalotiopsis* sp.		
				#11VVRLLK	*Aspergillus* sp.		
Pune, Maharashtra	*Vitis vinifera* (Merlot)	40	19	# 12VVLPM	*Arcopilus sp.*	47.5	27.5
				#37VVLPM	*Penicillium* sp.		
				#66VVLP	*Penicillium* sp.		
				#98VVSTPM	*Penicillium* sp.		
				#184VVSTPM	*Lasiodiplodia* sp.		
				#193VVSTPM	*Nigrospora* sp.		
				#19(P)VVLPM	*Epicoccum sp.*		
				#113(P)VVLPM	*Botrytis* sp.		
				#104(P)VVLPM	*Alternaria* sp.		
				#139(P)VVLPM	Unidentified		
				#140(P)VVLPM	*Colletotrichum* sp.		
				#33(P)VVLPM	Unidentified		
				#79(P)VVLPM	*Colletotrichum* sp.		

				#71(P)VVLPM	Unidentified		
				#117(P)VVLPM	Unidentified		
				#41(P)VVLPM	*Fusarium* sp.		
				#43(P)VVLPM	*Aspergillus* sp.		
				#141(P)VVLPM	*Fusarium* sp.		
				#59(P)VVLPM	Unidentified		
	Vitis vinifera (Wild)	40	4	#129(P)VVLPM	Unidentified	10	
				#111(P)VVLPM	*Botrytis* sp.		
				#55(P)VVLPM	*Xylaria* sp.		
				#131(P)VVLPM	Unidentified		
	Vitis vinifera (Pinot)	40	12	#19VVLPM	*Fusarium* sp.	30	
				#18(P)VVLPM	*Aurobasidium* sp.		
				#18VVLPM	*Botryosphaeria* sp.		
				#20(b)VVLPM	*Botryosphaeria* sp.		
				#21VVLPM	*Aspergillus* sp.		
				#23VVLPM	*Alternaria* sp.		
				#24VVLPM	*Alternaria* sp.		
				#23(P)VVLPM	*Fusarium* sp.		
				#15(P)VVLPM	*Xylaria* sp.		
				#133(P)VVLPM	Unidentified		
				#68(P)VVLPM	*Botrytis* sp.		
				#57(P)VVLPM	*Alternaria* sp.		
	Vitis vinifera (Shiraz)	40	10	#10VVLPM	*Botryosphaeria* sp.	25	
				#10(b)VVLPM	*Fusarium* sp.		
				#25VVLPM	*Fusarium* sp.		
				#27VVLPM	Unidentified		
				#51(P)VVLPM	*Xylaria* sp.		
				#10(P)VVLPM	*Phaeoacremonium* sp.		
				#22(P)VVLPM	*Xylaria* sp.		
				#107(P)VVLPM	*Phomopsis* sp.		
				#122(P)VVLPM	*Aspergillus* sp.		
				#42VVLPM	*Fusarium* sp.		
	Vitis vinifera (Muscat)	40	10	#19(b)VVLPM	*Epicoccum* sp.	25	
				#13(P)VVLPM	*Fusarium* sp.		
				#36(P)VVLPM	*Aspergillus* sp.		
				#103(P)VVLPM	Unidentified		
				#101(P)VVLPM	*Botrytis* sp.		
				#11VVSPM	*Fusarium* sp.		
				#12(b)VVLPM	*Aspergillus* sp.		
				##12(c)VVLPM	*Chaetomium* sp.		
				#16(b)VVLPM	*Fusarium* sp.		
				#17(b)VVLPM	Unidentified		
Nashik, Maharashtra	*Vitis vinifera* (Merlot)	30	16	#29VVLNM	Unidentified	53.3	36
				#29(b)VVLNM	*Aspergillus* sp.		
				#30(b)VVLNM	*Lasiodiplodia* sp.		

				#31VVLNM	*Colletotrichum* sp.		
				#33VVLNM	Unidentified		
				#35VVLNM	*Alternaria* sp.		
				#83VVLNM	*Aspergillus* sp.		
				#109VVLNM	*Alternaria* sp.		
				#105VVLNM	*Aspergillus* sp.		
				#75VVSSWN	*Botrytis* sp.		
				#71VVSSWN	*Alternaria* sp.		
				#68VVSSWN	*Botryosphaeria* sp.		
				#40VVLSWN	*Colletotrichum* sp.		
				#15VVLSWN	*Fusarium* sp.		
				#76VVSSWN	*Fusarium* sp.		
				#176VVSSWN	*Epicoccocum* sp.		
	Vitis vinifera (Wild)	30	6	#671VVLSWN	*Botryosphaeria* sp.	20	
				#45VVLSWN	*Lasiodiplodia* sp.		
				#49VVLSWN	*Botryosphaeria* sp.		
				#103VVLSWN	*Lasiodiplodia* sp.		
				#107VVLSWN	*Phomopsis* sp.		
				#721VVLSWN	*Alternaria* sp.		
	Vitis vinifera (Pinot)	30	6	#79VVLSWN	*Fusarium* sp.	20	
				#91VVLSWN	*Colletotrichum* sp.		
				#17VVLSWN	*Alternaria* sp.		
				#591VVLSWN	*Botryosphaeria* sp.		
				#515VVLSWN	Unidentified		
				#801VVLSWN	*Alternaria* sp.		
	Vitis vinifera (Shiraz)	30	20	#4(P)VVLNM	*Fusarium* sp.	66.7	
				#77VVLNM	*Aureobasidium* sp.		
				#19VVLSWN	Unidentified		
				#201VVLSWN	*Colletotrichum* sp.		
				#289VVLSWN	*Aspergillus* sp.		
				#61VVLSWN	*Phomopsis* sp.		
				#695VVLSWN	*Botryosphaeria* sp.		
				#31VVLSWN	*Alternaria* sp.		
				#21VVLSWN	Unidentified		
				#729VVLSWN	*Alternaria* sp.		
				#733VVLSWN	Unidentified		
				#720VVLSWN	*Fusarium* sp.		
				#751VVLSWN	*Botryosphaeria* sp.		
				#757VVLSWN	*Aspergillus* sp.		
				#761VVLSWN	*Aspergillus* sp.		
				#771VVLSWN	*Aspergillus* sp.		
				#779VVLSWN	Unidentified		
				#781VVLSWN	*Aspergillus* sp.		
				#791VVLSWN	*Alternaria* sp.		
				#799VVLSWN	*Aspergillus* sp.		

	Vitis vinifera (Muscat)	30	6	#315VVLSWN	*Chaetomium* sp.	20	
				#111VVLSWN	*Botryosphaeria* sp.		
				#115VVLSWN	*Alternaria* sp.		
				#8VVSSWN	Unidentified		
				#763VVLSWN	Unidentified		
				#805VVLSWN	*Botryosphaeria* sp.		
Bengaluru, Karnataka	*Vitis vinifera* (Merlot)	20	4	#20VVLBK	*Fusarium* sp.	20	18
				#20(a)VVLBK	*Botryosphaeria* sp.		
				#49(b)VVLBK	*Aspergillus* sp.		
				#101VVLBK	*Aspergillus sp.*		
	Vitis vinifera (Wild)	30	5	#13VVLBK	*Lasiodiplodia* sp.	16.7	
				#23VVSTBK	*Aspergillus* sp.		
				#53VVSTBK	*Aspergillus* sp.		
				#56VVLBK	Unidentified		
				#101VVSTBK	*Alternaria* sp.		

Table 5.2. Diversity indexes of endophytic fungi isolated from different locations according to different *Vitis vinifera* species (i.e. variety wise) and according to location.

Location	Variety	Taxa (S)		Shannon-Wiener diversity index (H')		Simpson's Diversity index (1-D)		Evenness (H'/ln S)		Species richness ($S/N^{1/2}$)	
		Variety wise	Total (location wise)	Variety wise	Total (location wise)	Variety wise	Total (location wise)	Variety wise	Total (location wise)	Variety wise	Total (location wise)
Alambagh, Lucknow	Merlot	4	11	1.33	2.15	0.9	0.89	0.96	0.89	1.79	2.12
	Wild	8		1.94		0.91		0.93		2.31	
	Pinot	4		1.39		1		1		2	
	Shiraz	3		1.10		1		1		1.73	
	Muscat	2		0.64		0.67		0.92		1.16	
Nashik, Maharashtra	Merlot	11	17	2.20	2.49	0.89	0.91	0.92	0.88	2.52	2.29
	Wild	3		1.04		0.83		0.95		1.5	
	Pinot	8		1.98		0.92		0.95		2.31	
	Shiraz	7		1.83		0.91		0.94		2.21	
	Muscat	6		1.69		0.89		0.95		1.89	
Pune, Maharashtra	Merlot	9	12	2.10	2.18	0.93	0.89	0.96	0.88	2.25	1.63
	Wild	4		1.33		0.87		0.96		1.63	
	Pinot	5		1.56		0.93		0.97		2.04	
	Shiraz	8		1.88		0.86		0.90		1.79	
	Muscat	4		1.33		0.87		0.96		1.63	
Bengaluru, Karnataka	Merlot	3	6	1.04	1.58	0.83	0.83	0.95	0.882	1.5	2
	Wild	4		1.33		0.9		0.96		1.79	

The rich diversity location was Nashik, Maharashtra where 17 different fungal genera were recovered and among the different varieties it was *Vitis vinifera* (Merlot=11) from Nashik, Maharashtra. The diversity of endophytic fungi isolated from different location was compared using alpha diversity indices i.e. (Simpson's diversity index and Shannon – Wiener index) and their components i.e. (Species richness and Evenness) (Table 5.2). The Simpson's Dominance or Concentration of Dominance of endophytic fungi was maximum in Nashik, Maharashtra (0.91) and the lowest was at Bengaluru, Karnataka (0.83) and among different varieties maximum was observed in *Vitis vinifera* (Pinot noir=1; Shiraz=1) from Alambagh, Lucknow and the lowest was in *Vitis vinifera* (Muscat=0.67) from Alambagh, Lucknow. On the other hand Shannon – Wiener diversity index too was also highest in Nashik, Maharashtra (2.49) while least in Bengaluru, Karnataka (1.58) and among different varieties maximum was observed in *Vitis vinifera* (Merlot=2.20) from Nashik, Maharashtra and the lowest was in *Vitis vinifera* (Wild=1.04) from Nashik, Maharashtra and *Vitis vinifera* (Merlot=1.04) from Bengaluru, Karnataka (Table 5.2).

The Evenness of endophytic fungi in Alambagh, Lucknow (0.89) was more uniform than rest of the location's and among different varieties *Vitis vinifera* (Pinot noir=1, Shiraz=1) from Alambagh, Lucknow was most uniform among the varieties than from those from locations (Table 5.2).

Table 5.3. Sorensen's index and Jaccard's index of similarity between different locations of endophytic fungi from *Vitis vinifera*.

S. No.	Location	Sorensen's index of similarity (2a/(b+c))	Jaccard's index ((w/(w+x+y))
1	Alambagh, Lucknow, Pune, Maharashtra	0.64	0.32
2	Alambagh, Lucknow, Nashik, Maharashtra	0.52	0.26
3	Alambagh, Lucknow, Bengaluru, Karnataka	0.59	0.29
4	Pune, Maharashtra, Nashik, Maharashtra	0.83	0.41
5	Pune, Maharashtra, Bengaluru, Karnataka	0.52	0.26
6	Nashik, Maharashtra, Bengaluru, Karnataka	0.6	0.33

The endophytic fungi isolated from Nashik, Maharashtra showed greater species richness than other locations viz. 2.29 and *Vitis vinifera* (Merlot=2.52) from Nashik, Maharashtra exhibited the greatest species richness amongst varieties than from different locations (Table 5.2).

The similarity between endophytic fungi of different location was calculated using Sorenson's similarity indices and Jaccard's Similarity indices. And the maximum Jaccard's and Sorenson's similarity was found between endophytic fungi isolated from Nashik, Maharashtra and Pune, Maharashtra (Sorenson's=0.83 and Jaccard's=0.41) and minimum similarity was between endophytic fungi isolated from Alambagh, Lucknow and Nashik, Maharashtra (Sorenson's=0.52 and Jaccard's=0.26) and Pune, Maharashtra and Bengaluru, Karnataka (Sorenson's=0.52 and Jaccard's=0.26) (Table 5.3).

Table 5.4. Sorensen's index of similarity between different *Vitis vinifera* species (i.e. variety wise).

Location	**Variety**									
	Merlot	Merlot	Merlot	Merlot	Wild	Wild	Wild	Pinot	Pinot	Shiraz
	Wild	Pinot	Shiraz	Muscat	Pinot	Shiraz	Muscat	Shiraz	Muscat	Muscat
Alambagh	0.50	0.50	0.29	0.33	0.50	0.18	0.4	0.29	0.67	0
Nashik	0.29	0.53	0.33	0.59	0.55	0.4	0.44	0.67	0.57	0.46
Pune	0.46	0.71	0.71	0.46	0.44	0.5	0.5	0.77	0.67	0.5
Bengaluru	0.29	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: NA represents not applicable.

Among different varieties the maximum Jaccard's and Sorenson's similarity was found between varieties Pinot noir and Shiraz (Sorenson's=0.77 and Jaccard's=0.39) from Pune, Maharashtra and minimum or no similarity was between varieties Shiraz and Muscat (Sorenson's=0 and Jaccard's=0) from Alambagh, Lucknow (Table 5.4, 5.5).

Table 5.5. Jaccard's index of similarity between different *Vitis vinifera* species (i.e. variety wise).

Location	**Variety**									
	Merlot	Merlot	Merlot	Merlot	Wild	Wild	Wild	Pinot	Pinot	Shiraz
	Wild	Pinot	Shiraz	Muscat	Pinot	Shiraz	Muscat	Shiraz	Muscat	Muscat
Alambagh	0.25	0.25	0.14	0.17	0.25	0.09	0.20	0.14	0.33	0
Nashik	0.14	0.26	0.17	0.29	0.27	0.2	0.22	0.33	0.29	0.23
Pune	0.23	0.36	0.35	0.23	0.22	0.25	0.25	0.39	0.33	0.25
Bengaluru	0.14	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: NA represents not applicable.

5.2. Preliminary screening of resveratrol producing endophytic fungi

Based on the three phytochemical tests for the detection of polyphenolic compounds viz. Liebermann; Acetic anhydride and Chromogenic reaction carried out on ethyl acetate extracts of all endophytic fungi, only 43 isolates exhibited the presence of polyphenolics presumably resveratrol out of 145 isolates. In the preliminary screening, the top four resveratrol producers were #12VVLPM, # 19VVLPM, #22(P)VVLPM and #4(P)VVLNM, which exhibited more than 80 % activity and were further quantified and confirmed by using HPLC (Table 5.6).

5.3. Quantification of resveratrol through high performance liquid chromatography (HPLC)

5.3.1. HPLC method validation

The standard graph of resveratrol in a concentration range of 0.1-1 mg/mL was constructed as linear (R^2=0.9986). The good fitting curve was indicated in this range due to the higher value of regression coefficient. Further the precision of fitting was thus confirmed based on the standard error (S.E.) at 95% confidence interval for the values of intercept (5E+07) and slope (-2E+06) (Figure 5.5 a)).

The accuracy of the proposed method at different levels of resveratrol concentrations: 0.10 mg/mL (lower concentration), 0.20 mg/mL (intermediate concentration) and 0.30 mg/mL (higher concentration) were prepared from the stock solutions and analyzed (n=10). The accuracy was assessed in terms of percentage accuracy and mean recovery (Table 5.6).

Thus, the result reveals that any small change in the concentration of resveratrol could be accurately determined by the proposed analytical method.

The calculated Limit of detection (LOD) and Limit of quantification (LOQ) value of the resveratrol were 0.0275 and 0.0917 mg/mL, respectively.

Table 5.6. Accuracy and precision data for the developed method of HPLC

Conc. Injected (mg/ml)	Predicted conc. (mg/ml) *	Mean recovery (%)	Accuracy (%)
0.10	0.097 ± 0.0098	97	-3.0
0.20	0.2034 ± 0.0098	101.7	1.7
0.30	0.2968 ± 0.0069	98.93	-1.07

*Data presented is mean ± standard deviation (n=10)

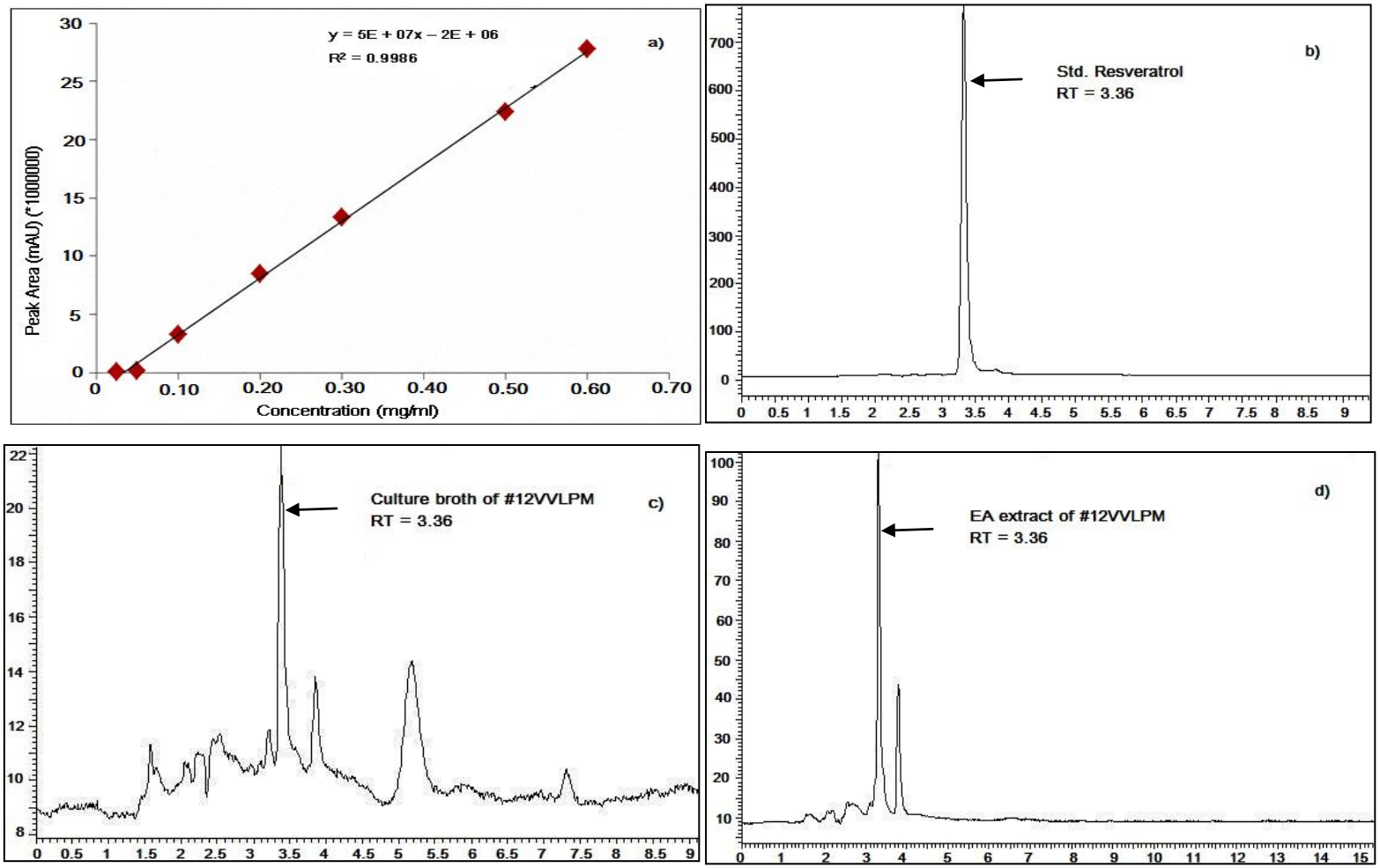


Figure 5.5. (a) Graph representing peak area versus resveratrol concentration; HPLC spectra of (b) Standard resveratrol, (c) crude residue of #12VVLPM, (d) EA extract of fungal resveratrol from #12VVLPM.

Table 5.7. Tentative identification and quantitative estimation of resveratrol production by endophytic fungi of *Vitis vinifera*.

Culture Code	Location	Culture identification (Tentative identification)	Biochemical screening			Quantification of Resveratrol by HPLC (µg/mL)
			Liebermann Test	Acetic Anhydride Test	Chromogenic reaction	
#6(b)VVLLK	Alambagh, Lucknow	Unidentified	+ +	+ +	+ +	$11.9^{l} \pm 0.15$
#9VVLLK		*Aspergillus* sp.	+	+	+	
#50(d)VVLLK		*Fusarium* sp.	+	++	+	
#1VVRLLK		Unidentified	++	+	+	
#1VVRSTL		*Lasiodiplodia* sp.	+	+	+	
#8VVGSTL		Unidentified	+	+ +	+ +	$13.2^{k} \pm 0.06$
#12VVLPM	Pune, Maharashtra	***Arcopilus* sp.**	+ + +	+ + +	+ + +	**$89.1^{a} \pm 0.10$**
#12(b)VVLPM		*Aspergillus* sp.	+	+	+	
##12(c)VVLPM		*Chaetomium* sp.	+	+	+	
#10(b)VVLPM		*Fusarium* sp.	+	+	+	
#16(b)VVLPM		*Fusarium* sp.	+	+	++	
#18VVLPM		*Botryosphaeria* sp.	+ +	+ +	+ + +	$30.3^{e} \pm 0.11$
#19(P)VVLPM		*Epicoccum* sp.	+	+	+	
#20(b)VVLPM		*Botryosphaeria* sp.	+	+ +	+ +	$15.3^{j} \pm 0.10$
#25VVLPM		*Fusarium* sp.	+	+	+	
#27VVLPM		Unidentified	+	+	+	
#15(P)VVLPM		*Xylaria* sp.	++	+	+	
#21VVLPM		*Aspergillus* sp.	+	++	+	
#18(P)VVLPM		*Auerobasidium* sp.	+	+	+	
#42VVLPM		*Fusarium* sp.	+	+	+	
#66VVLPM		*Penicillium* sp.	+	+	++	
#36(P)VVLPM		*Aspergillus* sp.	+	+	+	
# 98VVSTPM		*Penicillium* sp.	++	++	+	$21.9^{i} \pm 0.06$
#184VVSTPM		*Lasiodiplodia* sp.	+	+	+	
#10(P)VVLPM		*Phaeoacremonium* sp.	+	+	+	

#19VVLPM		***Fusarium* sp.**	+ + +	+ +	++	**52.3^{b} ± 0.10**
#27VVLPM		Unidentified	+	+	+	
#22(P)VVLPM		***Xylaria* sp.**	+ +	+ +	++	**35.4^{d} ± 0.10**
#193VVSTPM		*Nigrospora* sp.	+ +	+ +	+ +	25.2^{f} ± 0.10
# 29VVLNM	Nashik, Maharashtra	Unidentified	+	+	+	
#176VVSSWN		*Epicoccocum* sp.	+	+	+	
#591VVLSWN		*Botryosphaeria* sp.	+	+	+	
#671VVLSWN		*Botryosphaeria* sp.	+	+	+	
#4(P)VVLNM		***Fusarium* sp.**	+ +	+ + +	++	**31.3^{e} ± 0.15**
#30(b)VVLNM		*Lasiodiplodia* sp.	+	+	+	
#83VVLNM		*Aspergillus* sp.	+ +	+ +	+	4.4^{m} ± 0.20
#109VVLNM		*Alternaria* sp.	+	+ +	+ +	24.1^{g} ± 0.10
#105VVLNM		*Aspergillus* sp.	+	+	+	
#20(a)VVLBK	Bangalore, Karnataka	*Botryosphaeria* sp.	+	+	+	
#101VVLBK		*Aspergillus* sp.	+	+ +	+ +	22.4^{h} ± 0.06
#13VVLBK		*Lasiodiplodia* sp.	+	+	+	
#23VVSTBK		*Aspergillus* sp.	+	+	+	
#53VVSTBK		*Aspergillus* sp.	+ +	+ +	+ +	23.9^{g} ± 0.10

Note: (+) indicates moderate activity (50-80%),(++) indicates good activity (80-90%), (+++) indicates very good activity (> 90%). Data presented are mean ± standard deviation of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$).

5.3.2. Quantification of resveratrol

The confirmatory and concentration of the fungal resveratrol was confirmed by HPLC analysis, which exhibited a symmetrical peak at RT 3.36 min on C18 reverse phase column similar to that of the standard resveratrol (Figure 5.5). The UV absorption analysis showed a peak representing absorption at 302 nm. The peak area versus resveratrol concentration, obtained in case of the standard resveratrol was used to estimate the quantity of fungal resveratrol ($y = 5E + 07x - 2E + 06$, $R^2 = 0.9986$). Out of 43 isolate, 14 endophytic fungi produced resveratrol in the range of 4.4–89.1 mg/L in liquid culture which was confirmed by HPLC analysis (Table 5.7). Out of 14 Isolate, #12VVLPM showed maximum resveratrol production of 89.1 mg/L (Figure 5.5). There was a statistically significant difference in mean resveratrol concentrations of the different resveratrol producing endophytic isolates which was confirmed by one way ANOVA. Further #109VVLNM and #53VVSTBK did not exhibit any significant difference in their mean resveratrol concentration which was confirmed by Tukey's post-hoc analysis ($p<0.05$).

5.4. Identification of the top four resveratrol producing endophytic fungi

By Qualitative as well as Quantitative estimation we found that isolates the isolates#12VVLPM, #19VVLPM, #22(P)VVLPM and #4(P)VVLNM were found to be the top resveratrol producers hence these were identified and further the application studies were carried using these four endophytic fungi. The isolates #12VVLPM, #19VVLPM, #22(P)VVLPM and #4(P)VVLNM were identified on the basis of both classical morphotaxomony and molecular taxonomy tools.

5.4.1. Morphotaxonomy of top four resveratrol producing endophytic fungi

5.4.1.1.#12VVLPM (*Arcopilus* sp.)

The identification of resveratrol producing endophytic strain (#12VVLPM) was done on the basis of its specific morphological and reproductive characteristics. The endophytic fungus #12VVLPM produced red wine color and orange over PDA and MEA respectively (Figure 5.6 a). Colonies over PDA and MEA are rapidly growing (90 ± 0), young colonies are usually white by aerial mycelium becoming red or orange due to its ascomata and red pigment exudation (Figure 5.6 a). Over CMA colonies are golden in color from front and reverse side with flat margin. The fungus produced golden color soluble pigment with fruity smell (Figure 5.6b). Among microscopic characteristics hyphae were septate, thick, branched and brown in

color. Ascomata are olivaceous to brown, maturing within 15-20 days, dark grey green when old, ovate in shape with 130.9-160.2 X 154.3-190.6 µm diameter (Figure 5.7). Ascomatal hairs are apically circinate or curved, septate (Figure 5.7).

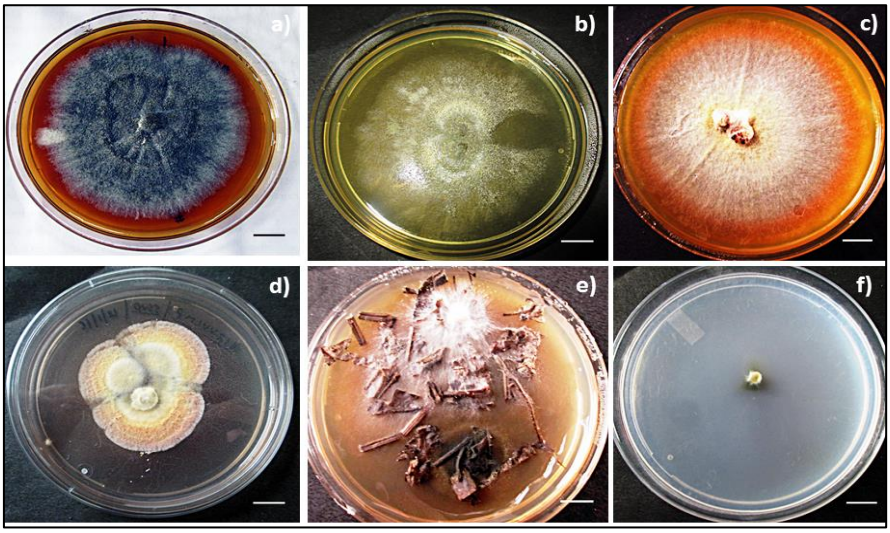


Figure 5.6. Colony morphology of (#12VVLPM) over different media a) PDA (pH 7) colonies with red wine color pigmentation, b) Colony over CMA with golden pigment formation, c) Colony over CDA ,d) PDA (pH 5) No red wine color pigment formation, e) - f) Colony over GLA and WA media respectively**.** Bar: 10 mm.

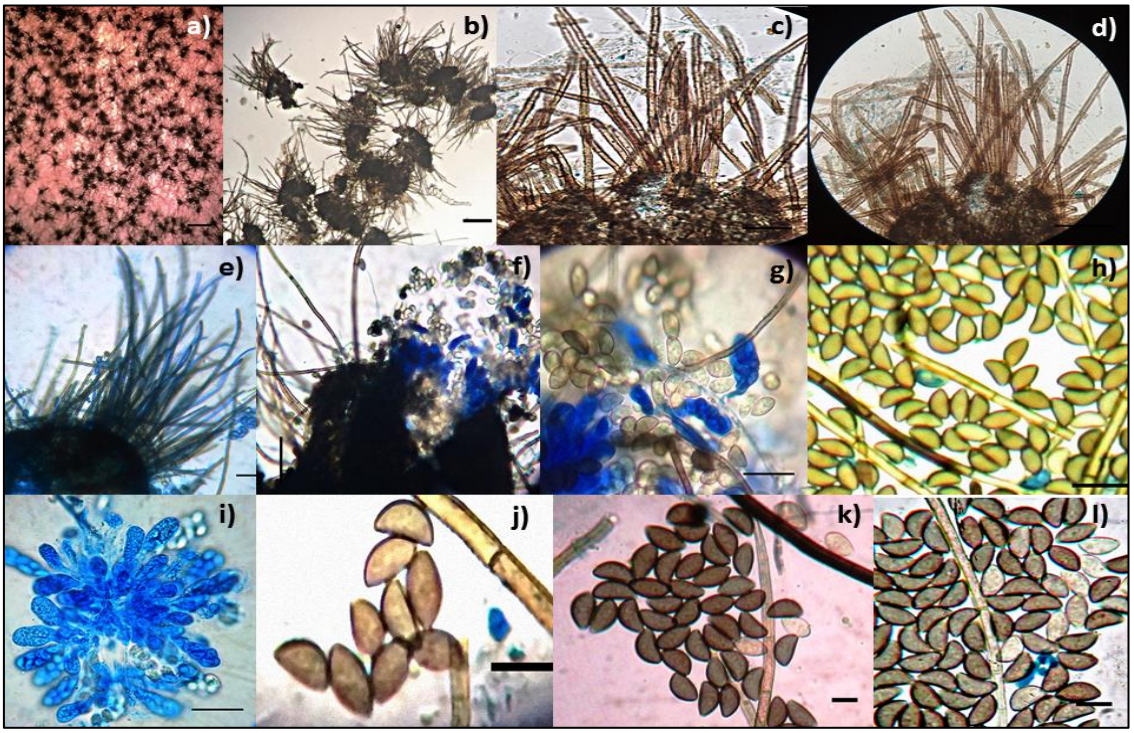


Figure 5.7. a) Microscopic features of (#12VVLPM) over different media, a-d: over PDA, a) colony photo of #12VVLPM, b-c) large dark brown to black mature ascomata on PDA, d)ascomatal hair, e)mature ascomata on CMA, f) close-up view of ascomata which demonstrates ostioles and contained asci, g) 6-8 ascospore arrange in irregular manner in an ascus, h) brown colored septate hyphae and ovate shaped ascospores, i) young asci, j-k) lunate, reniform shape ascospores on PDA, l) lunate, reniform shape ascospores on CMA. Bar 10: µm.

Asci are fasciculate, clavate in shape with 6-8 irregularly arranged ascospores per ascus (Figure 5.7i). Ascospores are ovate, lunate and reniform in shape with 8.6-10.0 X 11.4-13.5 µm diameter (Figure 5.7).

5.4.1.2. #4(P)VVLNM (*Fusarium* sp.)

The endophytic fungus #4(P)VVLNM produced white, moderate growing (75 ± 1mm) floccose aerial mycelium over PDA after 15 days of incubation with 12 h photoperiod (Figure 5.8). Initially white in color and later becomes orange floccose to puffy with smooth margin over PDA (Figure 5.8a). On PDA hyphae were thick, septate and branched.

Micro conidia are usually abundant, cylindrical to oval, one to two-celled and formed from long lateral phialides (8-16 x 2-4.5) µm diameter (Figure 5.8). Chlamydospores are hyaline, globose, and smooth to rough-walled, in pairs on short lateral hyphal branches, (6-10) µm diameter.

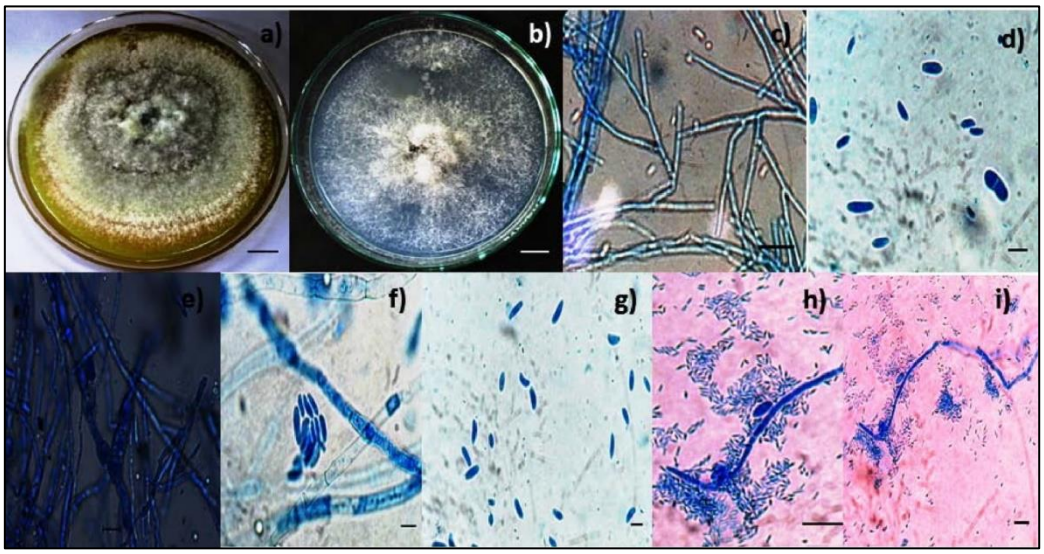


Figure 5.8. Colony morphology and microscopic features of #4(P)VVLNM (*Fusarium* sp.) a) PDA (pH 7) pale yellow to brown in color, b) over SNA media white in color, c) micro conidia developed on water agar, c) short lateral hyphal branches d-i) conidia growing over different media. Bars: a-b) 10 mm; c – i) 10 µm.

5.4.1.3. #22(P)VVLPM (*Xylaria* sp.)

The endophytic fungus #22(P)VVLPM produced red wine color and orange over PDA. Colonies over PDA are moderately growing (54 ± 2 mm), floccose to downy colonies on different media after 15 days of incubation with 12 h photoperiod (Figure 5.9). 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 5.9 b, 5.9 c, 5.9d). 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 5.9a).

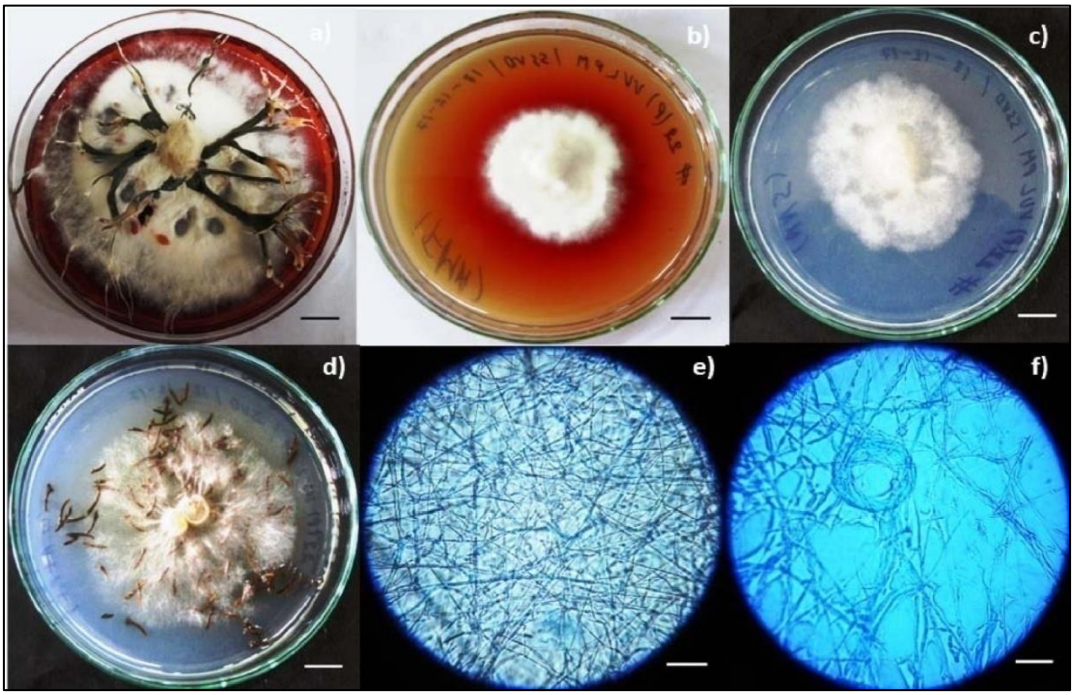


Figure 5.9. Colony morphology and microscopic features of #22(P)VVLPM (*Xylaria* sp.). a) PDA (pH 7) red wine in color, b) over FMA media reddish brown in color, c-d) over SNA and PLA media white in color, e-f) microscopic feature on SNA and PLA. Bars: a-d) 10 mm; e – f) 10 µm.

5.4.1.4. #19VVLPM (*Fusarium* sp.)

The endophytic fungus #19VVLPM produced white, fast growing (90 ± 1mm) floccose aerial mycelium over PDA after 15 days of incubation with 12 h photoperiod (Figure 5.10). Initially white in color and later become brown with smooth margin over PDA (Figure 5.10a). Over pine leaf agar (PLA) and water agar (WA) the margins were flat. On SNA medium it was white in color with wooly appearance (Figure 5.10b).On PDA and MEA hyphae were thick, septate and branched. Conidiophores were present in the aerial mycelium. Conidia developed over aerial conidiophores are generally fusiform to falcate in shape, usually 3-7 septate, only macro conidia were present (25.17-34.57 X 2.12-3.32) µm

diameter (Figure 5.10 c–5.10e). On WA and SNA medium, colonies were moderate to fast growing, white in color, hyphae were septate and thick, conidia were fusiform to slightly curved with foot shell, mostly 3-5 septate and only macro conidia (32.33-41.16 X 3.2-4.87) µm diameter were developed (Figure 5.10 f-5.10g).

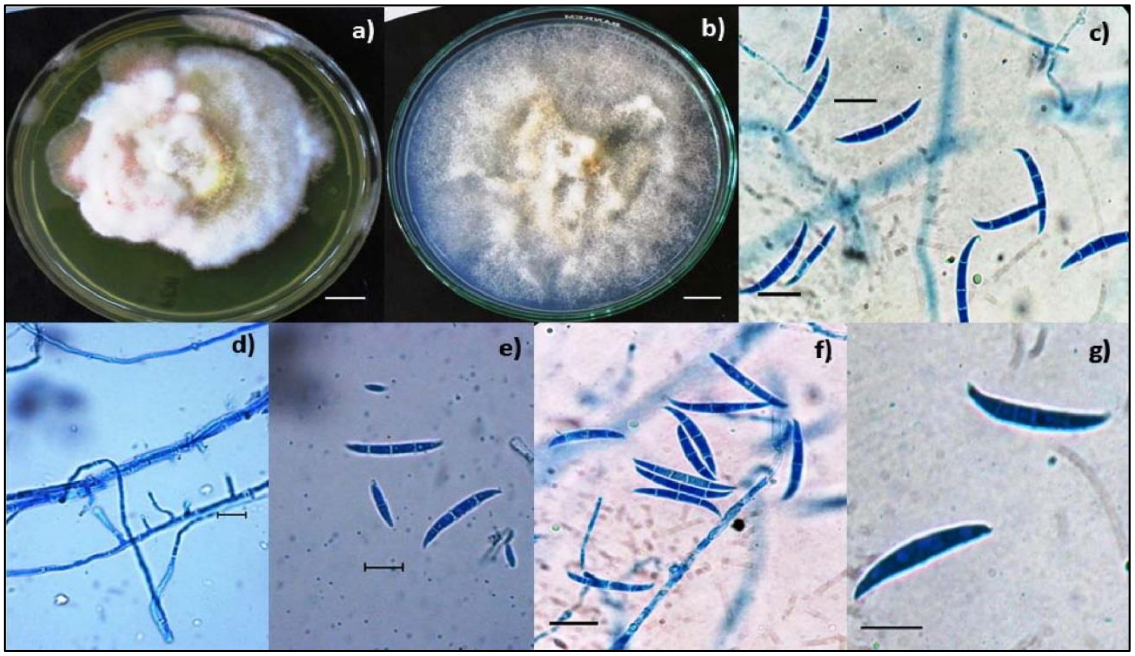


Figure 5.10. Colony morphology and microscopic features of #19VVLPM (*Fusarium* sp.) a-b) PDA & SNA (pH 7) white in color, c-g) Sickle shaped conidia developed on SNA & WA, d) conidia growing over monophialides. Bars: a-b) 10 mm; c – g) 10 µm.

5.4.2. ITS-rDNA based molecular taxonomy

The PCR amplification of DNA of the four cultures viz. #12VVLPM, #19VVLPM, #22(P)VVLPM and #4(P)VVLNM was carried out using ITS primers and a product of size around 500-600 bp was amplified in each case (Figure 5.11).

5.4.2.1. #12VVLPM (Arcopilus sp.*)*

The phylogenetic placement of the ITS sequence of #12VVLPM with type and non-type sequences recovered from BLAST analysis clearly indicated alignment with two isolate of *Arcopilus aureus* strain WW05 and *Arcopilus aureus* ATT 218 and one type strain *A. aureus* CBS 538.73 in the first clade (Figure 5.12). Thus, based on the phylogenetic tree of ITS sequence as well as on the basis of morphological characteristics the most probable species assignation of the isolate #12VVLPM is *Arcopilus aureus*. The strain has been

deposited at the national fungal culture collection of India (NFCCI-4191) and its rDNA in GenBank with accession no. MF597785.

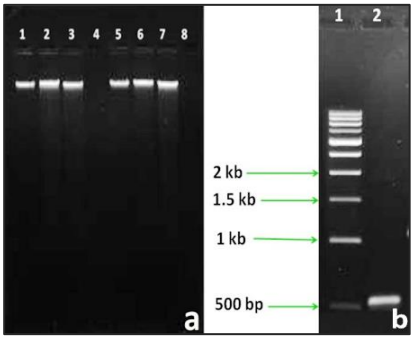


Figure 5.11.a) Lane1-2: Genomic DNA of #12VVLPM, Lane 3: Genomic DNA of #4(P)VVLNM, Lane 5-6: Genomic DNA of #22(P)VVLPM, Lane 7: Genomic DNA of #19VVLPM; b) The PCR product of size around 550bp

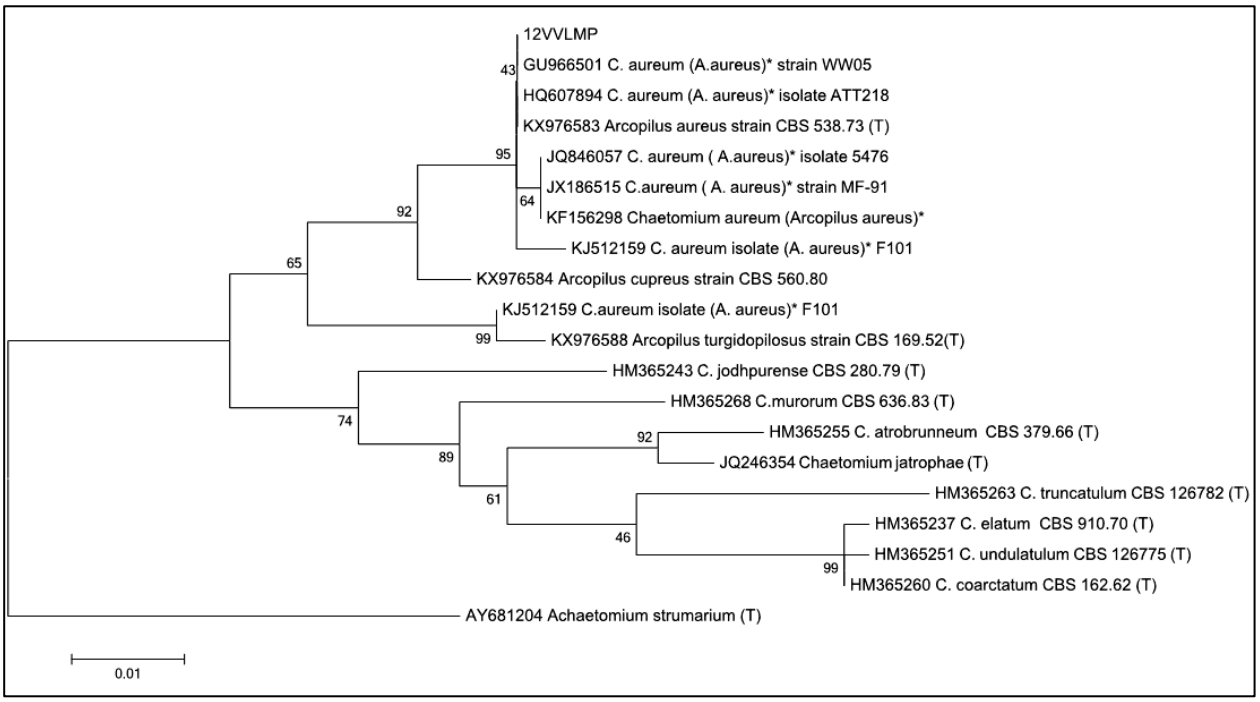


Figure 5.12. The mmaximum Likelihood tree based on the ITS1-5.8S-ITS2 region of #12VVLPM. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates)

5.4.2.2. #4(P)VVLNM (Fusarium sp.*)*

The ITS sequence of #4(P)VVLNM was analysed by using the BLAST search tool to find homologous sequences. Maximum likelihood tree based upon Tamura and Nei model exhibited #4(P)VVLNM clustering with homologous sequences of *Fusarium solani* (non-type and type) in clade I suggesting it to be an isolate of *Fusarium solani*. Hence #4(P)VVLNM based on molecular as well as morphological data was assigned the name *Fusarium solani* and was submitted in GenBank with Accession no.MH169229 (Figure 5.13).

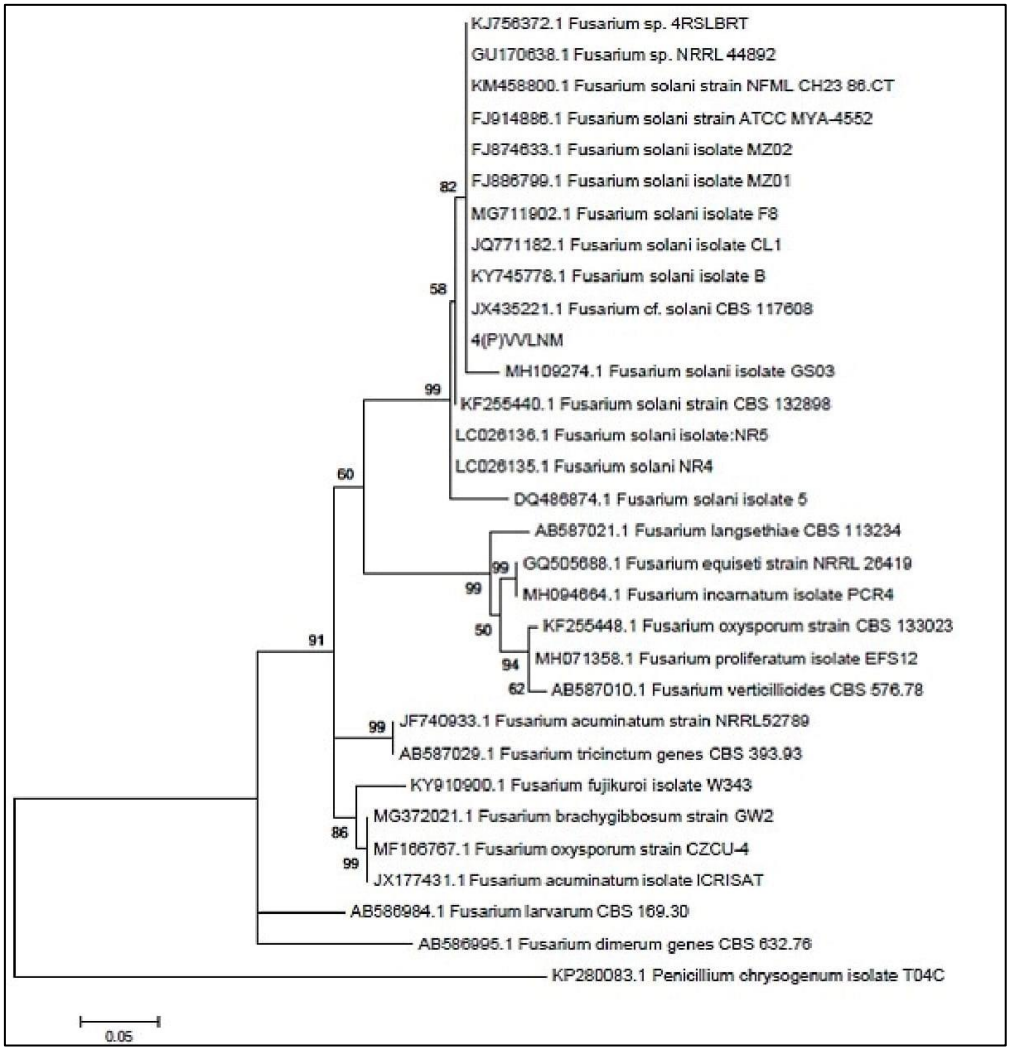


Figure 5.13. The maximum Likelihood tree based on the ITS1-5.8S-ITS2 region of #4(P)VVLNM. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates)

5.4.2.3. #22(P)VVLPM (Xylaria sp.*)*

BLAST analysis of ITS sequence of #22(P)VVLPM exhibited it 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 based on morphological as well as phylogenetic analysis #22(P)VVLPM was assigned the name *Xylaria psidii* (Figure 5.14). The ITS sequence of #22(P)VVLPM has been submitted in Genbank with accession no.MH142837

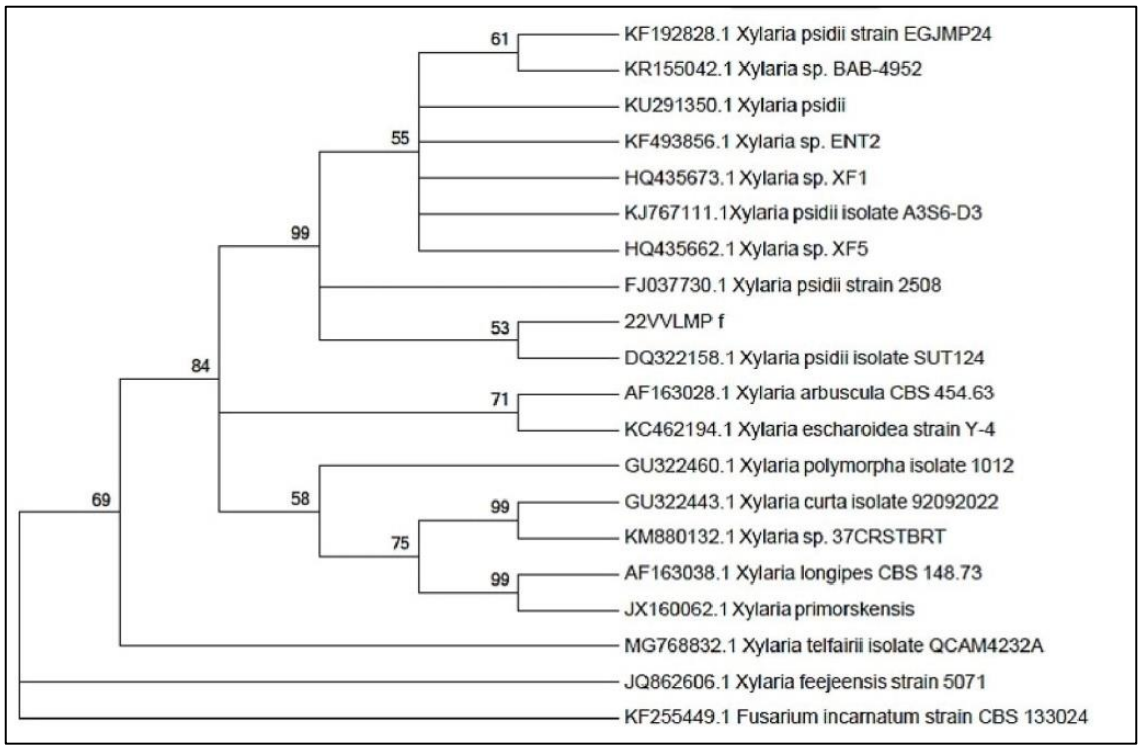


Figure 5.14. The maximum Likelihood tree based on the ITS1-5.8S-ITS2 region of #22(P)VVLPM. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates)

5.4.2.4. #19VVLPM (Fusarium sp.*)*

The ITS sequence of #19VVLPM was submitted in GenBank with Accession no. MH181157 and subsequently homologous ITS sequences were searched using BLAST. The phylogenetic association of homologous ITS sequences obtained by BLAST, ITS sequence of #19VVLPM and type species were analysed by preparation of a Maximum likelihood tree using Tamura and Nei model. In this phylogenetic tree, #19VVLPM clustered with *Fusarium equiseti* isolate TMC as well as *Fusarium equiseti*. Based on the molecular clustering of #19VVLPM as well as morphotaxonomic data it was assigned the name *Fusarium equiseti* (Figure 5.15).

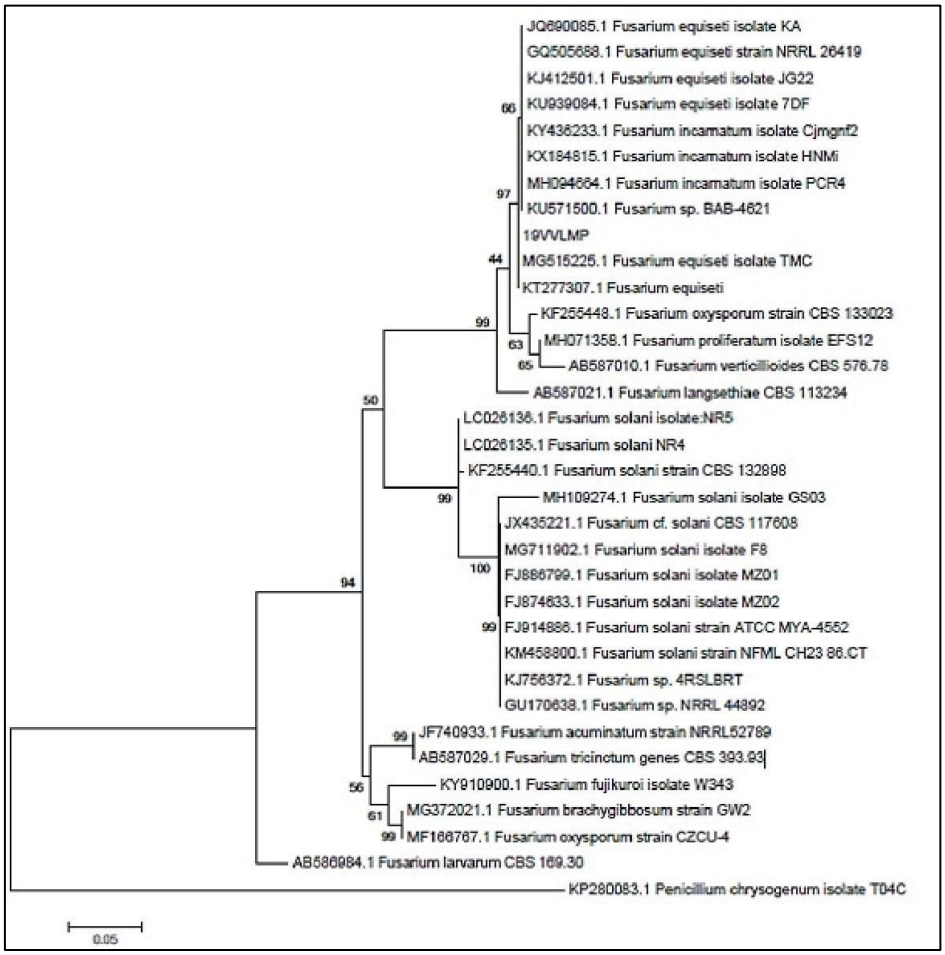


Figure 5.15. The mmaximum Likelihood tree based on the ITS1-5.8S-ITS2 region of #19VVLPM. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates)

5.5. PCR based molecular screening for resveratrol producing endophytic fungi

#12VVLPM (*Arcopilus aureus*), #4(P)VVLNM (*Fusarium solani*), #22(P)VVLPM (*Xylaria psidii*) and #19VVLPM (*Fusarium equiseti*) the resveratrol producing endophytic fungus was found to be possesses the *STS* gene which is responsible for the resveratrol biosynthesis (Figure 5.16).

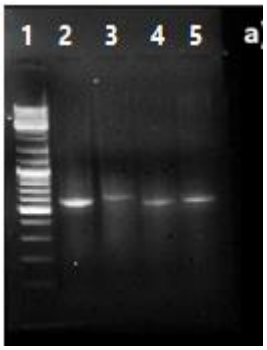


Figure 5.16. Screening for the presence of *STS* gene of resveratrol biosynthetic pathway. Lane 1: Ladder (100 bp), Lane 2: #12VVLPM, Lane 3: #4(P)VVLNM, Lane 4: #22(P)VVLPM and Lane 5: #19VVLPM shows specific band about 500-600 bp for *STS* gene in 1.5 % agarose gel.

5.6. In vitro antioxidant activities

Free radical damage has been implicated in pathogenic infections, inflammatory response, cardiovascular disorders, ageing and autoimmune disorders like rheumatoid arthritis. Different *in vitro* assays were used to assess the antioxidant activity. The *in vitro* antioxidant assay of top resveratrol producing endophytic fungi (#12VVLPM, #19VVLPM, #22(P)VVLPM and #4(P)VVLNM) was carried out using DPPH scavenging assay, trolox equivalent antioxidant capacity assay, metal ion chelating scavenging assay, nitric oxide radical scavenging assay and hydrogen peroxide radical scavenging assay.

5.6.1. DPPH Scavenging Assay

There was a statistically significant difference in the *in vitro* DPPH scavenging activity among the different fungal extracts by one-way ANOVA. Further, post-hoc comparisons using Tukey's HSD ($p<0.05$) indicated that maximum DPPH scavenging was carried out by a fungal extract of #12VVLPM with IC_{50} of 0.11 ± 0.01 mg/mL when compared to resveratrol which was used as a standard with IC_{50} of 0.1 ± 0.011 mg/mL. The least DPPH scavenging was found in the fungal extract of #4(P)VVLNM with IC_{50} of 4.96 ± 0.21 mg/mL (Table 5.8, Figure 5.17 a).

Table 5.8. IC_{50} of top four resveratrol producing isolates evaluated using DPPH, TEAC, Metal ion chelating, Nitric oxide radical scavenging and Hydrogen peroxide scavenging assays.

		IC_{50} (mg/ml)*				
S.No.	**SAMPLE**	**DPPH scavenging**	**Trolox equivalent antioxidant scavenging**	**Metal Ion Chelating Scavenging**	**Nitric Oxide Radical Scavenging**	**Hydrogen Peroxide Scavenging**
1.	Standard	0.1^{d} ± 0.011	0.16^{e} ± 0.03	0.33^{b} ± 0.02	0.37^{d} ± 0.02	0.38^{c} ± 0.01
2.	#12VVLPM	0.11^{d} ± 0.01	0.28^{d} ± 0.02	0.12^{c} ± 0.03	0.08^{e} ± 0.03	0.12^{d} ± 0.07
3.	#19VVLPM	0.48^{c} ± 0.02	0.82^{c} ± 0.01	0.42^{b} ± 0.02	0.61^{c} ± 0.01	0.26^{cd} ± 0.04
4.	#22(P)VVLPM	1.25^{b} ± 0.03	1.51^{b} ± 0.04	1.35^{a} ± 0.05	0.91^{b} ± 0.01	0.75^{b} ± 0.02
5.	#4(P)VVLNM	4.96^{a} ± 0.21	2.16^{a} ± 0.02	1.33^{a} ± 0.03	1.35^{a} ± 0.01	1.18^{a} ± 0.04

* Data presented are mean ± standard deviation of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

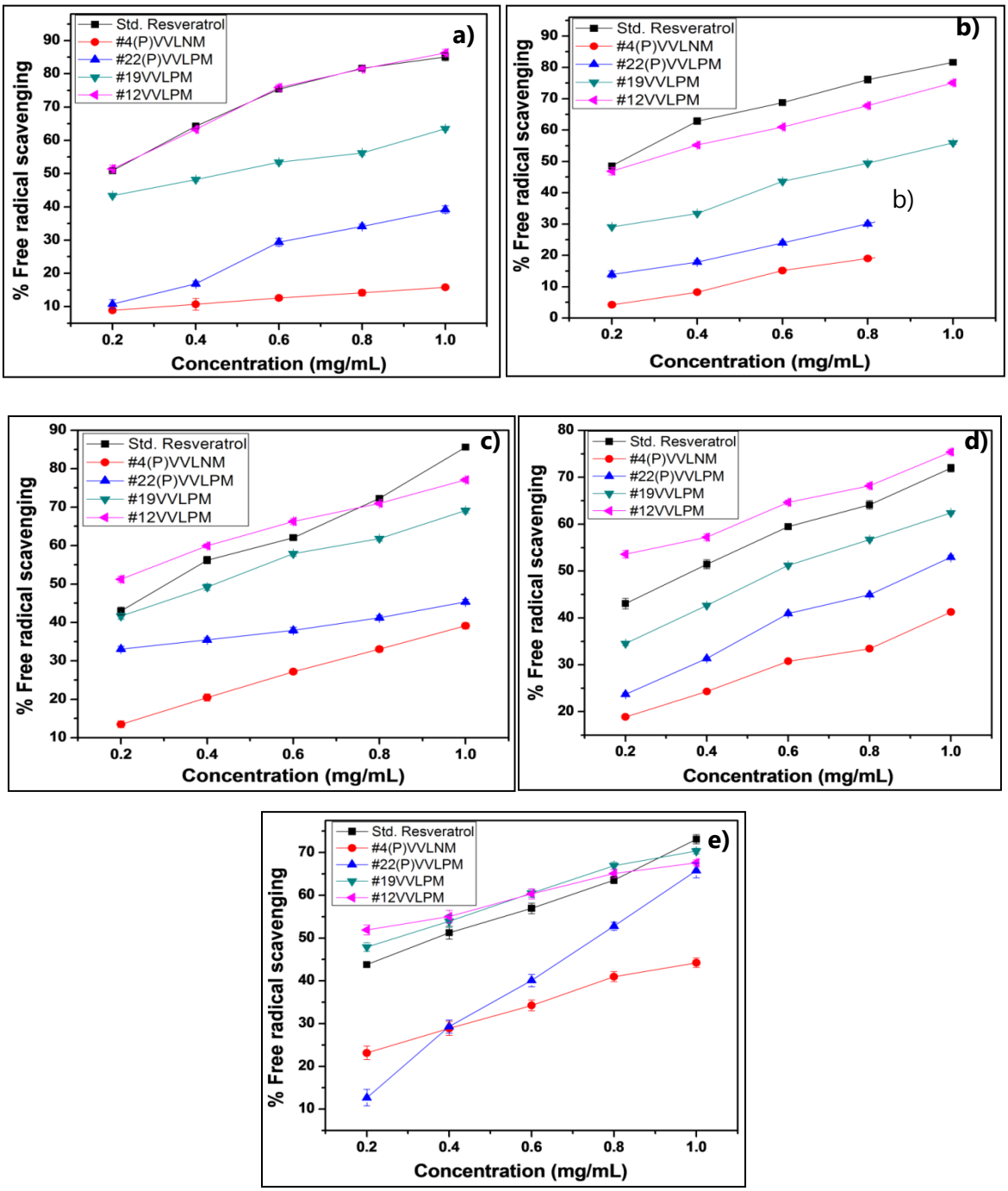


Figure 5.17. %free radical scavenging of top four resveratrol producing isolates evaluated using a) DPPH, B) TEAC, c) Metal ion chelating, d) Nitric oxide radical scavenging and e) Hydrogen peroxide scavenging assays. All results are mean ± SD (n=3)

5.6.2. Trolox equivalent antioxidant capacity (TEAC) assay

In case of *in vitro* $ABTS^{+}$ radical scavenging potential, there was a statistically significant difference among the different fungal extracts analysed by one way ANOVA. The highest scavenging was found in fungal extract (ethyl acetate fraction) of #12VVLPM with IC_{50} of 0.28 ± 0.02 mg/mL as compared to resveratrol used as a standard which exhibited IC_{50} of

0.16 ± 0.03 mg/mL, while the least scavenging was recorded in fungal extract of #4(P)VVLNM with IC_{50} of 2.16 ± 0.02 mg/mL (Table 5.8, Figure 5.17 b).

5.6.3. Metal ion chelating scavenging assay

In metal ion chelating assay, there was a significant statistical difference among different fungal extracts which was analysed using one-way ANOVA. The highest scavenging was present in the fungal extract of #12VVLPM with IC_{50} of 0.12 ± 0.03 mg/mL as compared to resveratrol which exhibited IC_{50} of 0.33 ± 0.02 mg/mL which was used as a standard. The least scavenging was obtained in the fungal extract of #22(P)VVLPM with IC_{50} of 1.35 ± 0.05 mg/mL. Further, #22(P)VVLPM and #4(P)VVLNM did not exhibit a significant difference in their meanIC_{50} which was confirmed by Tukey's post-hoc analysis ($p<0.05$) (Table 5.8, Figure 5.17 c).

5.6.4. Nitric oxide radical scavenging assay

In case of nitric oxide radical scavenging assay, there was a statistically significant difference among the different fungal extracts by one-way ANOVA. Further, post-hoc comparisons using Tukey's HSD ($p<0.05$) indicated that the mean nitric oxide radical scavenging was highest in #12VVLPM with IC_{50} of 0.08 ± 0.03 mg/mL as compared to resveratrol which was used as standard with IC_{50} of 0.37 ± 0.02 mg/mL and the least scavenging was exhibited by the fungal extract of #4(P)VVLNM with IC_{50} of 1.35 ± 0.01 mg/mL (Table 5.8, Figure 5.17 d).

5.6.5. Hydrogen peroxide radical scavenging assay

In this assay, the ability of fungal extract to scavenge hydrogen peroxide was assayed and it was observed that a significant statistical difference existed among the different fungal extracts by one-way ANOVA. Further, post-hoc comparisons using Tukey's HSD ($p<0.05$) indicated that the mean scavenging was highest in fungal extract of #12VVLPM with IC_{50} of 0.12 ± 0.07 mg/mL followed by #19VVLPM with IC_{50} of 0.26 ± 0.04 mg/mL as compared to resveratrol which was used as the standard with IC_{50} of 0.38 ± 0.01 mg/mL and the least scavenging was exhibited by #4(P)VVLNM with IC_{50} of 1.18 ± 0.04 mg/mL (Table 5.8, Figure 5.17 e).

5.7. DNA Protection assay

The assay was carried out to understand the possible role of the fungal extracts (ethyl acetate extract) on prevention of damage to DNA by free radicals. In this study, it was found that fungal extracts of resveratrol producing endophytic fungi exhibited good anti-oxidant activity and thus prevented the DNA damage (Figure 5.18).

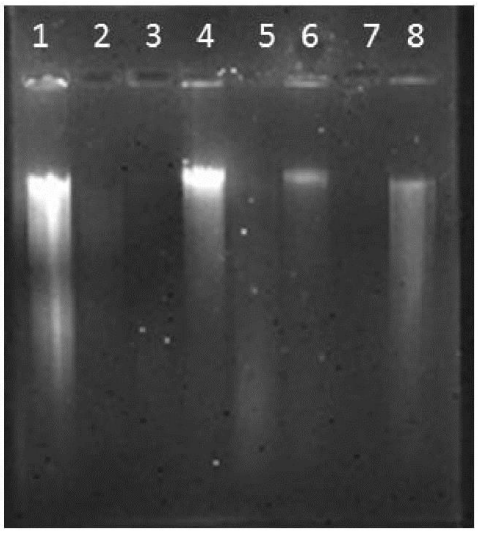


Figure 5.18. DNA protection assay; Lane 1: λ-DNA, Lane 2,3: λ-DNA+ Fenton Reagent (FR), Lane 4: λ-DNA+ #12VVLPM extract + FR, Lane 5: λ-DNA+ #22(P)VVLPM extract + FR, Lane 6,8: λ-DNA+ #19VVLPM extract + FR, Lane 7: λ-DNA+ #4(P)VVLNM extract + FR.

5.8. In vitro antifungal activity

All the resveratrol producing isolates significantly inhibited mycelial growth and conidial formation against *Botrytis cinerea* (MTCC 359), *Colletotrichum gloeosporioides* (MTCC 9623), *Cercospora beticola* (ATCC 24888) and *Rhizoctonia solani* (MTCC 4634), which were analyzed by one-way ANOVA. The highest inhibitory was observed in #12VVLPM against *B. cinerea* (57.8± 0.8%) followed by *Cercospora beticola* (ATCC24888) while the least inhibition was observed in #22(P)VVLPM and #4(P)VVLNM against *Botrytis cinerea*.

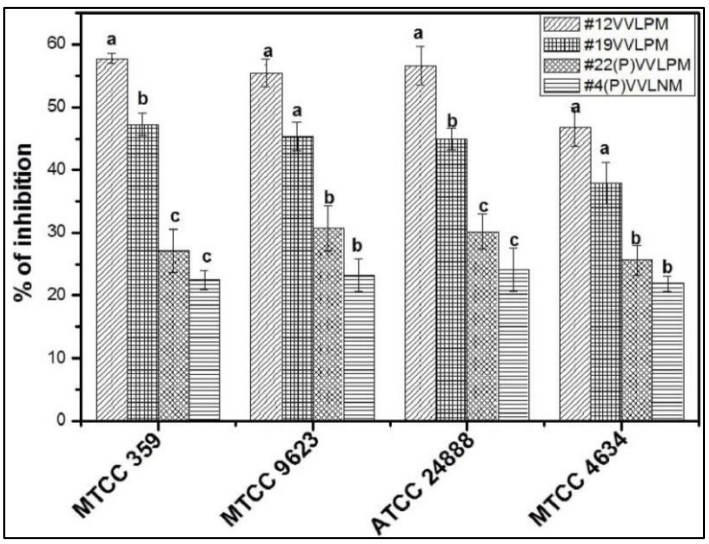


Figure 5.19. Antifungal activity of top four resveratrol producing endophytic fungi. All results are mean ± SD (n=3), Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$).

A non-significant inhibition was observed in the case of #22(P)VVLNM against the test panel isolates (Table 5.9, Figure 5.19).

Table 5.9. Antifungal activity of top four resveratrol producing endophytic fungi.

S. No.	Sample	% OF INHIBITION*			
		***Botrytis cinerea* (MTCC 359)**	***Colletotrichum gloeosporioides* (MTCC 9623)**	***Cercospora beticola* (ATCC 24888)**	***Rhizoctonia solani* (MTCC 4634)**
1	#12VVLPM	57.76^{a} ± 0.82	55.41^{a} ± 2.21	56.59^{a} ± 3.08	46.83^{a} ± 3.09
2	#19VVLPM	47.28^{b} ± 1.78	45.30^{a} ± 2.32	44.91^{b} ± 1.70	37.92^{a} ± 3.34
3	#22(P)VVLPM	27.08^{c} ± 3.45	30.68^{b} ± 3.60	30.19^{c} ± 2.84	25.66^{b} ± 2.38
4	#4(P)VVLNM	22.47^{c} ± 1.47	23.20^{b} ± 2.57	24.15^{c} ± 3.44	21.93^{b} ± 1.22

* Data presented are mean ± standard deviation of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

5.9. *In-vitro* anti-staphylococcal activity

The fungal resveratrol was subjected to screen for its anti-staphylococcal activity against a battery of five human pathogenic microorganisms comprising of clinical and standard isolates of *Sau* exhibiting methicillin and multidrug resistance. There was a statistically significant difference in the *in vitro* anti-staphylococcal activity among the different resveratrol producing endophytic fungi by one-way ANOVA.

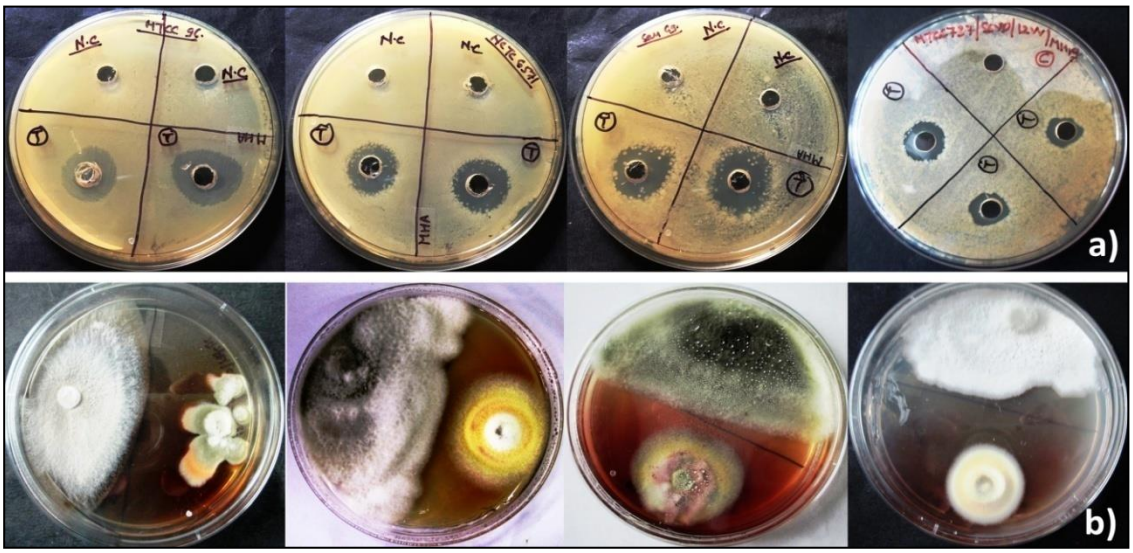


Figure 5.20. Plate photo of *In vitro* anti-staphylococcal and antifungal activity of the different resveratrol producing endophytic fungi

Further, post-hoc comparisons using Tukey's HSD ($p<0.05$) indicated that extent of inhibitory activity of #12VVLPM and #19VVLPM amongst all the isolates and against all the cultures tested was maximum (Table 5.10, Figure 5.20).

Table 5.10. Antibacterial activity of top four resveratrol producing endophytic fungi.

S. No.	Sample	% OF INHIBITION*				
		NCTC 6571	***Sau G9***	***MTCC 96***	***MTCC 737***	***Sau 902***
1.	Positive control	15.60^{a}± 0.10	15.70^{a}±0.10	17.20^{a}± 0.06	17.4^{a}± 0.10	17.00^{a}± 0.10
2.	#12VVLPM	10.67^{b} ± 0.15	10.33^{b} ± 0.06	11.00^{b} ± 0.10	11.67^{b} ± 0.15	12.00^{b} ±0.10
3.	#19VVLPM	10.33^{b} ± 0.15	9.67^{bc} ± 0.12	10.67^{b} ± 0.12	11.33^{b} ± 0.12	11.33^{b} ±0.06
4.	#22(P)VVLPM	9.33^{bc} ± 0.15	9.33^{bc} ± 0.15	12.00^{b} ± 0.10	11.67^{b} ± 0.15	11.67^{b} ±0.12
5.	#4(P)VVLNM	7.67^{c}± 0.15	8.33^{c} ± 0.06	7.67^{c} ± 0.15	7.67^{c} ± 0.10	9.67^{c}± 0.06

* Data presented are mean ± standard deviation of three replicates. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

5.10. Correlation between in vitro antioxidant and anti-microbial activity

To explore the quantitative relationship between *in vitro* antioxidant activity and anti-microbial activity a correlation analysis was carried out. The relationship between the above parameters was statistically evaluated and presented in Table 5.11. DPPH, TEAC, Metal ion chelating and Nitric oxide scavenging activity and antibacterial assay indicated a significant positive correlation. Similarly, the anti-fungal activity also exhibited a positive correlation with the different antioxidant assays. A similar correlation was observed by the resveratrol producing endophytic fungi between the antifungal activity and the antibacterial activity (Table 5.11).

The positive correlation occurred between the *in vitro* anti-microbial and the antioxidant activity (i.e., IC_{50}), despite different methods. In our study too it was found that lower the IC_{50} higher is the scavenging potential. #12VVLPM exhibited the highest scavenging ant-oxidant activity. Our study support the free radical scavenging property of resveratrol producing endophytic fungi relies upon the polyphenolic content. All the resveratrol producing endophytic fungi demonstrate positive and significant correlation between DPPH, TEAC, Metal ion chelating, Nitric oxide and Hydrogen peroxide as well as anti-microbial with coefficient in a range of 0.44-0.999 (Table 5.11).

Table 5.11. Correlation analysis between the data determined with all experimental samples (*n = 5*).

Parameter	RESV conc.	DPPH	TEAC	Metal ion chelating	Nitric oxide	Hydrogen peroxide	*B. cinerea*	MTCC 9623	ATCC 24888	MTCC 4634
RESV conc.		***0.3208*	*0.0832*	*0.0867*	*0.0514*	*0.1526*	*0.0605*	*0.0583*	*0.0416*	*0.0414*
DPPH	*-0.679		*0.0996*	*0.2803*	*0.1235*	*0.0709*	*0.2057*	*0.1551*	*0.1847*	*0.2032*
TEAC	-0.917	0.9		*0.0624*	*0.0109*	*0.0123*	*0.0239*	*0.0069*	*0.014*	*0.0199*
Metal ion chelating	-0.913	0.719	0.938		*0.0921*	*0.0751*	*0.01*	*0.0303*	*0.0264*	*0.0192*
Nitric oxide	-0.949	0.877	0.989	0.908		*0.0445*	*0.0423*	*0.0187*	*0.0206*	*0.0285*
Hydrogen peroxide	-0.847	0.929	0.988	0.925	0.956		*0.0414*	*0.0255*	*0.0405*	*0.0465*
B. cinerea	0.939	-0.794	-0.976	-0.989	-0.958	-0.959		*0.0056*	*0.0044*	*0.0024*
MTCC 9623	0.942	-0.845	-0.993	-0.969	-0.981	-0.974	0.994		*0.0017*	*0.0036*
ATCC 24888	0.958	-0.815	-0.986	-0.974	-0.979	-0.959	0.996	0.998		*0.0007*
MTCC 4634	0.958	-0.797	-0.98	-0.981	-0.972	-0.953	0.998	0.996	0.999	

* Pearson's correlation coefficient; **Significance level of Pearson's correlation coefficient (in italic).

5.11. Purification and Characterization of bioactive residue

5.11.1. TLC fractionation of bioactive residue

The crude ethyl acetate residue of #12VVLPM (red wine in color) was subjected to TLC using different combinations of mobile phases (Table 5.12). Methanol: DCM solvent system, in the ratio of 0.5:9.5 which resolved the extract into 8 different bands. The R_f value of Band 1 to Band 8 was 0.14, 0.27, 0.51, 0.69, 0.78, 0.83, 0.89 and 0.97, respectively. When the UV light was exposed (long wave, 365 nm) the resveratrol in the crude EA residue showed similar violet color as that of the standard resveratrol. The standard resveratrol exhibited R_f value of 0.69 which was same as that of R_f value of Band 4 of crude EA extract of #12VVLPM.

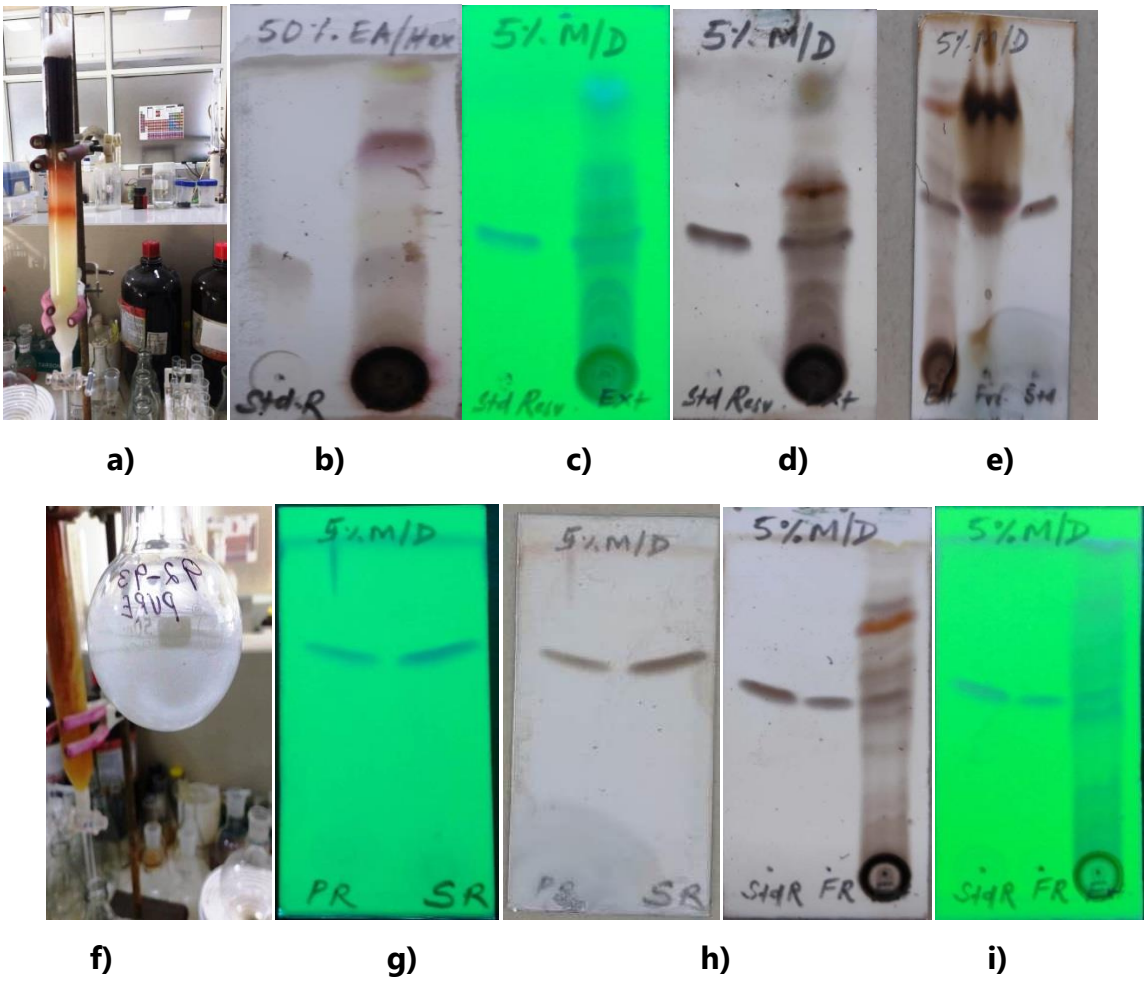
Table 5.12. Different mobile phases used for separation of crude residue of #12VVLPM on TLC

Solvents used	Ratio (v/v)	No of bands
Hexane: Ethyl acetate: Formic acid	2: 1: 2 drops	2
Hexane: Ethyl acetate: Formic acid	1: 1: 2 drops	3
Chloroform: Methanol	4.5: 0.5	4
Chloroform: Methanol	4:1	3
Chloroform: Methanol: Acetic acid	4: 1: 2 drops	4
Chloroform: Methanol: Acetic acid	3: 2: 5 drops	4
Dichloromethane: Methanol	4.5: 0.5	6
Dichloromethane: Methanol: Formic acid	2.0: 1.0:0.1	3
Dichloromethane: Methanol: Formic acid	3.5: 1.5:0.1	5
Dichloromethane: Acetonitrile	3.5: 1.5	2
Ethyl acetate: acetic acid	4: 0.1	3
Methanol: DCM	**0.5:9.5**	**8**
Ethyl acetate: Hexane	1:1	4

5.11.2. Purification of resveratrol by silica gel column chromatography

Based on the optimized TLC system for the detection of standard resveratrol with a comparison TLC of fractionated crude EA residue of #12VVLPM, the mobile phase for silica gel column chromatography was chosen. From the TLC optimization state, it was observed

that for column chromatography the mobile phase with 5% MeOH/DCM (0.5:9.5) is quite preferable over 50% ethyl acetate: Hexane (1:1) and MeOH/DCM (0.5:4.5) to isolate the pure resveratrol. Approximately, 30 g of silica was used as stationary phase for column chromatography in respect to 1g of fungal ethyl acetate extract and allowed to run the column starting from 100% DCM followed the gradient elution technique.



a) **b)** **c)** **d)** **e)**

f) **g)** **h)** **i)**

Figure 5.21. TLC profile of (a) Packed column, (b) Std RESV and 50% EA/Hexane (c) & (d)Std RESV and 5% MeOH and DCM; after charring with ceric ammonium sulfate (e) comparison of crude fungal RSEV with STD RSEV and 5% MeOH/DCM (f) Pure fungal RESV in RB (g) & (h) Pure fungal RESV with STD RESV; after charring with ceric ammonium sulfate (i) & (j) Comparison of pure fungal RESV with STD RESV and crude with 5%MeOH/DCM; after charring with ceric ammonium sulfate

Different fractions were collected with the respective increase in mobile phase and simultaneously observed the presence of compounds with standard resveratrol by TLC using optimized solvent system 5% MeOH/DCM. The fungal resveratrol was obtained in pure form by eluting the column with mobile phase of MeOH: DCM:: 1.75:98.25. Fungal

resveratrol (25 mg) was observed pure by TLC system 5% MeOH: DCM and concentrated by rotary evaporator (Figure 5.21).

5.11.3. HPLC analysis of purified fungal resveratrol

The homogeneity of the purified fungal resveratrol was confirmed by HPLC, which exhibited a single and the symmetrical peak at R_t 3.36 min on C_{18} reverse phase column like that of standard resveratrol (Figure 5.22). The peak area versus resveratrol concentration, obtained in case of the standard resveratrol was used to estimate the quantity of fungal resveratrol.

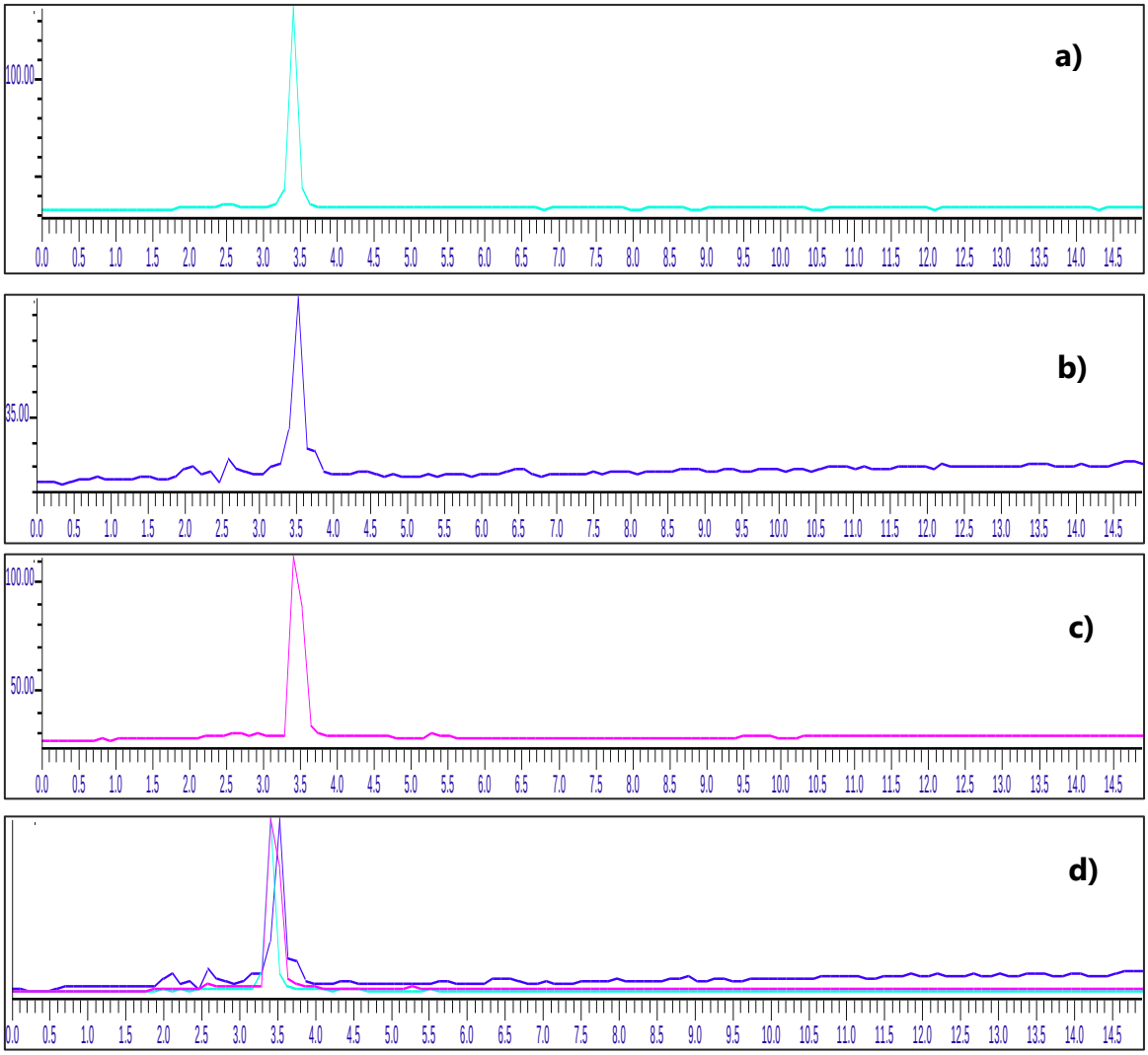


Figure 5.22. HPLC spectra of (a) Standard RESV, (b) crude ethyl acetate residue of #12VVLPM (c) purified fungal RESV from #12VVLPM (d) combined spectra of purified RESV along with standard RESV

5.11.4. Nuclear magnetic resonance spectroscopy (NMR)

In order to confirm the structure of fungal resveratrol, the isolated pure compound was analysed for structural identification with the help of ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) and compared with the ^{1}H-NMR of standard resveratrol. Later it was confirmed by ^{13}C-NMR. Both ^{1}H and ^{13}C-NMR spectra were measured on a JOEL 400 instrument using CD_3OD as the solvent.

^{1}H NMR (400 MHz, METHANOL-D3) δ 7.33 (dd, *J* = 17.8, 8.5 Hz, 2H), 6.94 (d, 2H, *J* = 16.4 Hz, 1H), 6.73 (m, 3H), 6.40 (m, 2H), 6.14 (m, 1H) (Figure 5.23). ^{13}C NMR (100 MHz, METHANOL-D3) δ 158.50, 158.42, 158.37, 139.90, 128.73, 127.90, 127.42 (2C), 125.39, 115.33 (2C), 104.35 (2C), 101.34 (Figure 5.24).

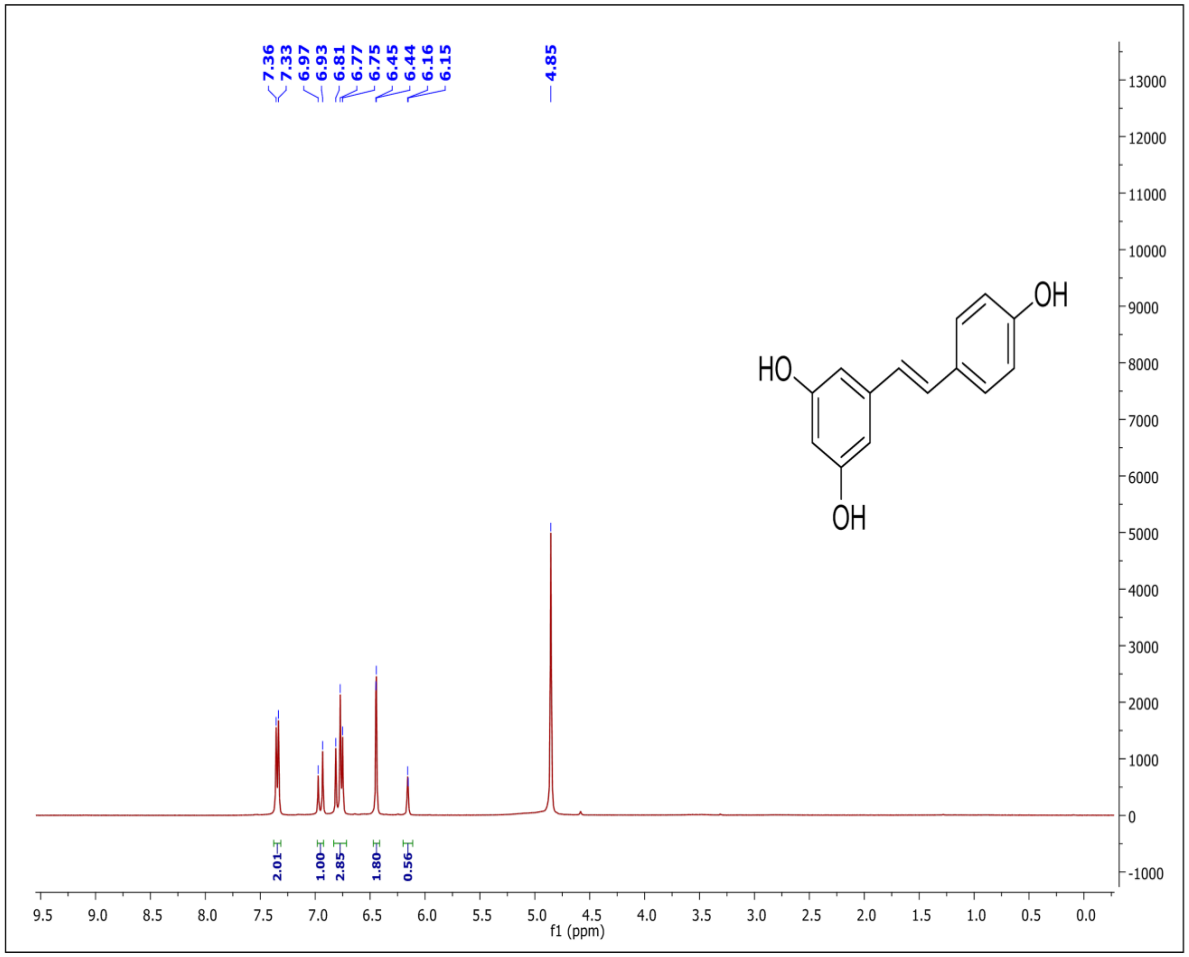


Figure 5.23. ^{1}H NMR analysis of purified fungal resveratrol

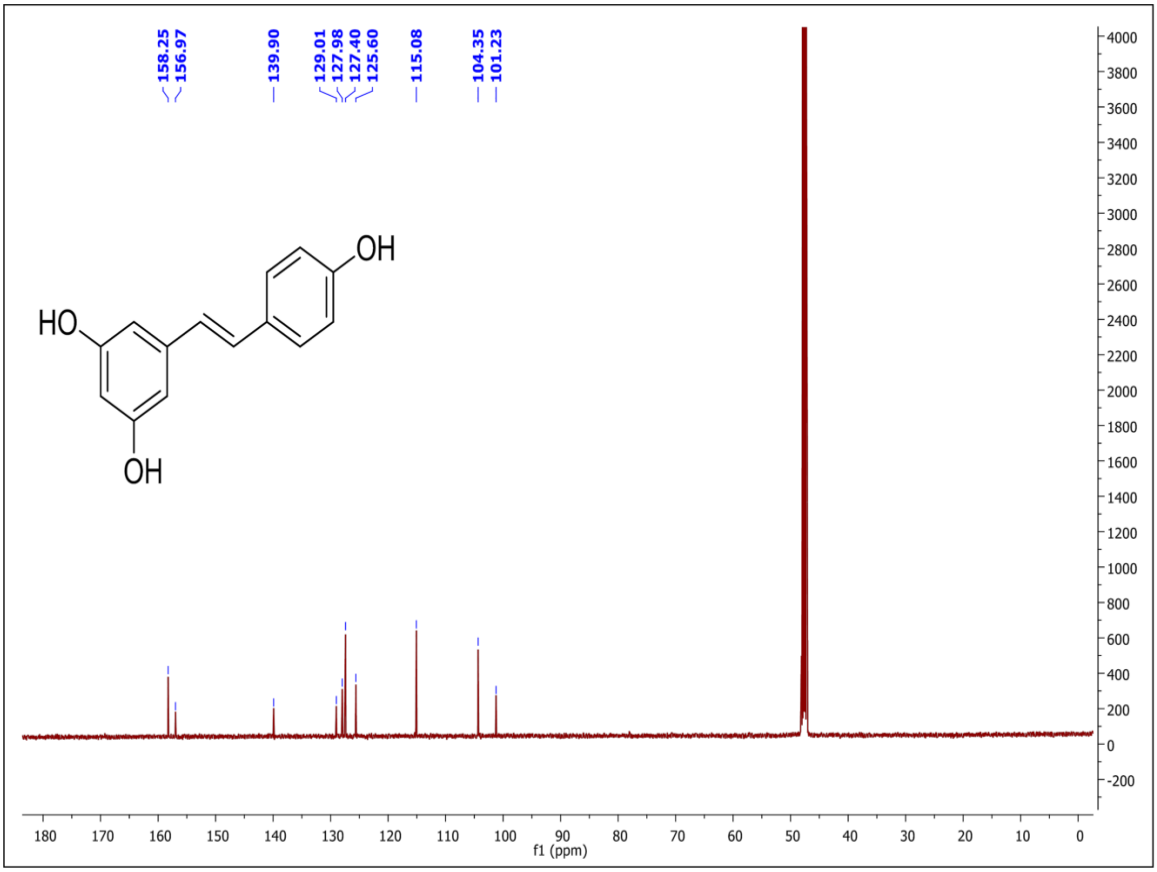
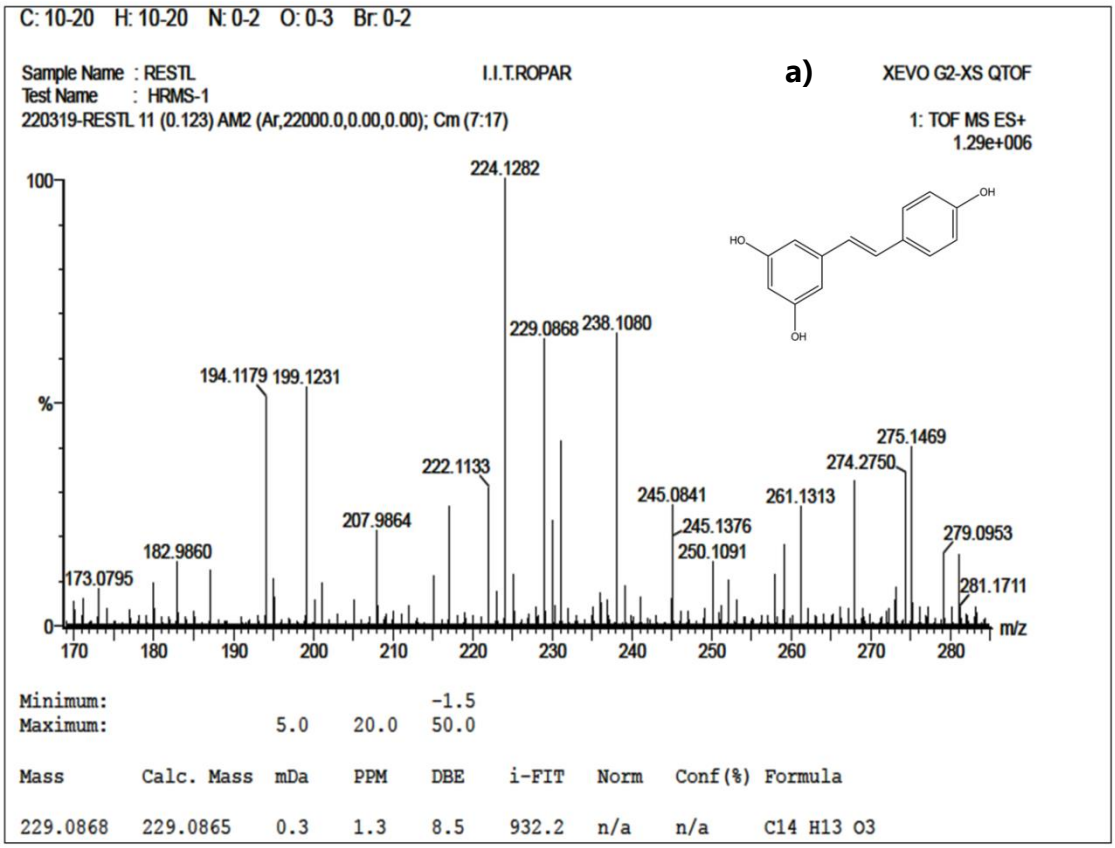


Figure 5.24. ^{13}C NMR analysis of purified fungal resveratrol

5.11.5. HR- MS analysis

HRMS analysis of standard resveratrol was observed HRMS*m/z* $[M+H]^+$calculated for $C_{14}H_{13}O_3$: 229.0865, found: 229.0858. The HRMS analysis of isolated fungal resveratrol is perfectly matching with the mass of standard which was found, HRMS*m/z* $[M+H]^+$calculated for $C_{14}H_{13}O_3$: 229.0865, found: 229.0868.



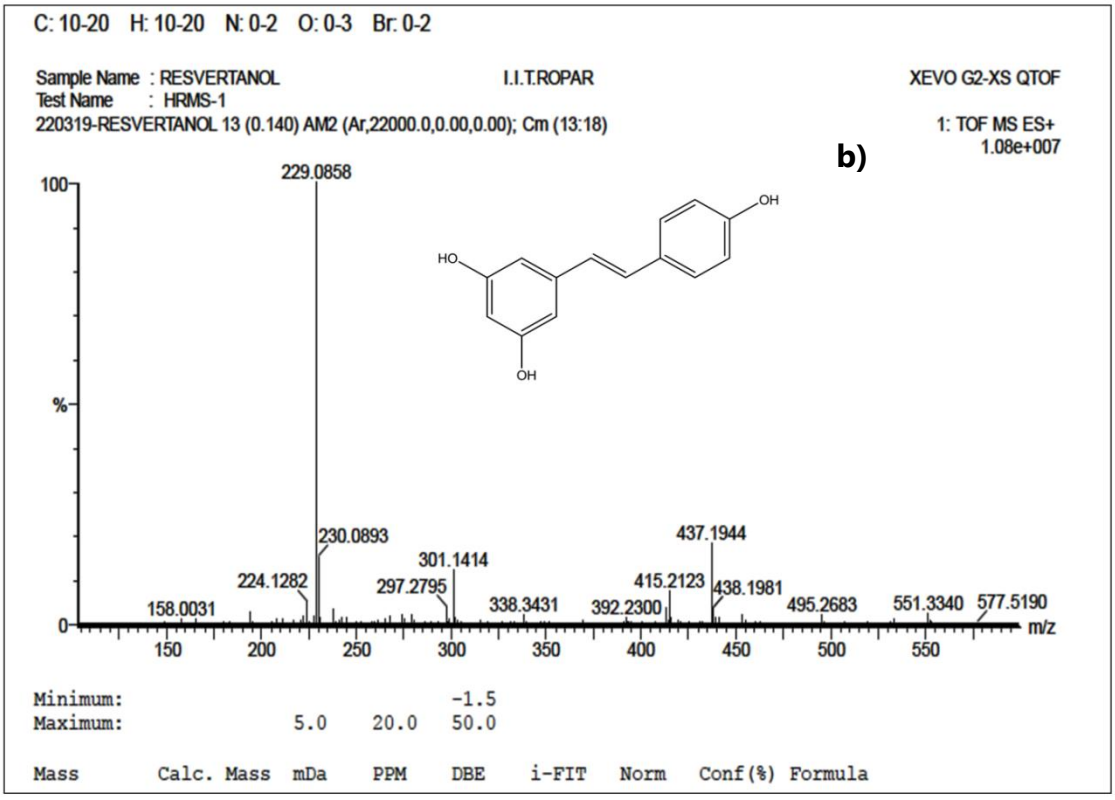


Figure 5.25. HRMS analysis of a) standard resveratrol, b) purified fungal resveratrol

5.12. Process optimization for maximum resveratrol production from endophytic fungus Arcopilus aureus

As it was envisaged that fungal resveratrol is novel and auspicious alternative for large scale production of drug in very efficient and economical way and in minimum period of time, so an attempt was made to enhance the resveratrol production by cost effective method from potent resveratrol producing endophytic fungus *Arcopilus aureus* with the help of optimization of different physiological and nutritional parameter as well as through response surface methodology. The different parameters to be optimized are mentioned in Table 5.13.

Table 5.13. Process optimization for enhancing resveratrol production from endophytic fungus *Arcopilus aureus*

Sr. NO	Parameters
1.	Growth Kinetics (Estimation of fungal biomass)
2.	Selection of best production medium for maximizing resveratrol production
3.	Optimization of medium-to-flask volume ratio (V_m/V_f ratio)
4.	Estimation of optimum pH Condition
5.	Estimation of optimum temperature Condition
6.	Estimation of optimum Agitation rate
7.	Estimation of optimum carbon source and their concentration
8.	Estimation of optimum nitrogen source and their concentration
9.	Experiment design using Response Surface Methodology (RSM)
10.	Box-Behnken Design (BBD)

5.12.1. "One variable at a time" approach (OVAT)

5.12.1.1. Optimization of basic parameters

Studies by many researchers have confirmed that fermentative production is affected by various physiological as well as nutritional parameters. The amount of resveratrol produced depends upon the type and growth conditions of fungus. For the process optimization this method is close ended system and is applied for the optimization of the process conditions and the media components.

i. Estimation of fungal biomass (Growth kinetics)

To study the growth kinetics of endophytic fungus, *Arcopilus aureus* was grown in PDB medium in 250 mL of Erlenmeyer flask and incubated at 30±2˚C for various time intervals (in days) and a significant difference in the biomass obtained and resveratrol yield was obtained by One-way ANOVA analysis. The mycelial growth of endophytic fungus showed that biomass increased exponentially till the 8th day after which it started decreasing (Table 5.13, Figure 5.26). Symmetrically an enhancement in resveratrol production was observed till the 8th day (89.1^{a} ± 0.08µg/mL) and further it fell to 28.83^{e} ± 1.23µg/mL. It was also observed that time period had significant impact on biomass as well as resveratrol production. Further, at 8th and 10th day also a similar biomass production was obtained based on Tukey's post-hoc analysis ($p<0.05$) but did not exhibited the same resveratrol production. Maximum resveratrol production (89.1^{a} ± 0.08µg/mL) and biomass production (125.33^{a} ± 8.18 g/L) was thus obtained on 8th day of growth cycle (Table 5.14, Figure 5.26).

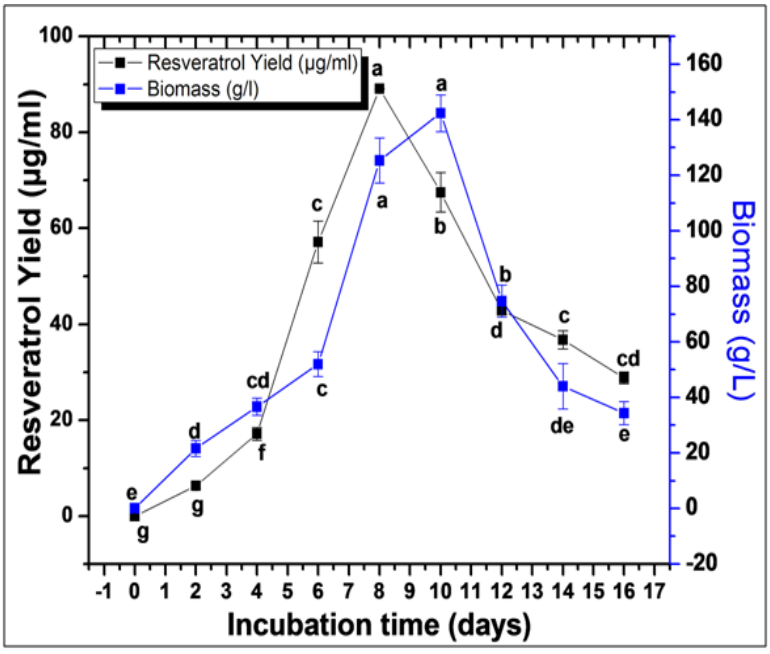


Figure 5.26. Effect of different time period on yield of resveratrol production and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean ± SD (n=3)

Table 5.14. Effect of incubation time (days) period on yield of resveratrol production and biomass of endophytic fungus *Arcopilus aureus*

Incubation time (days)	Biomass* (g/L)	Resveratrol yield* (µg/mL)
0	0.00^{e}	0.00^{g}
2	$21.67^{d} \pm 2.87$	$6.33^{g} \pm 0.56$
4	$36.67^{cd} \pm 3.09$	$17.13^{f} \pm 1.35$
6	$52.00^{c} \pm 4.55$	$57.12^{c} \pm 4.39$
8	$125.33^{a} \pm 8.18$	$89.1^{a} \pm 0.08$
10	$105.67^{a} \pm 4.11$	$67.52^{b} \pm 4.13$
12	$74.67^{b} \pm 5.79$	$42.85^{d} \pm 0.77$
14	$44.00^{c} \pm 8.16$	$36.77^{de} \pm 1.86$
16	$34.33^{cd} \pm 4.11$	$28.83^{e} \pm 1.23$

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

ii. Selection of best production medium for maximizing resveratrol production

A significant difference in the biomass as well as resveratrol concentration in different media was observed by one way ANOVA.

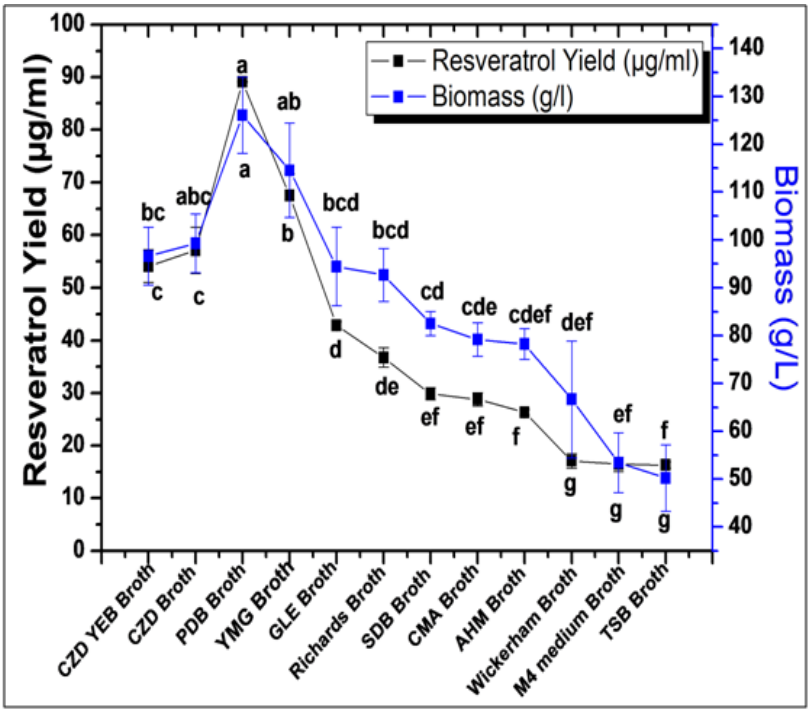


Figure 5.27. Optimization of different production medium of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

Further Tukey's post-hoc analysis confirmed that the selected endophytic fungus *Arcopilus aureus* amongst all the different media tested, produced maximum resveratrol production of 89.1[a] ± 0.08 µg/mL in PDB liquid medium as compared to YMG medium in which resveratrol production was observed to be 67.52[b] ± 4.13 µg/mL, symmetrically maximum biomass production (126.03[a] ± 7.98 g/L) was obtained in PDB medium. Hence PDB medium was further optimized for maximum resveratrol production (Table 5.15, Figure 5.27).

Table 5.15. Resveratrol production in different production medium

Media	Biomass* (g/L)	Resveratrol yield* (µg/mL)
CZD YEB Broth	96.62[bc] ± 6.02	54.12[c]± 3.16
CZD Broth	99.29[abc] ± 6.07	57.12[c] ± 4.39
PDB Broth	**126.03[a] ± 7.98**	**89.1[a] ± 0.08**
YMG Broth	114.52[ab] ± 9.85	67.52[b] ± 4.13
Grape Leaf Extract Broth	94.40[bcd] ± 8.21	42.85[d] ± 0.77
Richards Broth	92.70[bcd] ± 5.53	36.77[de] ± 1.86
SDB Broth	82.55[cd] ± 2.53	29.83[ef] ± 1.14
CMB Broth	79.19[cde] ± 3.51	28.83[ef] ± 1.23
Asthana Hawker Medium	78.23[cdef] ± 3.19	26.33[f] ± 0.56
Wickerham Medium	66.67[def] ± 12.25	17.13[g] ± 1.35
M4 Medium	53.44[ef] ± 6.22	16.46[g] ± 1.38
Tryptone Soy Broth	50.26[f] ± 6.96	16.33[g] ± 0.56

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

iii. Optimization of medium-to-flask volume ratio (V_m/V_f ratio)

A significant difference in the biomass as well as resveratrol concentration was observed in optimization of medium-to-flask volume ratio by one-way ANOVA. While optimizing the ratio of V_m to V_f, it is noted that no relative significant difference was observed in the fungal biomass from 0.2 to 0.4 V_m/Vf ratio by Tukey's post-hoc analysis. Thus, an increased fungal biomass was observed from 34.33d ± 4.11 g/L at V_m to V_f ratio of 0.12 to a reasonably high value of 124.67[a] ± 3.40 g/L at V_m to V_f ratio of 0.3, after that decreased to 90.00[b] ± 4.55 g/L at V_m to V_f ratio of 0.7. On the other hand resveratrol production continuously increased from 36.77[d]± 1.86 to 89.10[a] ± 0.08 µg/mL with an increase of V_m/V_f from 0.12 to 0.2, after that decreased to 6.33[f] ± 0.56 µg/mL at V_m to V_f ratio of 0.7 (Table 5.16, Figure 5.28).

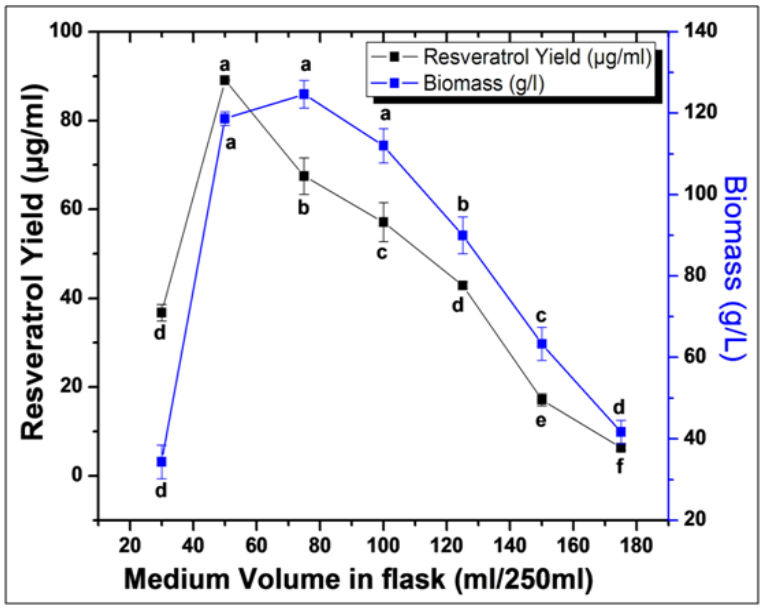


Figure 5.28. Effect of medium to flask volume ratio on yield of resveratrol production and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

Table 5.16. Effect of medium to flask volume ratio on yield of resveratrol production and biomass of endophytic fungus *Arcopilus aureus*

Medium to flask volume (V_m/V_f)	Media volume in flask (mL/250 mL flask)	Biomass (g/L)	Resveratrol yield (µg/mL)
0.12	30	34.33^{d} ± 4.11	36.77^{d} ± 1.86
0.2	**50**	**118.67^{a} ± 1.70**	**89.10^{a} ± 0.08**
0.3	75	124.67^{a} ± 3.40	67.52^{b} ± 4.13
0.4	100	112.00^{a} ± 4.24	57.12^{c} ± 4.39
0.5	125	90.00^{b} ± 4.55	42.85^{d} ± 0.77
0.6	150	63.33^{c} ± 4.11	17.13^{e} ± 1.35
0.7	175	41.67^{d} ± 2.87	6.33^{f} ± 0.56

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

Statistical analysis reveals that V_m to V_f ratio had significant impact on biomass as well as resveratrol production. Hence the optimal value of V_m to V_f ratio was found to be 0.2 which enhanced the resveratrol production.

5.12.1.2. Optimization of physiological parameters

i. Estimation of optimum agitation rate

The agitation rate influences the dissolved oxygen in the medium which affects the growth and production of any bioactive molecule and a significant difference was obtained by one-way ANOVA. Therefore, the endophytic fungal isolate was grown at different agitation

rates i.e. static, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 rpm to identify its effect on resveratrol production.

Further, by Tukey's post-hoc analysis it was observed that at rpm of 80 and 120 exhibited no relative significant difference in biomass was obtained. It is evident from the results in table 5.17 and figure 5.29 that a significant amount of resveratrol was produced at agitation rates 80rpm, 100rpm and 120rpm.

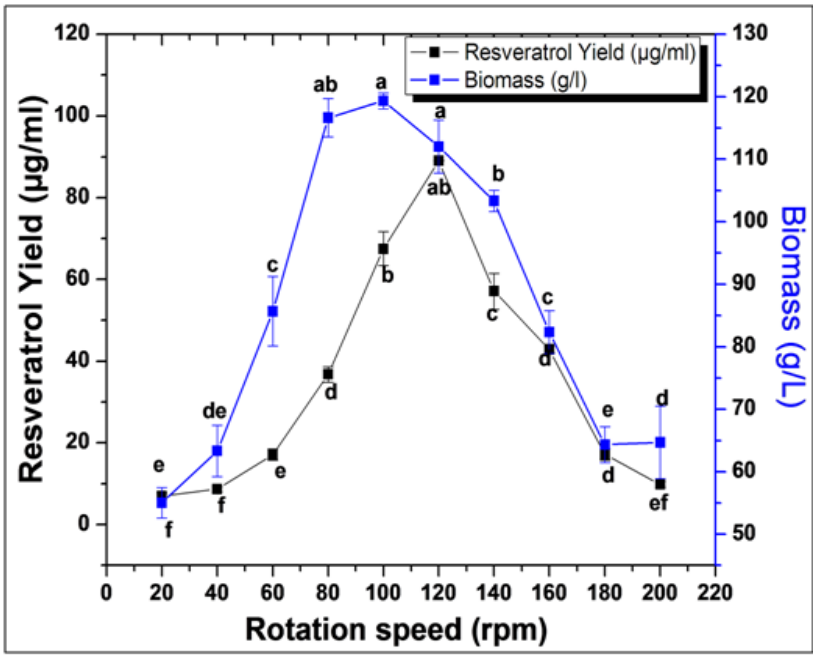


Figure 5.29. Effect of different agitation rate on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean ± SD (n=3)

Table 5.17. Effect of different agitation rate on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

Rpm	Biomass (g/L)	Resveratrol yield (µg/mL)
Static	48.67^{e} ± 2.87	6.33^{f} ± 0.56
20	55.00^{de} ± 2.45	7.00^{f} ± 0.70
40	63.33^{de} ± 4.11	8.63^{f} ± 0.60
60	85.67^{c} ± 5.56	17.13^{e} ± 1.35
80	116.67^{ab} ± 3.09	36.77^{d} ± 1.86
100	119.33^{a} ± 1.25	67.52^{b} ± 4.13
120	**112.00^{ab} ± 4.24**	**89.10^{a} ± 0.08**
140	103.33^{b} ± 1.70	57.12^{c} ± 4.39
160	82.33^{c} ± 3.40	42.85^{d} ± 0.77
180	64.33^{d} ± 2.87	17.13^{e} ± 1.35
200	64.67^{d} ± 5.80	9.84^{ef} ± 0.70

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

However, a maximum amount of resveratrol i.e. 89.10^{a} ± 0.08 µg/mL as well as biomass i.e. 112.00^{ab} ± 4.24 g/L was observed at 120 rpm. At lower agitation rates, there is a decline in resveratrol production. Under static conditions, very minute amount of resveratrol is produced, indicating the fact that agitation rate is essential for resveratrol production.

ii. Estimation of optimum pH

The pH of the any fermentative medium is a crucial factor that has an influence on the growth and production of any bioactive compound. Therefore, in this experiment, *Arcopilus aureus* was grown in the PDB medium at a different pH in the range of 3-10 (Table 5.18, Figure 5.30) at 30±2˚C and was maximized for resveratrol production and a significant difference was obtained by one way ANOVA. It is clearly evident from the figure 5.30 and table 5.18 that although resveratrol production was observed at pH range of 3-10, thus maximum resveratrol production of 92.20^{a} ± 1.68 µg/mL as well as biomass (125.33^{a} ± 8.18 g/L) was observed at neutral pH (7.0) and a significant difference was obtained when compared using Tukey's post-hoc analysis, production on increasing pH from 7 to 10.Hence neutral pH (7.0) was used as optimum pH for further experiments.

Table 5.18. Effect of different pH on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

pH	Biomass (g/L)	Resveratrol yield (µg/mL)
3	34.33^{f} ± 4.11	5.00^{f} ± 1.49
4	41.67^{ef} ± 2.87	16.13^{e} ± 2.08
5	90.00^{bc} ± 4.55	57.12^{b} ± 4.39
6	98.67^{b} ± 7.04	67.52^{b} ± 4.13
7	**125.33^{a} ± 8.18**	**92.20^{a} ± 1.68**
8	85.67^{bc} ± 4.11	42.85^{c} ± 0.77
9	74.67^{cd} ± 5.79	36.77^{cd} ± 1.86
10	57.33^{de} ± 4.71	28.83^{d} ± 1.23

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

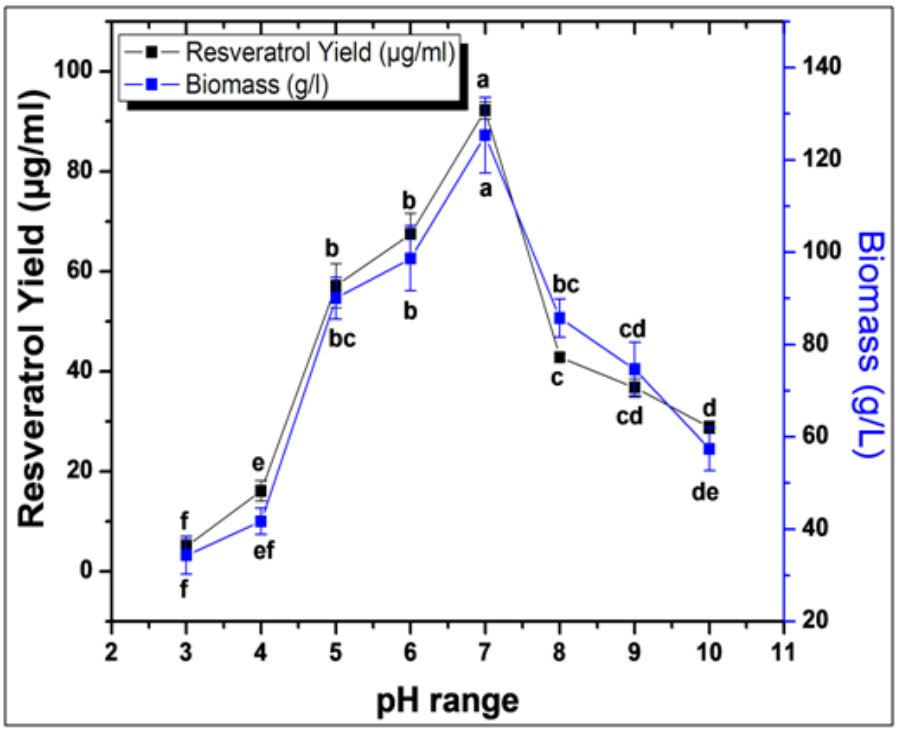


Figure 5.30. Effect of different pH on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

There was no growth observed at pH<3 and pH>10. There was decline in resveratrol

iii. Estimation of optimum temperature

Resveratrol production for the endophytic fungal isolate *Arcopilus aureus* was observed at temperature ranging from 12-39˚C and a significant difference was observed using one-way ANOVA.

Maximum enhancement of resveratrol production and biomass was observed at temperature 30˚C (Table 5.19, Figure 5.31), compared to minimum (12˚C) and maximum (39˚C) temperature and there was a significant difference amongst their means which was confirmed by Tukey's post-hoc analysis ($p<0.05$). There was major decline in the resveratrol production as temperature was increased above 30˚C. Hence further optimization was carried out at 30˚C.

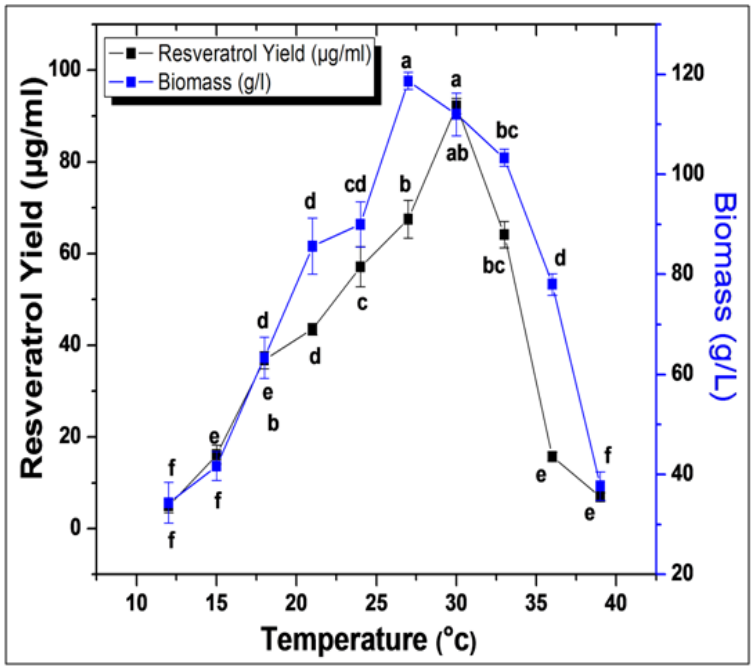


Figure 5.31. Effect of different temperature on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

Table 5.19. Effect of different temperature on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

Temperature (˚C)	Biomass (g/L)	Resveratrol yield (µg/mL)
12	34.33^{f} ± 4.11	5.00^{f}± 1.49
15	41.67^{f} ± 2.87	16.13^{e} ± 2.08
18	63.33^{e} ± 4.11	36.77^{d} ± 1.86
21	85.67^{d} ± 5.56	43.52^{d} ± 1.23
24	90.00^{cd} ± 4.55	57.12^{c} ± 4.39
27	118.67^{a} ± 1.70	67.52^{b} ± 4.13
30	**112.00^{ab} ± 4.24**	**92.20^{a} ± 1.68**
33	103.33^{bc} ± 1.70	64.17^{bc} ± 2.88
36	78.00^{d} ± 2.16	15.67^{e} ± 1.02
39	37.67^{f} ± 2.87	6.93^{ef} ± 0.94

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

5.12.1.3. Optimization of nutritional parameters

i. Estimation of optimum carbon source

As carbohydrates serve as sources of energy which not only plays a role in cellular maintenance and growth but also metabolite production. Hence different carbon sources i.e. sugars were evaluated for production of RESV. As evident from the studies maximum

RESV production of 97.77[a] ± 2.29 µg/mL was observed in glucose as a carbon source (Table 5.21, Figure 5.32).

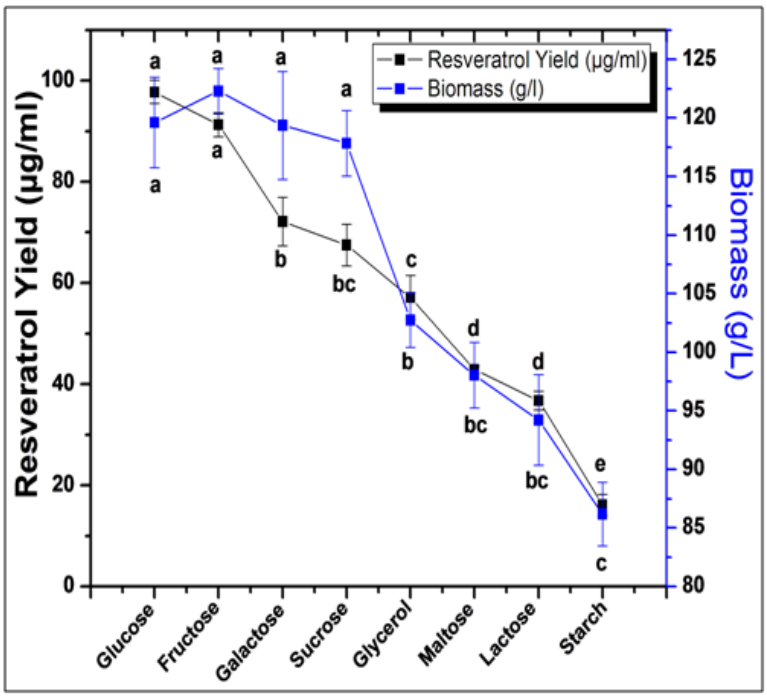


Figure 5.32. Effect of different carbon source on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

Table 5.20. Effect of different carbon source on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

Carbon source	Biomass (g/L)	Resveratrol yield (µg/mL)
Glucose	**119.62[a] ± 3.87**	**97.77[a] ± 2.29**
Fructose	**122.29[a] ± 1.93**	**91.30[a] ± 2.41**
Galactose	119.36[a] ± 4.61	72.17[b] ± 4.78
Sucrose	117.85[a] ± 2.78	67.52[bc] ± 4.13
Glycerol	102.73[b] ± 2.34	57.12[c] ± 4.39
Maltose	98.03[bc] ± 2.79	42.85[d] ± 0.77
Lactose	94.21[bc] ± 3.85	36.77[d] ± 1.86
Starch	86.18[c] ± 2.74	16.13[e] ± 2.08

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

There is statistically no significant difference between glucose (97.77[a] ± 2.29µg/mL) and fructose (91.30[a] ± 2.41 µg/mL) as a carbon source and their biomass (119.62[a] ± 3.87 and 122.29[a] ± 1.93g/L, respectively) by using Tukey's post-hoc analysis ($p<0.05$). Among the carbon source medium supplemented with monosaccharides showed significant higher

resveratrol and biomass as compared to disaccharides and polysaccharides (Table 5.20, Figure 5.32).

Minimum resveratrol production was found in starch (16.13^{e} ± 2.08µg/mL) and Lactose (36.77^{d} ± 1.86µg/mL). Hence, further optimization was carried out using glucose as the carbon source.

ii. Estimation of optimum carbon source concentration

After optimization of glucose as the carbon source, experiments with varying concentrations of glucose ranging from 0.25% to 5% was done to arrive to optimal glucose concentration for maximum resveratrol production and a significant difference was observed using one way ANOVA. The potent endophytic fungus was grown under optimized conditions obtained so far and the samples were analysed for resveratrol production (µg/mL), cell mass (g/L).

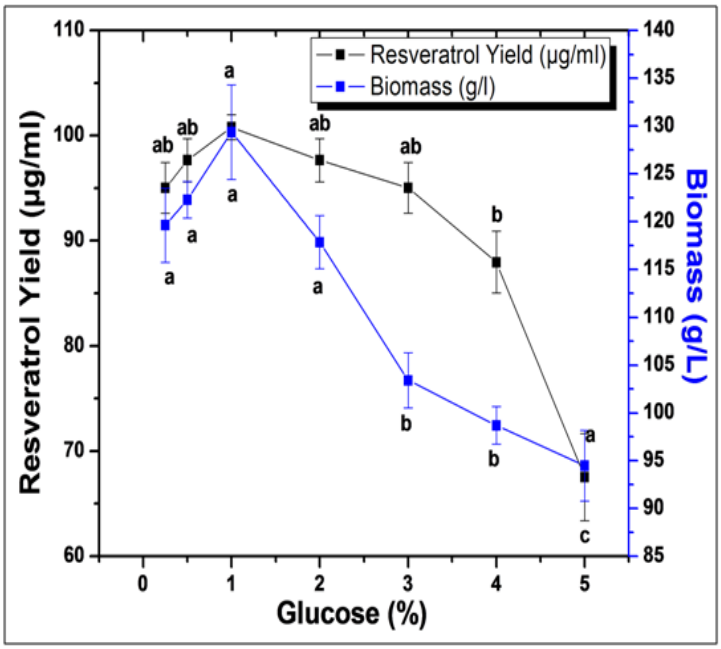


Figure 5.33. Effect of optimum carbon source concentration on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus.* Bars represent mean± SD (n=3)

The highest resveratrol yields i.e. 100.8^{a}±1.18 µg/mL was produced on the 8th day at glucose concentration of 1% (Table 5.21, Figure 5.33). There was major decline in the resveratrol production as concentration was increased above 1% and a relatively significant

difference was observed using Tukey's post-hoc analysis ($p<0.05$). Hence further optimization was carried out at 1% of glucose concentration.

Table 5.21. Effect of optimum carbon source concentration on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

Glucose conc. (%)	Biomass (g/L)	Resveratrol yield (µg/mL)
0.25%	$119.62^{a} \pm 3.87$	$95.03^{ab} \pm 2.40$
0.50%	$122.29^{a} \pm 1.93$	$97.66^{ab} \pm 2.05$
1%	**$129.36^{a} \pm 4.92$**	**$100.8^{a} \pm 1.18$**
2%	$117.85^{a} \pm 2.79$	$97.66^{ab} \pm 2.05$
3%	$103.39^{b} \pm 2.88$	$95.03^{ab} \pm 2.40$
4%	$98.70^{b} \pm 1.97$	$87.97^{b} \pm 2.95$
5%	$94.47^{b} \pm 3.71$	$67.52^{c} \pm 4.13$

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

iii. Estimation of optimum nitrogen source

Besides carbon, nitrogen is also the vital nutrient mandatorily required by the micro-organism for its growth, metabolism and the production of secondary metabolite.

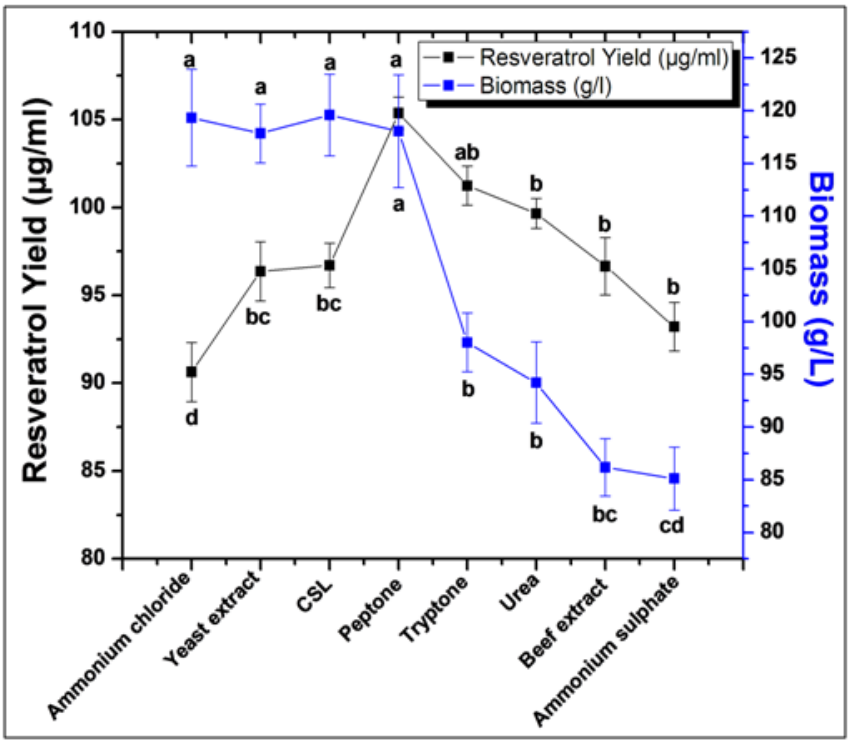


Figure 5.34. Effect of different nitrogen source on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

Table 5.22. Effect of different nitrogen source on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

Nitrogen source	Biomass (g/L)	Resveratrol yield (µg/ml)
Ammonium chloride	119.36^{a} ± 4.61	90.63^{d} ± 1.68
Yeast extract	117.85^{a} ± 2.79	96.37^{bc} ± 1.68
CSL	119.62^{a} ± 3.87	96.7^{bc} ± 1.27
Peptone	**118.07^{a} ± 5.35**	**105.37^{a} ± 0.92**
Tryptone	98.03^{b} ± 2.79	101.24^{ab} ± 1.10
Urea	94.21^{b} ± 3.85	99.66^{b} ± 0.84
Beef extract	86.18^{b} ± 2.74	96.66^{bc} ± 1.63
Ammonium sulphate	85.12^{b} ± 2.99	93.21^{cd} ± 1.39

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

It was observed that all the organic nitrogen sources used supported the growth of endophytic fungus as well as the resveratrol production (Table 5.22, Figure 5.34). Peptone was found to give the maximum resveratrol production i.e. 105.37^{a} ± 0.92 µg/mL as well as biomass (mycelia mass) i.e. 118.07^{a} ± 5.35 g/L. One way ANOVA analysis confirmed that nitrogen source had a statistically significant effect on production of resveratrol and fungal biomass.

Least resveratrol production was observed in ammonium sulphate i.e. 93.21^{cd} ± 1.39µg/mL and beef extract i.e. 96.66^{bc} ± 1.63 µg/mL. Hence, further optimization was carried out using Peptone as the nitrogen source and there was a statistical difference using Tukey's post-hoc analysis ($p<0.05$) (Table 5.22, Figure 5.34).

iv. Estimation of optimum nitrogen source concentration

After selection of peptone as nitrogen source in the production medium, different concentrations in the range of 0.25%-5% were used for enhancing the resveratrol production. Observations in terms of resveratrol production (µg/mL) and fungal Biomass (g/L) are given in table 5.23 and figure 5.35. A statistical difference was observed using one way ANOVA. It was also observed that high peptone concentration had a negative impact on resveratrol production.

Using Tukey's post-hoc analysis ($p<0.05$) there was no significant difference at 0.5% and 1% of peptone, but 0.5% of peptone was found to be optimum for resveratrol production.

Concentrations above this value also saw a decline in resveratrol production. Hence further optimization was carried out using 0.5% of peptone concentration.

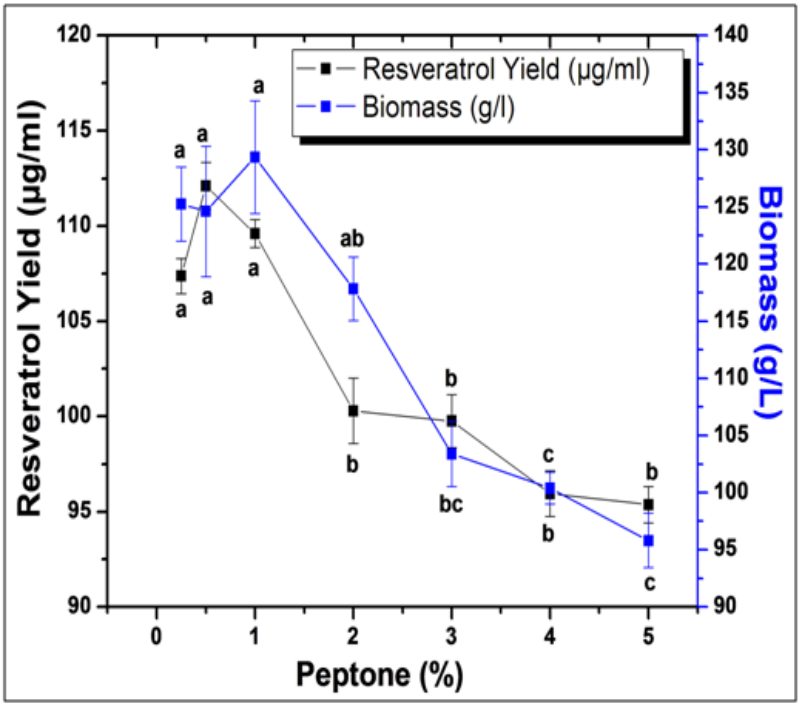


Figure 5.35. Effect of optimum nitrogen source concentration on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*. Bars represent mean± SD (n=3)

Table 5.23. Effect of optimum nitrogen source concentration on resveratrol yield and biomass of endophytic fungus *Arcopilus aureus*

Peptone conc. (%)	Biomass (g/L)*	Resveratrol yield (µg/mL)*
0.25%	125.25^{a} ± 3.25	107.37^{a} ± 0.92
0.50%	**124.62^{a} ± 5.72**	**112.10^{a} ± 1.23**
1%	129.36^{a} ± 4.92	109.61^{a} ± 0.74
2%	117.85^{ab} ± 2.78	100.29^{b} ± 1.71
3%	103.39^{bc} ± 2.88	99.76^{b} ± 1.40
4%	100.39^{c} ± 1.39	95.95^{b} ± 1.20
5%	95.81^{c} ± 2.39	95.37^{b} ± 0.95

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

The conditions optimized to produce resveratrol using one variable at time approach (OVAT) are summarized in table 5.24.

Table 5.24. Summary of optimized condition after "One Variable at time Approach (OVAT)"

Sr. No.	Parameters	Optimized Condition
1.	Growth Kinetics (Estimation of fungal biomass)	8th Day
2.	Selection of best production medium for maximizing resveratrol production	Potato Dextrose Broth
3.	Optimization of medium-to-flask volume ratio (V_m/V_f ratio)	50mL/250mL
4.	Estimation of optimum Agitation rate	120 rpm
5.	Estimation of optimum temperature Condition	30˚C
6.	Estimation of optimum pH Condition	7.0
7.	Estimation of optimum carbon source and their concentration and its concentration	Glucose – 1%
8.	Estimation of optimum nitrogen source and their concentration and its concentration	Peptone- 0.5%
9.	**Initial yield of Resveratrol**	**89.1^a ± 0.08 µg/mL**
10.	**Yield of resveratrol after "one-variable-at-a-time approach"**	**109.61^a ± 0.74 µg/mL**
11.	**Fold increase**	**1.23**

Optimization of different physiological and nutritional factors finally resulted in **109.61^a ± 0.74 µg/mL** of resveratrol which is **1.23 fold increases** in the resveratrol production. Resveratrol production was further optimized by evaluating the interactions amongst the most influential factors by the statistical approach i.e. response surface methodology.

5.12.2. Response surface methodology (RSM)

After using OVAT approach, the final yield of resveratrol 109.61 ± 0.74 µg/mL was obtained in presence of optimized physiological parameter (120 rpm, 30˚C, pH 7.0) and nutritional parameter (Glucose – 1%, Peptone- 1%) from this strain of *Arcopilus aureus* after 8 days of incubation. However, in this approach, process optimization of resveratrol production from *Arcopilus aureus* was carried out by using a statistical approach known as Response Surface Methodology (RSM). The design of the RSM was made using Box-Behnken design (BBD) [Design-Expert® 6.0.8 Stat-Ease, Inc., (Minneapolis, USA)] taking into consideration the interactions between the most influencing factors obtained from OVAT approach, for maximizing the resveratrol production.

5.11.2.1. Box-Behnken design (BBD)

BBD is used in the final stage of process optimization to obtain a set of conditions which describes the behavior of system inside the limits of the regions studied. This help in understanding the interactions occurring between the most influential factors taken into consideration.

i. Experimental

The optimization for resveratrol production by OVAT approach showed that the most influential factors for resveratrol production were:

1. RPM,
2. Temperature,
3. Incubation days,
4. pH,
5. Glucose
6. Peptone

Thus, the interactions amongst these factors were investigated with a view to further increase the resveratrol production from this isolate of *Arcopilus aureus* by applying RSM using Box-Behnken design (BBD). The optimum values of these factors obtained by OVAT method were taken as the "zero level" and accordingly, the ranges for each of these factors -1,0,+1 were selected. The actual values and the coded values of these factors are presented in the table 4.1 (refer section 4.15.2.1). Other factors such as incubation period, agitation rate, temperature etc. were kept at their optimal level as obtained by "one-variable-at-a-time" method.

*ii. *Optimum values obtained after one-variable-at-a-time approach*

Based on the Design-Expert® 6.0.8 Stat-Ease, Inc., (Minneapolis, USA), a set of 54 experiments was generated (Table 5.25). As per to the design matrix data, a set of 54 experiments in 250 mL size Erlenmeyer flask containing 50 mL of the PDB medium in different permutations and combinations of the selected variables were prepared in triplicates. These flasks were set with different according to the design matrix data. After

the desired incubation period, the culture was harvested, and each sample was analyzed for resveratrol production by the help of HPLC. Table 5.25 presents the mean observed value of resveratrol production and mean predicted value of resveratrol. The experimental values of resveratrol production were results of triplicates and predicated values were obtained from the following quadratic polynomial equation analyzed by the standard analysis of Variance (ANOVA), which resulted out following equation:

$$Y = 133.53 + 2.09 * A - 2.26 * B + 1.71 * C - 2.52 * D + 1.39 * E + 0.69 * F - 1.42 * A^2 - 36.58 * B^2 - 8.75 * C^2 - 14.33 - 3.04 * E^2 - 4.80 * F^2 + 0.62 * A * -0.10 * A * -0.40 * A * D - 2.67 * A * E - 2.13 * A * F - 2.01 * B * C + 1.34 * B * D - 0.55 * B * E + 1.02 * B * F - 3.65 * C * D + 0.16 * C * E + 0.14 * C * F + 5.47 * D * E + 0.46 * D * F + 1.37 * E * F$$

Where Y= resveratrol production, A= RPM, B= Temperature, C= Incubation days, D= pH, E= Glucose, F= Peptone

Results exhibit that interaction of the most influential factors resulted in a resveratrol production of 133.53µg/mL in 8.5 days of incubation, rpm-115, temp-30ºC, pH 7.0 at glucose concentration of 1.25% and peptone-0.625%.This is approximately **1.5 fold increase** in resveratrol production against the un-optimized conditions where 89.1 µg/mL of resveratrol was produced.

Table 5.25. Box-Behnken design (BBD)of the variables with resveratrol production

Run	Coded levels						Response	
	RPM	**Temperature**	**Incubation days**	**pH**	**Glucose**	**Peptone**	**Experimental Values (µg/mL)**	**Predicted Values (µg/mL)**
1	115.00	30.00	12.00	9.00	1.25	1.00	101.68	102.49
2	140.00	20.00	8.50	9.00	1.25	0.63	79.63	80.67
3	140.00	30.00	8.50	5.00	2.00	0.63	104.95	113.00
4	115.00	30.00	5.00	5.00	1.25	1.00	108.76	102.91
5	115.00	40.00	8.50	7.00	0.50	1.00	85.69	86.36
6	115.00	30.00	8.50	7.00	1.25	0.63	133.53	133.59
7	115.00	30.00	8.50	7.00	1.25	0.63	133.53	134.54
8	115.00	30.00	5.00	5.00	1.25	0.25	98.91	102.71
9	140.00	30.00	12.00	7.00	1.25	1.00	130.95	120.96
10	140.00	20.00	8.50	5.00	1.25	0.63	91.95	89.18
11	90.00	30.00	8.50	9.00	2.00	0.63	119.98	120.07

12	90.00	40.00	8.50	9.00	1.25	0.63	69.79	75.45
13	140.00	30.00	8.50	9.00	2.00	0.63	117.67	118.10
14	90.00	30.00	12.00	7.00	1.25	0.25	116.63	115.32
15	115.00	30.00	8.50	7.00	1.25	0.63	132.51	133.43
16	115.00	20.00	12.00	7.00	2.00	0.63	91.33	93.23
17	90.00	30.00	8.50	5.00	0.50	0.63	113.73	116.19
18	115.00	20.00	8.50	7.00	2.00	1.00	94.6	94.34
19	115.00	20.00	5.00	7.00	0.50	0.63	84.66	81.93
20	90.00	30.00	8.50	9.00	0.50	0.63	111.96	101.02
21	115.00	20.00	8.50	7.00	2.00	0.25	93.95	92.26
22	90.00	30.00	12.00	7.00	1.25	1.00	121.69	121.25
23	115.00	30.00	8.50	7.00	1.25	0.63	132.51	133.89
24	90.00	20.00	8.50	5.00	1.25	0.63	89.85	85.44
25	90.00	20.00	8.50	9.00	1.25	0.63	76.54	78.54
26	140.00	30.00	5.00	7.00	1.25	0.25	124.79	120.62
27	90.00	30.00	8.50	5.00	2.00	0.63	115.54	113.37
28	140.00	40.00	8.50	5.00	1.25	0.63	82.35	83.24
29	115.00	30.00	12.00	5.00	1.25	0.25	110.47	113.15
30	140.00	30.00	8.50	9.00	0.50	0.63	104.68	109.74
31	90.00	40.00	8.50	5.00	1.25	0.63	80.95	77.02
32	140.00	30.00	8.50	5.00	0.50	0.63	129.5	126.51
33	90.00	30.00	5.00	7.00	1.25	0.25	106.59	111.97
34	115.00	20.00	8.50	7.00	0.50	0.25	88.95	91.13
35	115.00	40.00	8.50	7.00	2.00	0.25	89.33	84.67
36	115.00	40.00	12.00	7.00	0.50	0.63	79.51	81.61
37	115.00	40.00	5.00	7.00	0.50	0.63	83.39	82.52
38	115.00	30.00	5.00	9.00	1.25	0.25	103.69	104.06
39	115.00	20.00	12.00	7.00	0.50	0.63	88.96	89.04
40	115.00	40.00	5.00	7.00	2.00	0.63	84.99	83.88
41	115.00	20.00	5.00	7.00	2.00	0.63	86.54	85.47
42	140.00	40.00	8.50	9.00	1.25	0.63	78.56	80.08
43	115.00	30.00	12.00	9.00	1.25	0.25	98.67	99.91
44	115.00	30.00	8.50	7.00	1.25	0.63	133.53	133.51
45	115.00	30.00	8.50	7.00	1.25	0.63	133.50	133.56
46	90.00	30.00	5.00	7.00	1.25	1.00	109.73	117.34
47	115.00	40.00	8.50	7.00	2.00	1.00	91.93	90.78
48	115.00	40.00	8.50	7.00	0.50	0.25	86.43	85.67
49	115.00	30.00	12.00	5.00	1.25	1.00	109.66	113.90
50	115.00	40.00	12.00	7.00	2.00	0.63	81.91	83.61
51	115.00	30.00	5.00	9.00	1.25	1.00	113.37	106.08
52	115.00	20.00	8.50	7.00	0.50	1.00	81.99	87.74
53	140.00	30.00	12.00	7.00	1.25	0.25	126.56	123.56
54	140.00	30.00	5.00	7.00	1.25	1.00	111.54	117.46

iii. ANOVA analysis

In order to evaluate the significance and accuracy of BBD model, an analysis of variance (ANOVA) was evaluated (Table 5.26). F value measures the variation of the data about its mean value. The higher the F value or lower the P value is, the optimum is the significance

of the any model. The Model F-value of 21.76 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case D, B^2, C^2, D^2, F^2, DE are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.On the other side, the models goodness of fit can be validated with the help of determination coefficent (R^2) as well as correlation coefficient (R). The closer the R^2 value is to 1, the better the correlation between the experimental and predicted values is. In the present study, the R^2 value (0.9576) demonstrates that 95.76% variation in the model is caused by three variables and only 4.24% variation could not be explained by the model. The "Pred R-Squared" of 0.7786 is in reasonable agreement with the "Adj R-Squared" of 0.9136. These results show that the model is an adequate predictor of the experimental conditions.

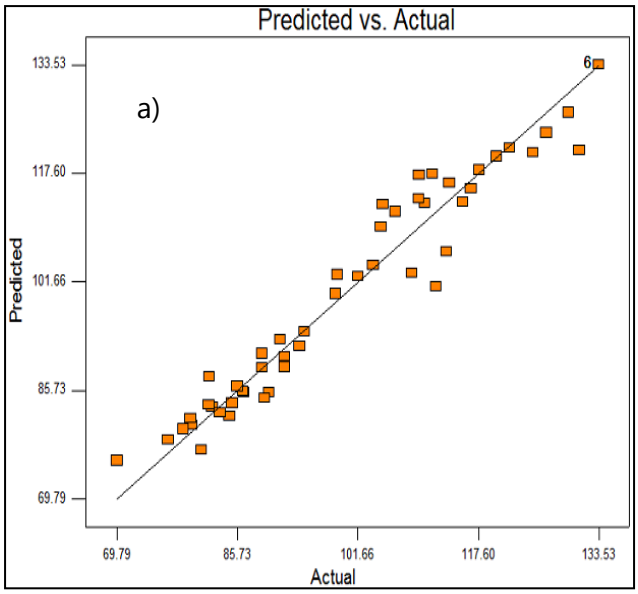
Table 5.26. ANOVA for Response Surface Quadratic Model

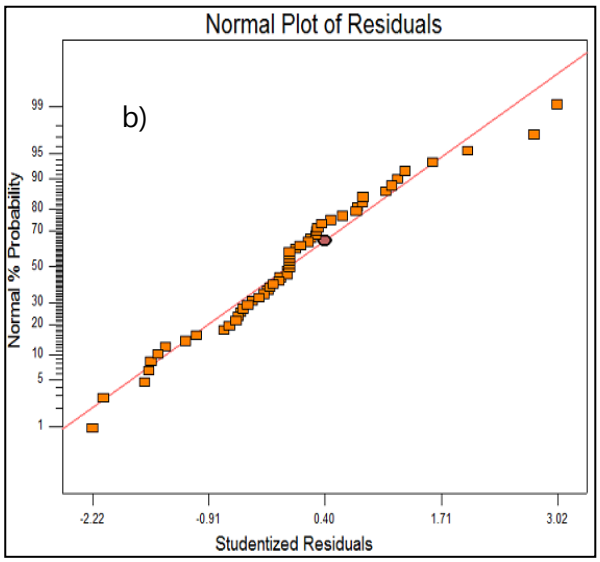
Source	**Sum of Squares**	**DF**	**Meanof Square**	**F Value**	**P Value**
Model	17608.77	27	652.18	21.76	< 0.0001
A	104.79	1	104.79	3.50	0.0728
B	122.04	1	122.04	4.07	0.0540
C	70.25	1	70.25	2.34	0.1379
D	152.01	1	152.01	5.07	0.0330
E	46.12	1	46.12	1.54	0.2259
F	11.51	1	11.51	0.38	0.5409
A^2	20.67	1	20.67	0.69	0.4139
B^2	13766.10	1	13766.10	459.32	< 0.0001
C^2	787.50	1	787.50	26.28	< 0.0001
D^2	2111.05	1	2111.05	70.44	< 0.0001
E^2	94.74	1	94.74	3.16	0.0871
F^2	237.23	1	237.23	7.92	0.0092

AB	3.10	1	3.10	0.10	0.7503
AC	0.084	1	0.084	2.804E-003	0.9582
AD	2.57	1	2.57	0.086	0.7721
AE	57.19	1	57.19	1.91	0.1789
AF	36.38	1	36.38	1.21	0.2807
BC	32.20	1	32.20	1.07	0.3095
BD	14.26	1	14.26	0.48	0.4965
BE	4.75	1	4.75	0.16	0.6937
BF	8.34	1	8.34	0.28	0.6022
CD	106.36	1	106.36	3.55	0.0708
CE	0.21	1	0.21	6.940E-003	0.9342
CF	0.31	1	0.31	0.010	0.9197
DE	239.26	1	239.26	7.98	0.0090
DF	1.67	1	1.67	0.056	0.8155
EF	14.99	1	14.99	0.50	0.4858
Residual	779.24	26	29.97		
Lack of Fit	779.24	21	37.11		
Pure Error	0.000	5	0.000		
Cor Total	18388.01	53			

Adequate Precision value measures the signal to noise ratio. A ratio greater than 4 is desirable. In the present study ratio of 14.732 indicates an adequate signal. This model can be used to navigate the design space. The BBD model showed standard deviation, mean and predicted residual sum of squares (PRESS) value of 5.47, 102.90, and 4071.15 respectively.

The three-dimensional response surface curves were then plotted to study the interaction among different physico-chemical factors used and to find out the optimum concentration of each factor for resveratrol production. The 3-D response surface plot is a function of two factors at a time; and maintaining all other factors at fixed levels (Figure 5.36, Figure 5.37). These plots clarify both the individual and the interaction effects of these two factors.





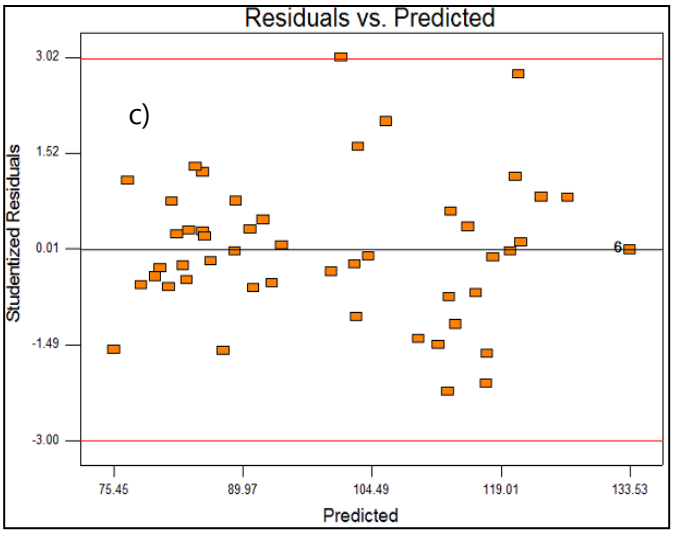
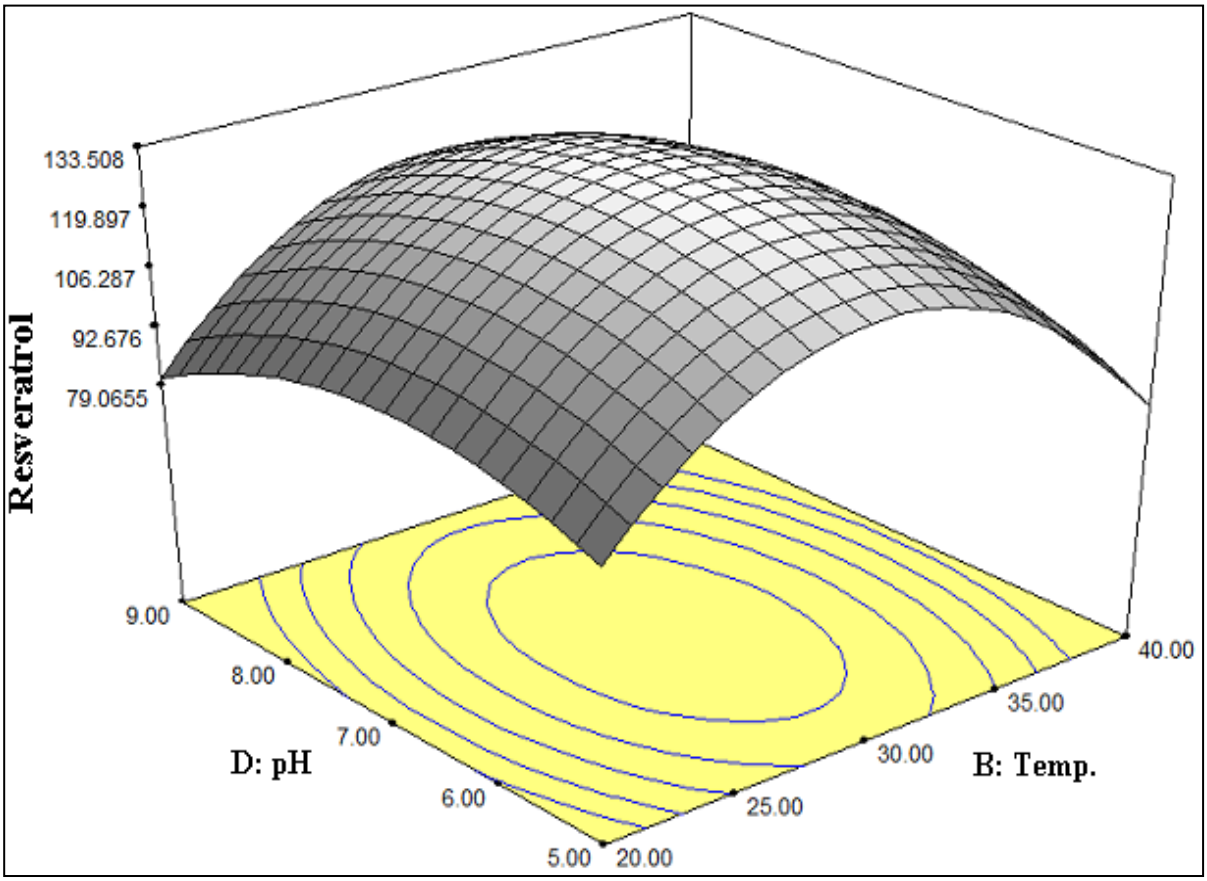
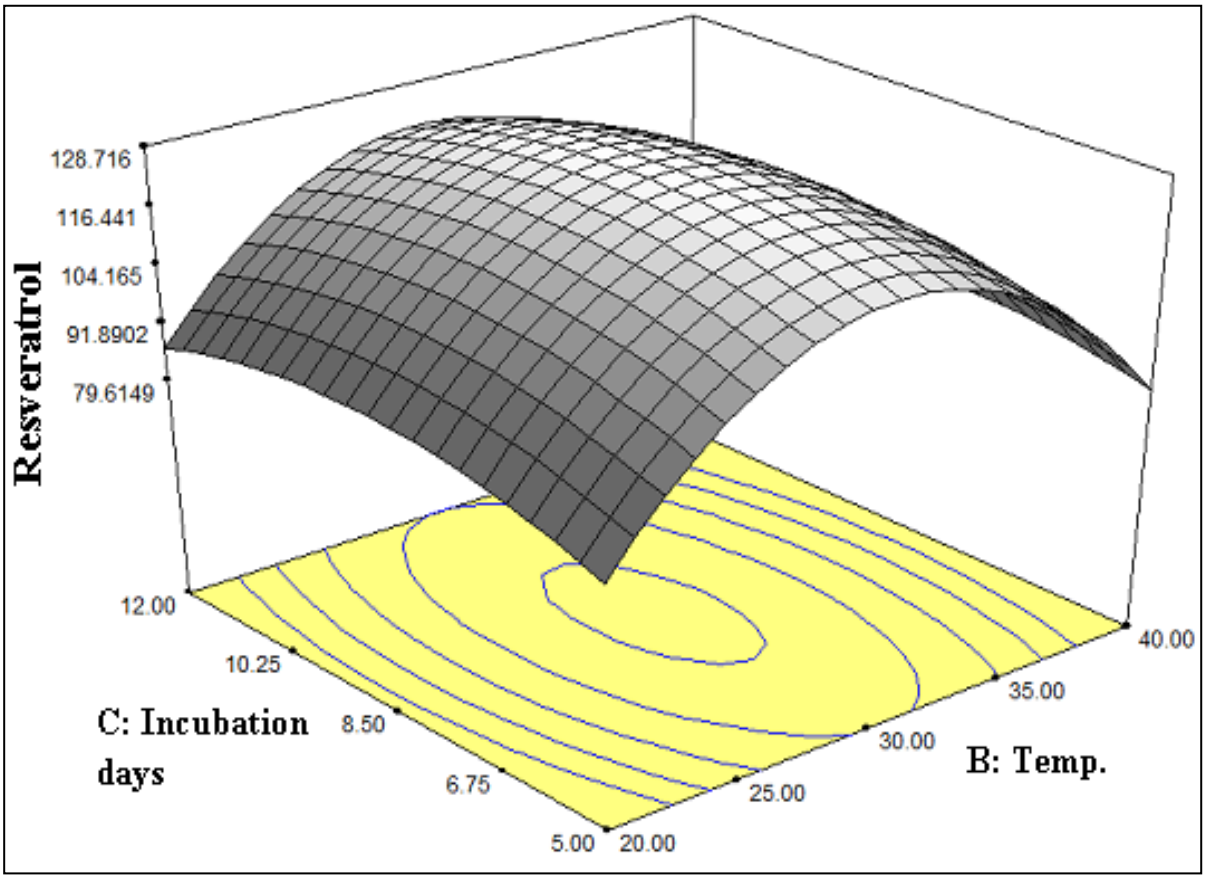
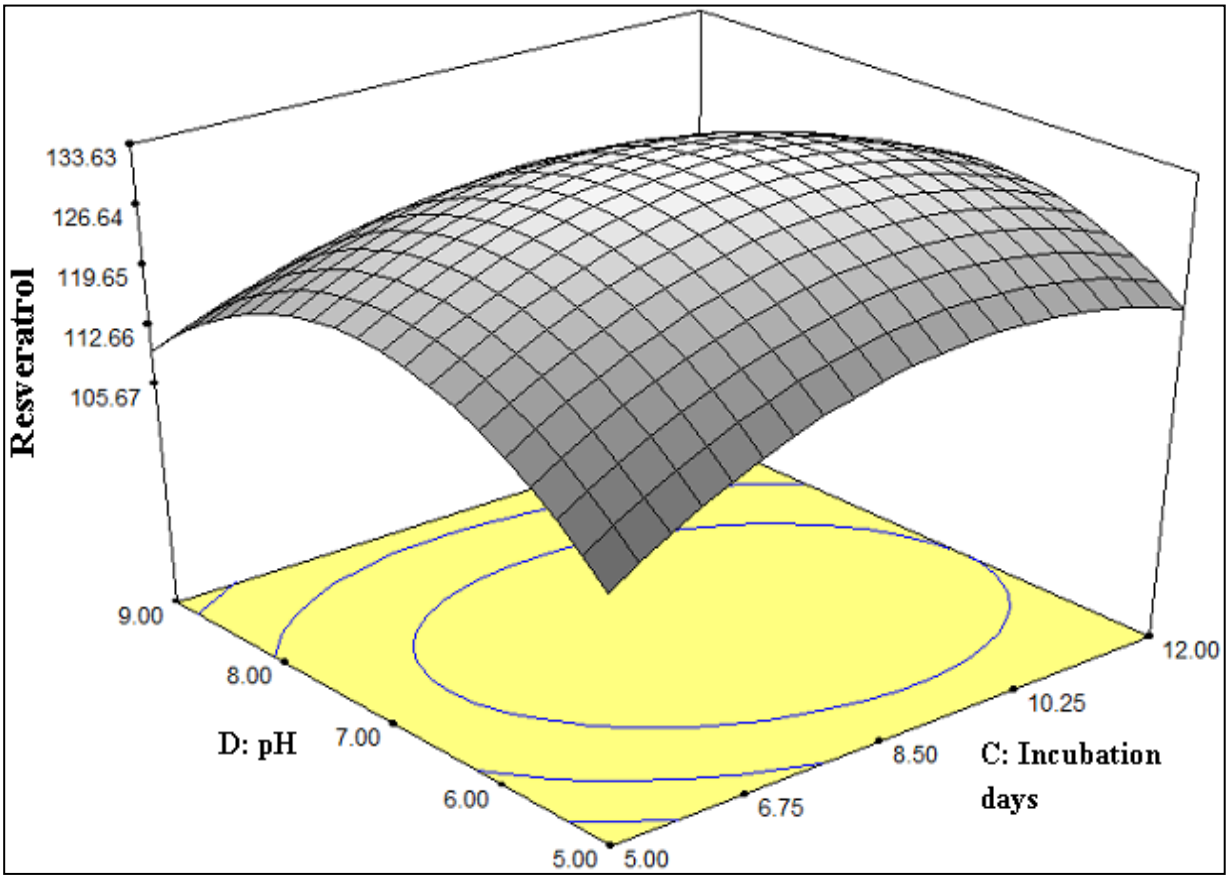
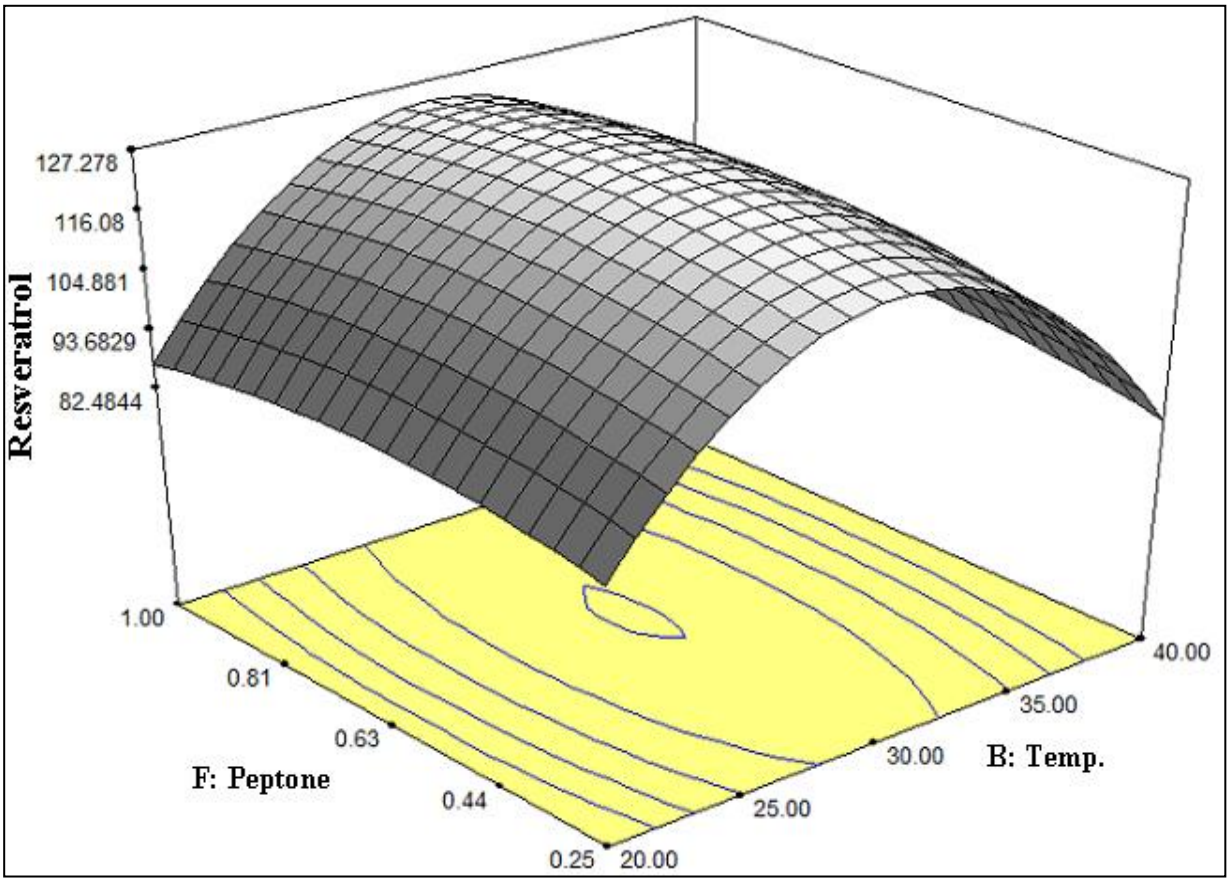


Figure 5.36. (a) The predicted vs actual graph, (b) normal plot of residuals for resveratrol production, (c) residuals vs predicted graph for resveratrol production.


Resveratrol
133.508
119.897
106.287
92.676
79.0655
9.00
8.00
7.00
6.00
5.00
D: pH
20.00
25.00
30.00
35.00
40.00
B: Temp.


Resveratrol
128.716
116.441
104.165
91.8902
79.6149
12.00
10.25
8.50
6.75
5.00
C: Incubation days
20.00
25.00
30.00
35.00
40.00
B: Temp.


Resveratrol
133.63
126.64
119.65
112.66
105.67
9.00
8.00
7.00
6.00
5.00
D: pH
5.00
6.75
8.50
10.25
12.00
C: Incubation days


Resveratrol
127.278
116.08
104.881
93.6829
82.4844
1.00
0.81
0.63
0.44
0.25
F: Peptone
20.00
25.00
30.00
35.00
40.00
B: Temp.

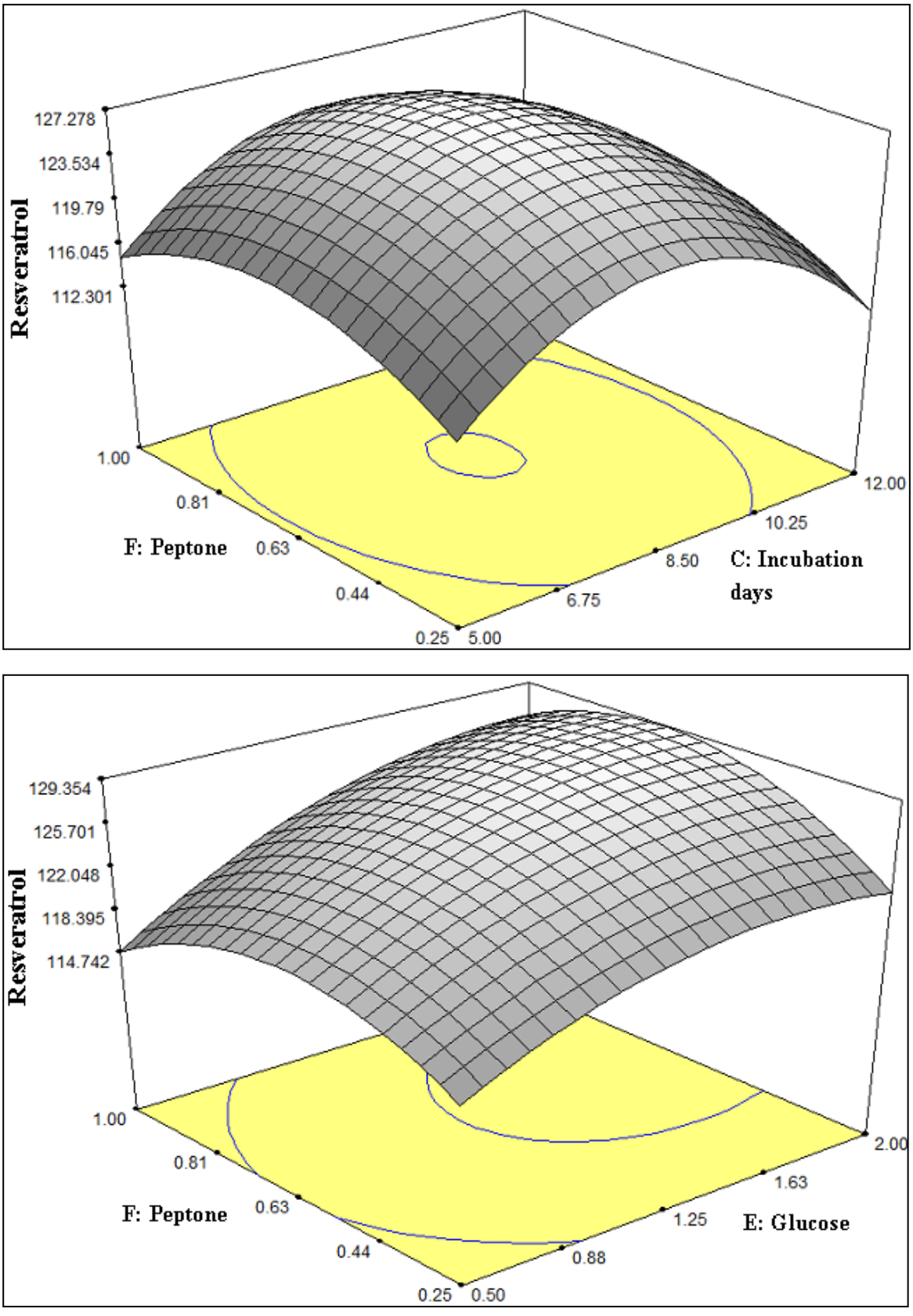


Figure 5.37. 3D surface and contour plots showing the effect of different variables on resveratrol production.

iv. Validation of Model

In order to test the accuracy of model whether the result predicted by the model is consistent with experimental results, 6 sets of experiments were performed with optimized fermentation conditions and the same 6 variables viz- RPM (A), Temp (B), Incubation days (C), pH (D), Glucose (E) and Peptone (F) which were optimized for RSM design. Table 5.27 presents the resveratrol production of each individual experiment along with predicted response. The results verify the previous model that glucose at 1.25%, peptone 0.63%, pH-7.0, incubation days-8.5, temperature-30 C and rpm- 115 as the best combination for obtaining the maximum resveratrol production. The maximum yield, 133.53 µg/mL was obtained experimentally, and this was closer to the predicted value of 133.59 µg/mL. The predicted results matched well with the experimental results obtained using optimal conditions, which validates the RSM models with good correlation.

Table 5.27. Validation of Box-Behnken design (BBD) using different levels of Physical and physiological parameter

Run	Coded levels						Response	
	RPM	Temperature	Incubation days	pH	Glucose	Peptone	Experimental Values (µg/mL)	Predicted Values (µg/mL)
1	110	29	8.96	6.95	1.57	0.71	132.98	133.61
2	111	29	8.81	7.06	1.66	0.70	132.87	133.52
3	136	28	8.90	6.61	0.89	0.51	133.49	134.66
4	136	29	9.31	6.94	0.91	0.50	133.51	134.18
5	120	29	9.12	6.85	1.56	0.68	131.97	133.79
6	115	30	8.50	7.00	1.25	0.63	133.53	133.59

Thus, it can be calculated that the process optimization for resveratrol production from the strain of *Arcopilus aureus* resulted with the final yield of **133.59 µg/mL** on 8.5th day in the medium containing 1.25% of glucose, 0.63% Peptone at neutral pH (7.0).The medium was inoculated and incubated at 30°C, 115 rpm for 8.5 days (Table 5.28).

Table 5.28. Summary of Optimized condition after "RSM"

Sr. NO	Parameters	Optimized Condition
1.	Growth Kinetics (Estimation of fungal biomass)	8.5th Day
4.	Estimation of optimum Agitation rate	115 rpm
5.	Estimation of optimum temperature Condition	30ºC
6.	Estimation of optimum pH Condition	7.0
7.	Estimation of optimum carbon source and their concentration and its concentration	Glucose – 1.25%
8.	Estimation of optimum nitrogen source and their concentration and its concentration	Peptone- 0.63%
9.	**Initial yield of Resveratrol**	**89.1 µg/mL**
10.	**Yield of resveratrol after "one-variable-at-a-time approach"**	**109.61µg/mL**
	Final Yield	**133.53 µg/mL**
11.	**Predicted yield**	**133.59 µg/mL**
12.	**Fold increase**	**1.49 fold**

5.12. Development of epigenetic variants

5.12.1. Sub- culturing of Arcopilus isolates on AZA and SAHA PDA plates

The fungal isolates were inoculated on PDA plates supplemented with different concentrations of AZA and SAHA. They produced different morphological characteristics over different concentrations. Changes in colony diameter of the subcultured #12VVLPM over PDA plates with different concentrations of AZA and SAHA were noted after the incubation of 8.5 days (Figure5.38, Table 5.29). It was observed that the diameter of the colony decreased after the concentration of 30µM of AZA and 50 µM SAHA due to the toxicity at higher concentrations (Figure 5.38).

5.12.2. Production and extraction of culture filtrates

It was found that the volume of spent broth reduced with the noticeable change in colour and characteristic smell of culture filtrates due to the production of secondary metabolites. The culture filtrates were filtered after 8.5 days of incubation using Whatman filter paper 4. The biomass of culture filtrates was also noted (Figure 5.39, Figure 5.40).

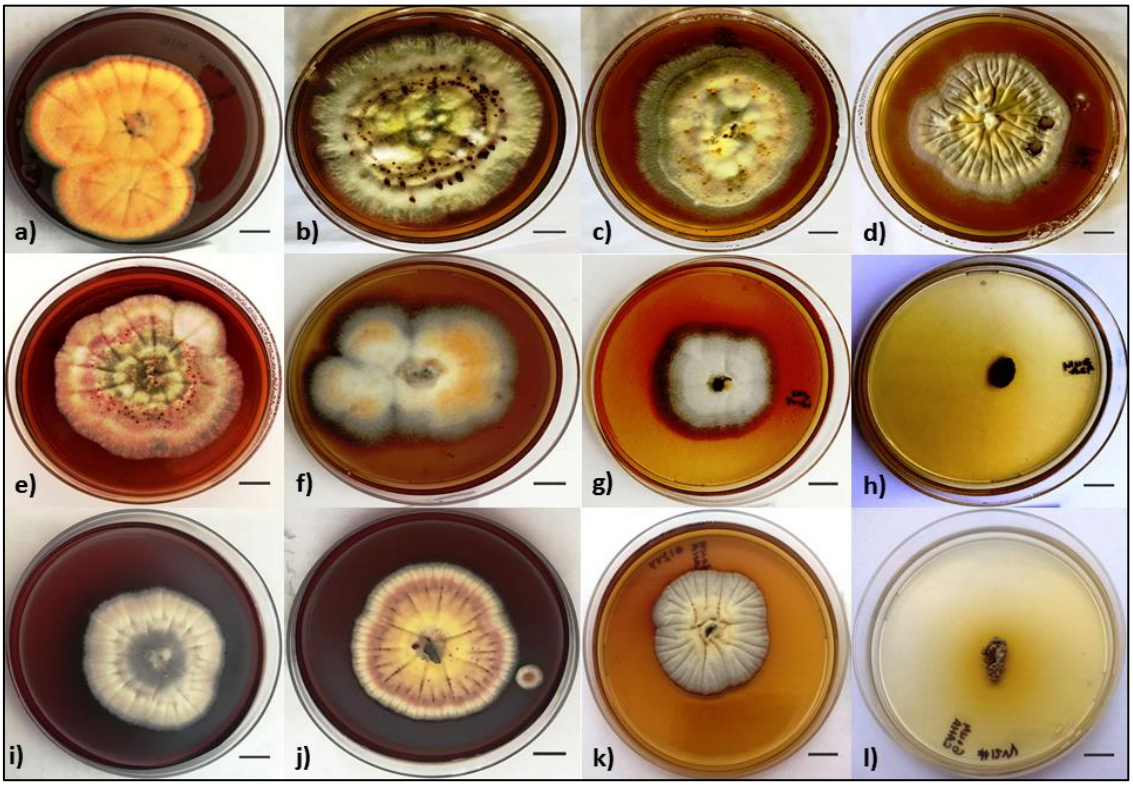


Figure 5.38. Epigenetic variants of *Arcopilus aureus*,(a) control, (b) AZA 10µm, (c) AZA5µm, (d) AZA 30µm, (e) AZA & SAHA (1:5µm), (f) AZA & SAHA (10:1µm),(g) AZA & SAHA (10:5µm), (h) AZA 90µm, (i) SAHA 10µm, (j) SAHA 5µm, (k) SAHA 30µm, (l) SAHA 90µm,(Bar: 10 mm)

Table 5.29. Morphological Characteristics of the epigenetic variants of *Arcopilus aureus* as compared to the wild #12VVLPM strain

Characteristics	Wild (#12VVLPM)	AZA (10 µm)	SAHA (5 µm)	AZA and SAHA (1:5 µm)
Diameter (mm)	70.67 ± 0.58	75.3± 0.58	82.3± 0.58	71.0± 1.00
Color (Fungi)	Peach pink with green spore at center	Reddish Pink	Reddish pink with green spore at center	Whitish pink with green spore
Color (Base)	Dark green	Red-wine color	Red-wine color	Reddish color
Pigment	Dark amber color	Amber color	Amber color	Dark brown color
Margin	Smooth margin	Cottony smooth margin	Cottony smooth margin	Cottony smooth margin
Odour	Fruity smell	Phenolic smell (Sweet and Terry)	Phenolic smell (Sweet and Terry)	Fruity smell

The results presented are a compilation of data from at least three cultures of each organism. Numerical values are means±SD

It was found that the weight of the biomass increased up to the concentration of 5µM and then decreased from 10µM as the concentration increases (Figure 5.34). It was also observed that there is a correlation between the colony diameter and the dry weight of biomass.

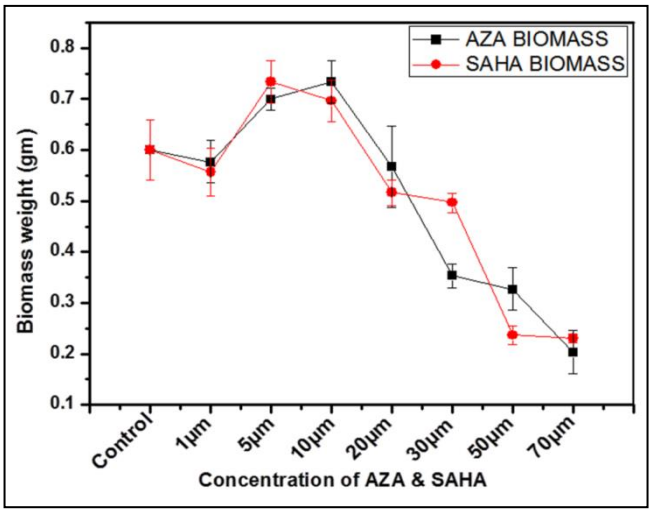


Figure 5.39. Weight of biomass of *Arcopilus aureus* on different concentration of AZA and SAHA.

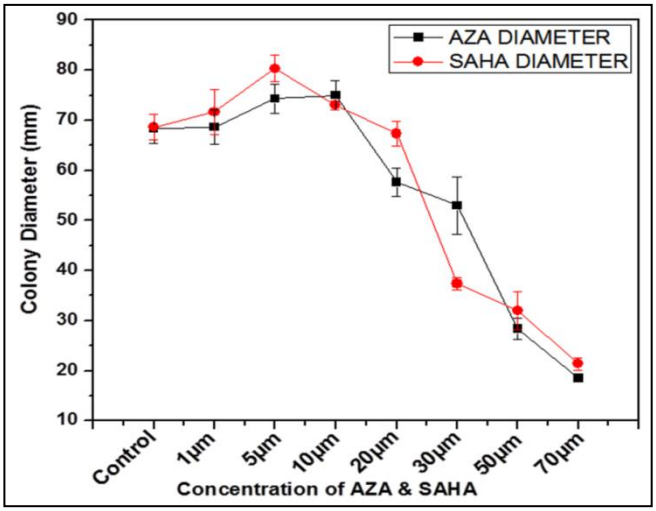


Figure 5.40. Colony diameter of *Arcopilus aureus* on different concentration of AZA and SAHA.

5.12.3. Estimation of resveratrol production through High-Performance Liquid Chromatography (HPLC)

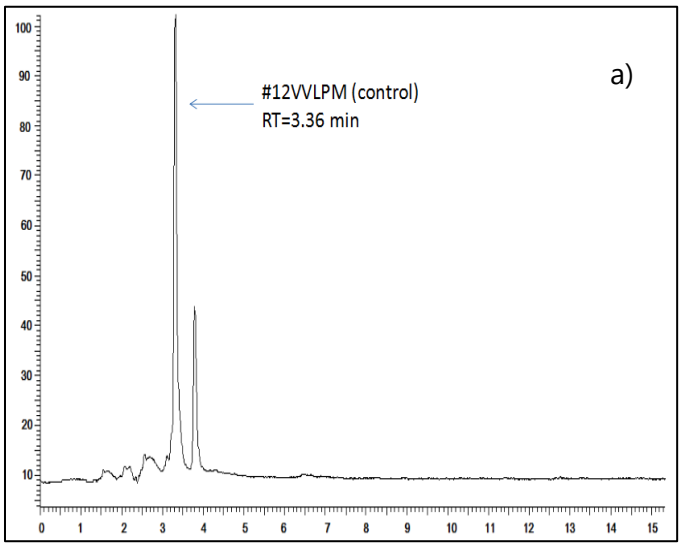
A symmetrical peak at RT 3.36 min on C18 reverse phase column like that of standard resveratrol was confirmed (Figure 5.41). The peak area versus resveratrol concentration, obtained in case of the standard resveratrol was used to estimate the quantity of fungal

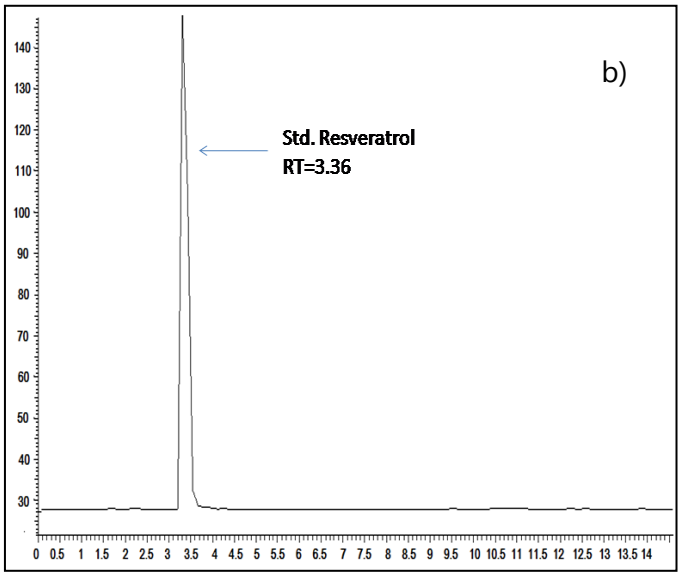
resveratrol. HPLC analysis also reveals the secretion of many cryptic compounds which were not seen in the untreated sample. Through HPLC it was observed that the resveratrol concentration was significantly enhanced in case of modification done using AZA and SAHA. Out of 66 epigenetic variants of #12VVLPM which were obtained after treatment with different concentrations of AZA and SAHA it was found that 10µM of AZA and 5µM SAHA exhibited highest resveratrol concentration of 153.18 µg/mL and 165.0 µg/mL, respectively as compared to control (89.1 µg/mL and 133.53 µg/mL (optimized using RSM) and on increasing the concentration of AZA and SAHA the decrease in resveratrol concentration was observed (Table 5.30, Table 5.31). The decrease in resveratrol concentration was observed after the treatment of AZA and SAHA at the concentration of 5 µM and 20 µM respectively (Table 5.30, Table 5.31). It was also observed that the concentration of resveratrol produced by the combinations of AZA and SAHA was less than the individual mutants (Table 5.32).

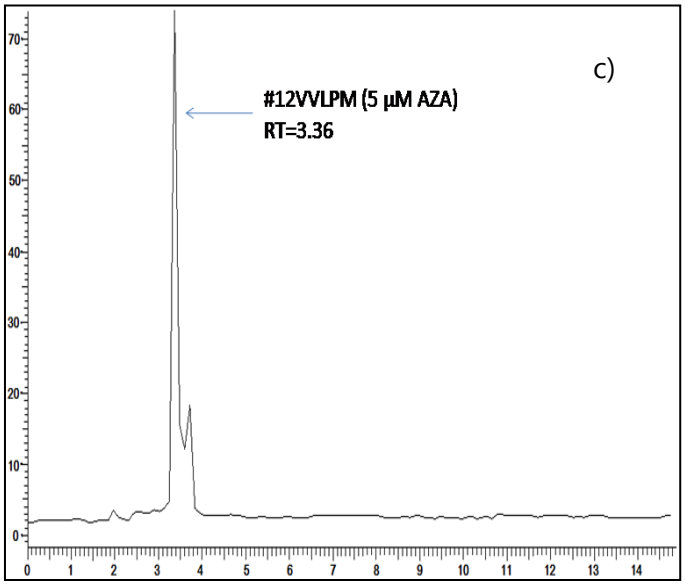
Table 5.30. The resveratrol concentration of AZA epigenetic modifiers of #12VVLPM

S.No.	AZA concentration	Biomass (g/L)*	Resveratrol yield (µg/mL)*	Colony diameter (mm)*
1.	CONTROL	119.08^{ab} ± 5.72	89.10^{g} ± 0.08	70.67^{a} ± 2.50
2.	1µM	120.16^{ab} ± 5.78	124.63^{e} ± 0.91	72.67^{a} ± 1.25
3.	5µM	124.44^{ab} ± 1.85	143.19^{c} ± 1.10	74.00^{a} ± 0.82
4.	**10µM**	**131.71^{a} ± 2.40**	**153.18^{a} ± 0.93**	**75.33^{a} ± 2.05**
5.	20µM	125.33^{a} ± 2.68	147.92^{b} ± 0.73	75.00^{a} ± 0.82
6.	30µM	110.63^{b} ± 1.17	132.66^{d} ± 0.49	70.67^{a} ± 2.05
7.	50µM	89.08^{c} ± 2.59	97.01^{f} ± 2.16	43.00^{b} ± 1.63
8.	70µM	49.53^{d} ± 2.63	68.82^{h} ± 0.97	25.67^{c} ± 2.87

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)


a)
#12VVLPM (control)
RT=3.36 min


b)
Std. Resveratrol
RT=3.36


c)
#12VVLPM (5 µM AZA)
RT=3.36

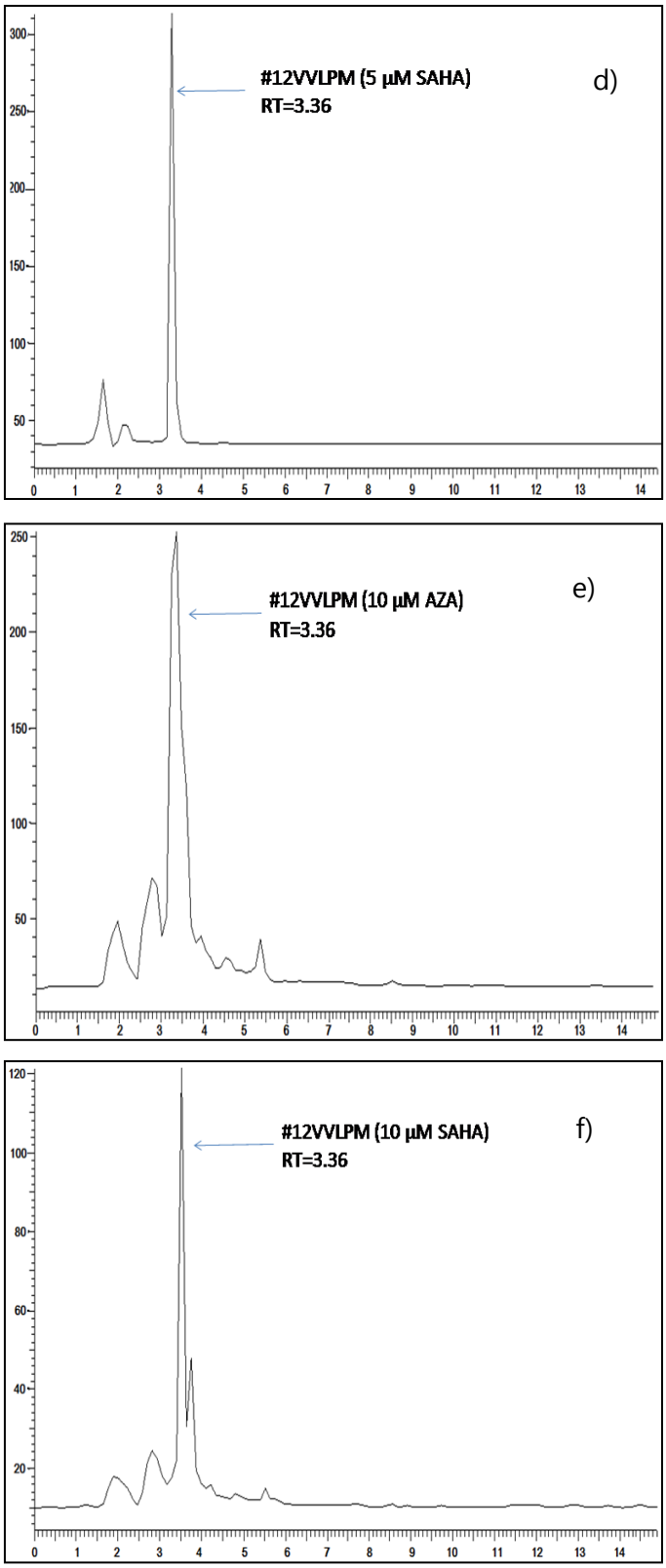


Figure 5.41. HPLC spectra of (a) #12VVLPM (control), (b) Standard resveratrol, (c) #12VVLPM + treatment with 5 µM AZA, (d) #12VVLPM + treatment with 5 µM SAHA, (e) #12VVLPM + treatment with 10 µM AZA, (f) #12VVLPM + treatment with 10 µM SAHA

Table 5.31. The resveratrol concentration of SAHA epigenetic modifiers of #12VVLPM

S.No.	SAHA concentration	Biomass (g/L)*	Resveratrol yield (µg/mL)*	Colony diameter (mm)*
1.	CONTROL	$119.08^{bc} \pm 5.72$	$89.10^{f} \pm 0.08$	$70.67^{ab} \pm 2.49$
2.	1µM	$129.41^{ab} \pm 1.98$	$156.51^{b} \pm 0.64$	$73.00^{ab} \pm 1.63$
3.	**5µM**	**$138.75^{a} \pm 2.18$**	**$165.55^{a} \pm 0.89$**	**$78.00^{a} \pm 0.82$**
4.	10µM	$130.74^{ab} \pm 2.01$	$155.17^{b} \pm 1.32$	$73.33^{ab} \pm 1.25$
5.	20µM	$116.40^{bc} \pm 3.16$	$142.34^{c} \pm 1.13$	$72.00^{ab} \pm 2.16$
6.	30µM	$107.69^{cd} \pm 5.36$	$133.67^{d} \pm 1.15$	$65.67^{b} \pm 2.05$
7.	50µM	$94.43^{d} \pm 2.50$	$100.41^{e} \pm 1.07$	$51.67^{c} \pm 2.49$
8.	70µM	$64.99^{e} \pm 2.72$	$68.72^{g} \pm 0.45$	$24.67^{d} \pm 2.87$

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

Table 5.32. The resveratrol concentration of epigenetic modifiers of #12VVLPM using possible combination of AZA and SAHA

Expt. No.	Combinations of AZA and SAHA	Biomass (g/L)*	Resveratrol yield (µg/mL)*	Colony diameter (mm)*
1.	AZA 1µM + SAHA 1µM	$101.77^{fghij} \pm 0.86$	$132.3^{ef} \pm 0.46$	$66.67^{bc} \pm 1.25$
2.	AZA 5µM + SAHA 1µM	$116.22^{cd} \pm 3.26$	$145.9^{c} \pm 0.66$	$70.33^{ab} \pm 1.70$
3.	AZA 10µM + SAHA 1µM	$120.49^{abc} \pm 0.87$	$149.1^{b} \pm 0.39$	$75.33^{a} \pm 1.25$
4.	AZA 20µM + SAHA 1µM	$109.18^{def} \pm 1.71$	$139.9^{d} \pm 0.71$	$69^{abc} \pm 1.63$
5.	AZA 30µM + SAHA 1µM	$99.56^{hij} \pm 0.5$	$109.9^{i} \pm 0.32$	$66^{bc} \pm 0.82$
6.	AZA 50µM + SAHA 1µM	$94.31^{jkl} \pm 2.07$	$87.5^{mn} \pm 0.71$	$62.67^{cde} \pm 1.25$
7.	AZA 70µM + SAHA 1µM	$75.21^{opqr} \pm 1.20$	$51.68^{tu} \pm 0.72$	$58.67^{def} \pm 1.70$
8.	AZA 1µM + SAHA 5µM	$125.89^{a} \pm 1.20$	$152.7^{a} \pm 0.73$	$76^{a} \pm 1.63$
9.	AZA 5µM + SAHA 5µM	$124.36^{ab} \pm 5.33$	$129.3^{f} \pm 0.60$	$72.67^{ab} \pm 1.25$
10.	AZA 10µM + SAHA 5µM	$116.1^{cd} \pm 1.68$	$122.6^{gh} \pm 0.93$	$69^{abc} \pm 0.82$
11.	AZA 20µM + SAHA 5µM	$106.83^{efgh} \pm 4.86$	$112.5^{i} \pm 0.73$	$70.33^{ab} \pm 1.70$
12.	AZA 30µM + SAHA 5µM	$96.71^{ijkl} \pm 1.72$	$91.81^{l} \pm 0.50$	$63^{cde} \pm 1.63$
13.	AZA 50µM + SAHA 5µM	$79.99^{nop} \pm 0.93$	$66.91^{qr} \pm 0.37$	$36.67^{kl} \pm 2.05$
14.	AZA 70µM + SAHA 5µM	$63.01^{tu} \pm 1.34$	$44.10^{w} \pm 0.65$	$31^{lmno} \pm 1.63$
15.	AZA 1µM + SAHA 10µM	$117.54^{bc} \pm 2.46$	$144.2^{c} \pm 0.37$	$72.67^{ab} \pm 1.25$
16.	AZA 5µM + SAHA 10µM	$108.51^{defg} \pm 2.60$	$119.9^{h} \pm 0.76$	$72.67^{ab} \pm 2.05$
17.	AZA 10µM + SAHA 10µM	$101.77^{fghij} \pm 0.77$	$106.0^{j} \pm 0.22$	$66^{bc} \pm 2.16$
18.	AZA 20µM + SAHA 10µM	$94.83^{jkl} \pm 1.44$	$90.62^{lm} \pm 1.02$	$63^{cde} \pm 1.63$
19.	AZA 30µM + SAHA 10µM	$90.55^{klm} \pm 0.66$	$80.7^{o} \pm 0.92$	$55.33^{fg} \pm 2.49$
20.	AZA 50µM + SAHA 10µM	$72.52^{pqrs} \pm 1.67$	$64.85^{qr} \pm 0.83$	$52.33^{fgh} \pm 1.25$
21.	AZA 70µM + SAHA 10µM	$63.75^{tu} \pm 2.66$	$39.93^{x} \pm 0.74$	$34.67^{lm} \pm 1.25$
22.	AZA 1µM + SAHA 20µM	$104.59^{fghi} \pm 1.60$	$134.7^{e} \pm 0.60$	$69^{abc} \pm 1.63$
23.	AZA 5µM + SAHA 20µM	$113.19^{cde} \pm 2.66$	$112.6^{i} \pm 0.65$	$70.67^{ab} \pm 2.05$
24.	AZA 10µM + SAHA 20µM	$90.87^{klm} \pm 0.95$	$99^{k} \pm 0.76$	$56.33^{efg} \pm 2.49$
25.	AZA 20µM + SAHA 20µM	$98.21^{ijk} \pm 1.18$	$84.99^{n} \pm 0.72$	$67.33^{bc} \pm 1.70$
26.	AZA 30µM + SAHA 20µM	$89.97^{lm} \pm 1.21$	$75.48^{p} \pm 0.76$	$57^{efg} \pm 1.63$
27.	AZA 50µM + SAHA 20µM	$68.89^{qrst} \pm 1.23$	$54.25^{t} \pm 0.48$	$38^{kl} \pm 0.82$
28.	AZA 70µM + SAHA 20µM	$54.81^{vw} \pm 1.82$	$36.43^{yz} \pm 0.63$	$29.33^{mnop} \pm 1.70$
29.	AZA 1µM + SAHA 30µM	$116.02^{cd} \pm 2.75$	$123.5^{g} \pm 0.83$	$70.33^{ab} \pm 2.05$
30.	AZA 5µM + SAHA 30µM	$90.52^{klm} \pm 1.41$	$98.55^{k} \pm 0.56$	$53.67^{fg} \pm 2.05$

31.	AZA 10µM + SAHA 30µM	$85.55^{mn} \pm 1.77$	$79.76^{o} \pm 0.91$	$50^{ghi} \pm 0.82$
32.	AZA 20µM + SAHA 30µM	$81.15^{no} \pm 1.01$	$74.32^{p} \pm 0.47$	$46.33^{hij} \pm 1.70$
33.	AZA 30µM + SAHA 30µM	$76.51^{opq} \pm 1.76$	$63.86^{r} \pm 1.09$	$43^{ijk} \pm 1.63$
34.	AZA 50µM + SAHA 30µM	$67.12^{rst} \pm 2.97$	$47.37^{v} \pm 1.1$	$36^{klm} \pm 1.63$
35.	AZA 70µM + SAHA 30µM	$44.03^{xy} \pm 2.31$	$30.98^{A} \pm 0.72$	$22.67^{pqr} \pm 1.25$
36.	AZA 1µM + SAHA 50µM	$100.56^{ghij} \pm 1.37$	$88.01^{mn} \pm 0.73$	$65.67^{bcd} \pm 2.49$
37.	AZA 5µM + SAHA 50µM	$76.52^{opq} \pm 0.86$	$67.42^{q} \pm 0.93$	$42.33^{jk} \pm 2.05$
38.	AZA 10µM + SAHA 50µM	$70.85^{qrst} \pm 0.86$	$60.63^{s} \pm 1.11$	$34.33^{lm} \pm 2.49$
39.	AZA 20µM + SAHA 50µM	$69.99^{qrst} \pm 1.54$	$49.01^{uv} \pm 0.42$	$33^{lmn} \pm 1.63$
40.	AZA 30µM + SAHA 50µM	$54.81^{vw} \pm 1.27$	$39.39^{xy} \pm 0.57$	$26.67^{nop} \pm 2.05$
41.	AZA 50µM + SAHA 50µM	$47.77^{wx} \pm 1.85$	$25.91^{BC} \pm 0.50$	$24^{opq} \pm 0.82$
42.	AZA 70µM + SAHA 50µM	$37.88^{y} \pm 1.76$	$16.73^{D} \pm 0.74$	$23^{pqr} \pm 1.63$
43.	AZA 1µM + SAHA 70µM	$64.56^{stu} \pm 1.04$	$49.58^{uv} \pm 1.1$	$32.33^{lmn} \pm 1.70$
44.	AZA 5µM + SAHA 70µM	$57.7^{uv} \pm 0.96$	$39.2^{xy} \pm 0.86$	$27^{nop} \pm 0.82$
45.	AZA 10µM + SAHA 70µM	$51.11^{vwx} \pm 0.97$	$37.43^{xy} \pm 0.95$	$27^{nop} \pm 1.63$
46.	AZA 20µM + SAHA 70µM	$48.66^{wx} \pm 0.82$	$34.18^{z} \pm 0.55$	$23.33^{pq} \pm 1.70$
47.	AZA 30µM + SAHA 70µM	$47.69^{wx} \pm 0.97$	$28.88^{AB} \pm 0.83$	$24^{opq} \pm 1.63$
48.	AZA 50µM + SAHA 70µM	$21.49^{z} \pm 0.65$	$23.4^{C} \pm 1.05$	$19.33^{qr} \pm 1.25$
49.	AZA 70µM + SAHA 70µM	$15.74^{z} \pm 0.91$	$9.33^{E} \pm 0.39$	$16^{r} \pm 0.82$

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p<0.05$)

5.12.4. Stability studies

To determine the stability of the epigenetic mutants AZA 10µM and SAHA 5µM the mutated culture was subculture up to 5 generations (Table 5.33). The concentration of resveratrol was further analysed by using HPLC. It was observed that even after five generations the concentration of resveratrol produced by the mutant AZA 10µM and SAHA 5µM did not decreased.

Table 5.33. The stability study of production of resveratrol by the epigenetic mutant 5 µM AZA and 10 µM SAHA

	Number of culture generation					**Resveratrol conc.* (µg/mL)**
	1	**2**	**3**	**4**	**5**	
5µM AZA[RSEV (µg/mL)]	165.55	165.98	164.34	166.28	165.79	165.59± 0.67
10µM SAHA[RSEV (µg/mL)]	154.07	155.01	156.09	154.97	155.88	155.20± 0.72

*data is represented as mean ± SD

After treatment of *Arcopilus aureus* with 5 µM SAHA, resulted in enhancement in yield of resveratrol viz. 165.55 µg/mL (1.86 fold increases from the wild strain and 1.49 fold increases in resveratrol concentration obtained after the optimized conditions by RSM). 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.

Chapter- 6

Discussion

6.0. DISCUSSION

Resveratrol a tri-hydroxy stilbene derivative ($C_{14}H_{12}O_3$) has been identified as a phytoalexin, produced by plants de novo to protect him against infections caused by pathogenic microbe's especially *B. cinerea*. Trans-RSEV has been associated to multifarious applications like cardioprotective activity, anti-cancer, anti-inflammatory, diabetes, anti-aging and anti-oxidant potential (Saxena and Srivastava 2014; Turrini et al., 2018; Moes et al., 2018; Chien et al., 2018; Dwibedi and Saxena, 2019). Interest in resveratrol, a stilbenoid often garnered as the important polyphenol in red grape wine as well as *Polygonum* exploded in year 2003 when Hubbard (Hubbard and Sinclair, 2014) has found that *trans*-resveratrol has ability to increase the shelf lifespan of *Saccharomyces cerevisiae*. According to Sinclair finding resveratrol could activate a gene known as *Sirtuin 1* which is also activated during calorie restriction in various species, including monkeys. Resveratrol for nutritional and wellness supplements is predominantly resourced from the plant *Polygonum cuspidatum* (syn. *Fallopia japonica*) root extracts (Chien et al., 2018; Dwibedi and Saxena, 2019). The leading producer of trans-resveratrol from root extracts is China using an extraction process. The level of trans-resveratrol purity in these extracts varies between 49%-99% (Zhang et al., 2006; Fan et al., 2010). For the same reason researchers are seeking for an alternate source for obtaining resveratrol, which can in-turn protect the limited and endangered resources which will minimize the cost of this billion-dollar drug. Exploration of micro-organisms capable to produce trans-resveratrol is comparatively latest, after all it was known as medicinal molecule with various health benefits (Saxena and Srivastava, 2014; Turrini et al., 2018; Moes et al., 2018). There is a very limited data on production of resveratrol from endophytic fungi therefore exploring endophytic fungi for this purpose is a new area. Powell (1994) was the first person to isolate resveratrol from endophytic genus *Acremonium* sp., further endophytic fungi including *Alternaria* sp., *Aspergillus niger*, *Penicillium* sp. and *Botryosphaeria* sp. are amongst the recently reported producers of resveratrol (Shi et al., 2012; Wang et al., 2013; Liu et al., 2016).

Endophytic fungi since last twenty years have gain impressive perception in field of natural drug discovery as producers as well as eco-friendly and cost-effective source of exclusive and disparate bio-active drug molecule which can be directly or indirectly used as drug candidates

for combating major worldwide diseases (Strobel et al., 2003; Zhang et al., 2006). Endophytic fungi generally coexist inside plant internal tissues and provide a beneficial role in promoting host growth and resistance to different physical and physiological stresses (Bacon and White, 2016). Market value of natural product drug is estimated to account for 17% of the top 100 best-selling drugs all over the world and approx. value of US$29 billion. It has been well documented that endophytic fungi co-evolve with the anchor plant thereby attaining the host biosynthetic capabilities thereby producing phytomedicinals which are commercially used such as paclitaxel (Ismaiel et al., 2017), vinblastine (Palemet al., 2015), camptothecin (Kusari et al., 2008; Ran et al., 2017), podophyllotoxin (Liang et al., 2015), huperzine A (Wang et al., 2011) and resveratrol (Shi et al., 2012; Dwibedi et al., 2018; Dwibedi et al., 2019). Fungal endophytes produce a variety of compounds of different classes such as alkaloids, polyphenols, terpenoids, quinones, steroids etc. (Strobel et al., 2003; Zhang et al., 2006; Kusari et al., 2008; Ismaiel et al., 2017).

Thus, the present study was oriented towards exploring culturable endophytic fungi associated with different *Vitis vinifera* varieties from different locations in India, for resveratrol production. Now days scientist give preference to culture independent methods like next generation sequencing (NGS) approach over culture dependent methods to isolate the true endophytic diversity (Tedersoo et al., 2010; Unterseher et al., 2013; Singh et al., 2017). However, these methods are not error proof and have been criticised for overvaluation (Tejesvi et al., 2011; Unterseher et al., 2013; Singh et al., 2017). Thoughtful ambiguity arises when dominant microbial species or community present in culture dependent isolation and absent or differ in culture independent methods like NGS (Setati et al., 2015; Felber et al., 2016; Singh et al., 2017). Many studies performed on comparing the traditional culture dependent isolation-based diversity and NGS based diversity of the same host plant and revealed that culture dependent based methods alone can reveal the accurate picture of microbial endophytic diversity (Unterseher et al., 2013; Setati et al., 2015; Esmaeel et al., 2019). Thus, culture dependent methods is only method to get culture isolate for future invitro lab use and to upgrade reference taxonomic database (Setati et al., 2015; Felber et al., 2016; Singh et al., 2017).

Hence in this study we undertook studies on the culturable diversity of fungal endophytes of different varieties of *Vitis vinifera*.

The different *Vitis vinifera* varieties comprised of Merlot, Pinot noir, Wild, Shiraz and Muscat from four different geographical locations examined in this study to explore their endophytic colonization. Collectively, 145 isolates were obtained belonging to eighteen different fungal genera from 525 segments. Gonzalez et al., (2011) presented isolation of endophytic fungus from six different locations in Spain and Brum et al., (2012) isolated 550 fungal endophytes from grape sp. It was analysed that different locations have different type of fungal endophytes. One interesting observation also found that the make-up of fungal endophytes and the isolation rate varied according to anchor plant, its characteristics viz. plant life, elevation, types of plant internal tissue, vulnerability (Bills and Polishook, 1994; Rodriguez et al., 2009; Christian et al., 2016; David et al., 2016) and age of tissue (Arnold et al., 2003; Arnold et al., 2007; Vermaet al., 2007; Hardoim et al., 2015).

The fungal endophytes associated with grape plant tissue in this study are dominated by hyphomycetes, which represented about 58.8 % of the endophytes isolated. Whereas, in earlier study the mycota isolated from various grape cultivars from the Madrid location of central Spain was dominated with ascomycetes (González et al., 2011). Colony frequency of Basidiomycota was very less in present study which is correlates with other studies (Márquez et al., 2010; González et al., 2011; Kernaghan et al., 2017; Dwibedi et al., 2019; Hall et al., 2019). In the present study the higher frequency of isolates observed were belonging to genera *Aspergillus*, *Fusarium*, *Alternaria* and *Botryosphaeria*, and these findings were accordance with the work of Varanda et al., (2016), Felber et al., (2016) who have also reported similar results.The dissimilarity in endophytes isolated from analogues plant from different locations is associate commonly to the retrieval of accidental and unique species with more detachment circulation, otherwise approximately a continual dimension of mycota are recovered from all samples (Bills et al., 1994; Christian et al., 2016; Ding and Melcher, 2016; Glynou et al., 2016; Griffin et al., 2019). The occurrence of the rare and incidental species, which were represented occasionally, was also significantly observed in our study. *Arcopilus* sp., *Quambalaria* sp. and *Xylaria* sp. isolated from *V. vinifera* were recovered as an incidental species has not been reported earlier. The occurrence

of incidental and rare species is said to be proportional to the site and the intensity of sampling, besides host itself (Mostert et al., 2000; Chagnon et al., 2016; Jin et al., 2017).

The biochemical screening of resveratrol (traditional method) is by phytochemical tests which basically detect the polyphenolic compounds (Shi et al., 2012; Liu et al., 2016; Wang et al., 2016). Scientists also used HPLC, LC-MS, UPLC and centrifugal force LC-MS (CFLC-MS) for screening of resveratrol production (Shi et al., 2012; Liu et al., 2016; Wang et al., 2016).

In the present study total of 145 isolates, 43 (29.66%) exhibited positive result in the preliminary screening. HPLC investigation performed a crucial role in recognition of potent bioactive compounds from endophytic fungi. The main keystones for analysis of resveratrol in the solvent extract have been HPLC which has also been performed in previous research (Shi et al., 2012; Liu et al., 2016; Wang et al., 2016). Of these 14 (9.66%) displayed maximum activity and hence these isolates were subjected for confirmatory analysis using HPLC. All the isolates under analysis were resveratrol producers which was confirmed by the HPLC chromatogram obtained. The potent resveratrol endophytes in the study (9.66%) were comparatively low than that found by Liu et al. (2016) and Shi et al. (2012) which were 19.44% and 32.3%, respectively. The principle foundation for analysis of resveratrol in the solvent extracts has been HPLC which has also been used in previous studies (Shi et al., 2012; Wang et al., 2013; Liu et al., 2016). Of these 14 isolates we selected four potent resveratrol producers viz. #12VVLPM, #19VVLPM, #22(P)VVLPM and #4(P)VVLNM which were identified as *Arcopilus aureus*, *Fusarium* sp., *Xylaria* sp. and *Fusarium* sp. respectively, using morphotaxomony. Further, for molecular phylogeny we focussed on these four isolates to reconfirm their identification (Dwibedi and Saxena, 2018; 2019). After that, for species level identification of endophytic fungus, ITS based molecular taxonomy was used worldwide which correlated with the detection of microscopy. 5.8 sRNA based molecular identification is the worldwide accepted technique for species level identification of the any of fungus (Saitou et al., 1987; White et al., 1990; Tamura et al., 2013). Previously reported resveratrol producing endophytic fungus *Alternaria* sp. MG 1 and *Aspergillus niger* also has been identified using ITS based molecular identification (Brum et al., 2012; Shi et al., 2012).

Resveratrol is synthesized via the Shikimate pathway, where stilbene synthase (*STS*) catalyzes the formation of simple monomeric stilbene (resveratrol) (Shi et al., 2012; Dwibedi et al., 2019). In

the present study molecular marker based (PCR) screening approach for the top four resveratrol producing endophytic fungi using *STS* specific primers has also been performed (Dwibedi and Saxena, 2019), and for this we chose end step gene i.e. *STS* gene which is vital gene involved in resveratrol biosynthesis in *Vitis vinifera* (Hasan and Bae, 2017; Zheng et al., 2017).

The resveratrol productions by these isolates were stable over several passages. The activity of these fungal endophytes found same after serval passaging and did not drop down as analysed in Shi et al, 2012 in *Alternaria* MG1 and Liu et al, 2016 in *Botryosphaeria* sp. YG3. But in case of 4 cultures viz. #83VVLNM, #6(b)VVLLK, #8VVGSTL, #20(b)VVLPM production of resveratrol drop down very fast on passaging (Dwibedi and Saxena, 2018; 19). The drop down in production of resveratrol some endophytic fungus could possibly be due to irregular behavior of STS gene expression which responsible for resveratrol biosynthesis in plants and fungi. Study on different crucial gene biosynthesis which is responsible for production of this bioactive compound may provide crucial scientific data which leads to exact mechanism behind the loss of production of this bioactive compound (Shi et al., 2012; Liu et al., 2016; Lu et al., 2016; Liu et al., 2017; Dwibedi and Saxena, 2018). By Qualitative as well as Quantitative estimation we found that isolates the isolates#12VVLPM, #19VVLPM, #22(P)VVLPM and #4(P)VVLNM were found to be the top resveratrol producers hence these were used for further application studies.

Since ancient times, people are using natural products as a source of medicine to alleviate and treat different diseases (Yuan et al., 2016). Researchers are still trying to investigate the mechanism of action of stilbenes relating to the free radical scavenging effect. However, conflicting results have been obtained concerning their *in vitro* antioxidant and free radical scavenging assays (Berman et al., 2017; Simioni et al., 2018). Resveratrol is a nutraceutical compound which is gaining much research attention due to its multifarious application and has been widely researched at the level of preclinical studies (Berman et al., 2017; Dwibedi and Saxena, 2018; Simioni et al., 2018). In our study exhibited a strong antioxidant activity in different anti-oxidant assays such as DPPH (IC_{50} of 0.11- 4.96 mg/mL), TEAC (IC_{50} of 0.28- 2.16 mg/mL), Metal ion chelating (IC_{50} of 0.12 – 1.33 mg/mL), Nitric oxide radical (IC_{50} of 0.08- 1.35 mg/mL) and Hydrogen peroxide (IC_{50} of 0.12- 1.18 mg/mL). The two resveratrol derivatives produced by endophytic *Alternaria* sp. also displayed moderate antioxidant activity by DPPH

scavenging (Wang et al., 2014). Khanduja and Bhardwaj (2003) in their study found that resveratrol showed stronger antioxidant potential than catechin, myricetin, and fisetin. Shrikanta et al., (2015) also observed antioxidant property which was attributed to the presence of resveratrol in grapes, and jamun. Apart from DPPH scavenging potential, resveratrol also exhibited hydrogen peroxide and superoxide anion radical scavenging activities (Gülçin et al., 2010). Zhou et al., (2018) performed the DPPH and ABTES scavenging assay of all fungal extract using four different media and found that more than 80% of isolates were showing good scavenging activity.

DPPH method is traditionally used for analyzing the preliminary free radical scavenging potential of microbes and plant extract and it also corroborates to the inhibition of lipid peroxidation. Antioxidant molecule acts as an electron donor to DPPH, by which it neutralizes its free radical character (Shrikanta et al., 2015; Brasanac-Vukanovic et al., 2018). Plant and endophytic fungi act as an electron donor due to the presence of phenolic compounds in them. This is also a justification due to which endophytic fungi exhibit the potential to scavenge DPPH free radical scavenging property. The DPPH scavenging has been widely used as a, fast, reliable as well as a reproducible parameter to measure the *in vitro* antioxidant capacity of any biological compound (Brasanac-Vukanovic et al., 2018; Zhou et al., 2018). Reducing potential of any biological compound could serve as an indicator of potential antioxidant property. The two resveratrol derivatives produced by endophytic *Alternaria* sp. also displayed moderate antioxidant activity by DPPH scavenging (Boue et al., 2013; Oh et al., 2017; Tang et al 2018; Zhou et al., 2018).

ABTES antioxidant assay is a well-known fast screening method for determination of antioxidant potential of any compound and it may be a useful tool for high throughput screening of foods for natural antioxidants. The decolonization of ABTES ions is measured by the reduction of free radical cation which is interpreted as percentage inhibition at absorbance of 734 nm (visible range) (Tang et al., 2018; Zhou et al., 2018). In the presence of particular chemical bonds in the endophytic fungal extract it inhibits the activity of potassium persulfate by which it inhibits the production of ABTES ions. The fungal resveratrol in our study exhibited highest scavenging was found in fungal extract (ethyl acetate fraction) of #12VVLPM with IC50

of 0.28 ± 0.02 mg/mL as compared to resveratrol used as a standard which exhibited IC50 of 0.16 ± 0.03 mg/mL, while the least scavenging was recorded in fungal extract of #4(P) VVLNM with IC50 of 2.16 ± 0.02 mg/mL. Zhou et al., (2018) performed the DPPH and ABTS scavenging assay of all fungal extract using four different media and found that more than 80% of isolates are showing good scavenging activity.

Metal ions play an important biochemical and physiological role in living systems. They help in transportation of oxygen, apart from being cofactors of many enzymes to optimize their catalytic activity. Metals also participate in catalysis reaction and results in oxidative change in lipids, proteins as well as many other cellular bio components. The metal ion chelating potential of endophytic fungal extract was measured by the development of ferrous ion ferrozine complex. Ferrozine binds with metal ions and produce a red color complex which is detected at absorbance at 562 nm (Thaipong et al., 2006; Dani et al., 2008; Ahmadi et al., 2018). Our study exhibited highest scavenging in the fungal extract of #12VVLPM with IC_{50} of 0.12 ± 0.03 mg/mL as compared to resveratrol which exhibited IC_{50} of 0.33 ± 0.02 mg/mL which was used as a standard. The least scavenging was obtained in the fungal extract of #22(P)VVLPM with IC_{50} of 1.35 ± 0.05.

In nitric oxide radical scavenging activity nitric oxide produced from sodium nitroprusside reacts with free oxygen from nitrite ion, which is inhibited by the antioxidant molecules. Sulfanilamide is converted to a diazonium salt by competing with nitrite in acidic medium. This diazonium salt reacts with NED (N-1- naphthyl-ethylenediamine), resulting in formation of an azo dye that can be measured quantitatively in visible range using a UV-Vis spectrophotometer (Medina et al., 2010; Shrikanta et al., 2015; Brasanac-Vukanovic et al., 2018; Fia et al 2018). The fungal resveratrol in our study exhibited highest scavenging in #12VVLPM with IC_{50} of 0.08 ± 0.03 mg/mL as compared to resveratrol which was used as standard with IC_{50} of 0.37 ± 0.02 mg/mL and the least scavenging was exhibited by the fungal extract of #4(P)VVLNM with IC_{50} of 1.35 ± 0.01 mg/mL.

The inhibition potential of the cell free culture extract from resveratrol producing endophytic fungi on the oxidative cell damage produced by hydrogen peroxide and hydroxyl ion with the help of Fenton reagent was assayed through peroxidase assay. In biological systems many

metabolic end molecule of lipid peroxidation like MDA is an excellent authority of cell membrane, abolition and damage of various cell (Chen et al., 2015; Lançon et al., 2016; Zhang et al., 2019). In present assay scavenging was highest in fungal extract of #12VVLPM with IC_{50} of 0.12 ± 0.07 mg/ml followed by #19VVLPM with IC_{50} of 0.26 ± 0.04 mg/mL as compared to resveratrol which was used as the standard with IC_{50} of 0.38 ± 0.01 mg/mL and the least scavenging was exhibited by #4(P)VVLNM with IC_{50} of 1.18 ± 0.04 mg/mL.

In this study, it was found that fungal extracts of resveratrol producing endophytic fungi exhibited good anti-oxidant activity and thus prevented the DNA damage. Based on result we can conclude that resveratrol producing isolates from *Vitis vinifera* have ability as a preservative molecule against various oxidative stresses caused by de novo. Our analysis also match with various research which demonstrates that polyphenols and stilbenes can protect biological from oxidative stresses or enhance the resistance to damages create by free radicals (Chen et al., 2015; Lançon et al., 2016; Zhang et al., 2019).

The emerging and re-emerging appearance of fungal disease encountered in the course of allogenic bone marrow transplantation, organ transplantation, and cancer therapy demands the screening of novel natural dietary component as an antifungal compound with better efficacy and elevates compatibility. There are defined numbers of naturally isolated antifungal compounds in practice against various fungal infections. Considering the massive hidden potential of natural products from endophytes as an alternative source of novel antifungal drug molecule for different applications remains a largely under explored area. Therefore, in this experiment, all the resveratrol producing endophytes were screened for their antagonistic potential against a battery of plant pathogenic fungi (Paulo et al., 2010; Houille et al., 2014; Chen et al., 2019; Singh et al., 2019; Jia et al., 2019; Martínez et al., 2019).

Further, in present study *in vitro* antifungal activity was conducted by co-cultivation against common pathogen of fruits and vegetables viz. *B. cinerea* (MTCC 359), *Colletotrichum gloeosporioides* (MTCC 9623), *Cercospora beticola* (ATCC 24888) and *Rhizoctonia solani* (MTCC 4634). The maximum inhibition was exhibited by #12VVLPM against *B. cinerea* (57.76 ± 0.82%). Houille et al., (2014) demonstrated that dimethoxy resveratrol derivatives exhibited antifungal activity against *C. albicans*. The inhibitory effect of different compounds of resveratrol on conidia

germination was found to be about 70%. Paulo et al., (2010) demonstrated that resveratrol possessed antimicrobial properties.

Multidrug resistant (MDR) microbes are becoming more and more prominent in both developed as well as underdeveloped countries due to their refractory behavior and are implicated in chronic infections. One of the deadliest bacteria which have seen a geometric rise and global spread is MRSA (Methicillin resistant *Staphylococcus aureus*). This demands exploration of new drug scaffold in order to combat with MRSA and its refractory variants in the hospital and community settings. Endophytic fungi have already been proven to be fountainheads of novel bioactive compounds (Ma et al., 2018; Chen et al., 2019; El-Readi et al., 2019; Singh et al., 2019). Further, in the present study fungal resveratrol showed potent anti-staphylococcal activity against a battery of *Staphylococcus aureus* (NCTC 6571, MTCC 96, MTCC 737, Sau G9, and Sau 902). Paulo et al., (2010) demonstrated that resveratrol possessed antimicrobial properties against a battery of Gram-positive and Gram-negative bacteria. The resveratrol was found to exhibit antimicrobial activity against surrogate, methicillin sensitive and methicillin-resistant *S. aureus*. Similarly, Ma et al., (2018) also demonstrated that resveratrol displayed potent antimicrobial activity against foodborne pathogen. The present study clearly indicates that resveratrol producing endophytic fungi possesses strong antimicrobial activity against all the tested clinical isolates as well as plant pathogenic fungi. As the demand for natural and safe chemical entities in agriculture and medicine is growing for applications in agriculture sector as well as therapeutic entities, endophytic fungi offer to be a prolific resource of these bioactive compounds which could replace synthetic chemicals (Houille et al., 2014; Deshmukh et al., 2015; Mora-Pale et al., 2015; Deshmukh et al., 2018, Ma et al., 2018; El-Readi et al., 2019; Martínez et al., 2019; Singh et al., 2019). In this context, fungal resveratrol also hold a promise for use as a therapeutic moiety as well as a crop protection agent i.e. biofungicide.

The correlation activity was performed to regulate the correlation between the resveratrol content, antioxidant and anti-microbial potential of the different resveratrol producing endophytes. The positive correlation occurred between the *in vitro* anti-microbial and the antioxidant activity (i.e., IC_{50}), despite different methods. In our study too it was found that lower the IC_{50} higher is the scavenging potential. #12VVLPM exhibited the highest scavenging ant-

oxidant activity. This was in accordance to the earlier study by Balik et al., (2008) wherein the maximum correlation was found between the antioxidant potential, various method performed, and TPC content was (0.8336–0.9952 mg/kg; $p<0.0001$). A comparatively distinguish range of the correlation coefficient was estimated between antioxidant potential and some polyphenolic content report presented by Fernandez-Pachon et al., (2004). The antimicrobial activity was positively correlated to the resveratrol content, and it was in accordance to earlier studies wherein resveratrol has been shown to exhibit activity against *B. cinerea* and *C. albicans* (Houille et al., 2014). Among the resveratrol producing endophytic isolates again #12VVLPM was the potent resveratrol producing as well as exhibited the best inhibitory activity against all the cultures tested in the panel. Hence, endophytic isolate #12VVLPM (*Arcopilus aureus*) was taken for further purification and optimization process.

Very huge amounts of polyphenolic compound occur in nature, hence there is not a single procedure may validate their separation and purification. Each group of polyphenolic compounds differ in their chemical structure, on basis of polarity, by spectral characteristics which demand convenient analytical and separation techniques (Khan et al., 1986; Walle et al., 2004; Commodari et al., 2005; Jerkovic et al., 2005; Sivakumar et al., 2013). Nowadays various techniques like TLC, HPLC, GC, MS-MS, LC-MS and NMR are widely used for separation and identification of various phenolics and stilbenes compounds (Pezzuto et al., 2008). To characterize a molecule purification is very important and basically it relies upon physiochemical properties. Here in present study purification of endophytic isolate #12VVLPM (*Arcopilus aureus*) was carried out using paper chromatography, column chromatography as well as thin layer chromatography. Firstly liquid-liquid extraction technique was performed to resolve the complex mixture into smaller fraction. Thus, bioassay guided fractionation is the most pertinent technique to narrow down and isolate the polyphenolic compounds (Zgoda-Pols et al., 2002; Lelono et al., 2013; Cunha et al., 2017; Kuo et al., 2017; Zenthoefer et al., 2017; Nalli et al., 2019). Various stilbene compounds have been identified from microbial source using bioassay guided compound isolation methods (Shi et al., 2012; Wang et al., 2014; Liu et al., 2016; Yang et al., 2016; Braga et al., 2018; Dwibedi and Saxena, 2018). In the current study, TLC fractionation of the crude ethyl acetate extract was separated Methanol: DCM solvent system, in the ratio of

0.5:9.5 which resolved the extract into 8 different bands. The R_f value of Band 1 to Band 8 was 0.14, 0.27, 0.51, 0.69, 0.78, 0.83, 0.89 and 0.97, respectively. In earlier reports also TLC analysis and bio-autographic assay has been used for identification as well as purification of resveratrol from different natural sources (Casagrande et al., 2019; Koeduka et al., 2019; Nalli et al., 2019; Sasikumar et al., 2019).

Based on the optimized TLC system for the detection of standard resveratrol with a comparison TLC of fractionated crude EA residue of #12 VVLPM, the mobile phase for silica gel column chromatography was chosen. From the TLC optimization state, it was observed that for column chromatography the mobile phase with 5% MeOH/DCM (0.5:9.5) is quite preferable over 50% ethyl acetate: Hexane (1:1) and MeOH/DCM (0.5:4.5) to isolate the pure resveratrol. Different fractions were collected with the respective increase in mobile phase and simultaneously observed the presence of compounds with standard resveratrol by TLC using optimized solvent system 5% MeOH/DCM. The fungal resveratrol was obtained in pure form by eluting the column with mobile phase of MeOH: DCM:: 1.75:98.25. Fungal resveratrol (25 mg) was observed pure by TLC system 5% MeOH: DCM and concentrated by rotary evaporator (Mei et al., 2015; Che et al., 2016; Liu et al., 2017; Dwibedi and Saxena 2018; Dwibedi et al., 2019). In order to confirm the structure of fungal resveratrol, the isolated pure compound was analyzed for structural identification with the help of ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) and compared with the ^{1}H-NMR of standard resveratrol. Later it was confirmed by ^{13}C-NMR. Both ^{1}H and ^{13}C-NMR spectra were measured on a JOEL 400 instrument using CD_3OD as the solvent. The peak of HPLC and m/z of HR-MS of fungal resveratrol similar to the molecular ions of standard resveratrol, which verify and confirm the presence of resveratrol in endophytic fungi (Zgoda-Pols et al., 2002; Jerkovic et al., 2010; Mei et al., 2015; Nandagopal et al., 2018; Samaradivakara et al., 2018; Nalli et al., 2019). These techniques NMR, FTIR, HPLC, and LC-MS have been used for structure elucidation of different stilbenes which has been isolated from the different endophytic fungi viz. *Xylaria*, *Cheatomium*, *Fusarium*, *Collectotricum*, *Alternaria* and *Aspergillus* (Mei et al., 2015; Che et al., 2016; Liu et al., 2017; Dwibedi and Saxena 2018; Dwibedi et al., 2019; Kumar et al., 2019). So from current study we have established that endophytic fungi have a resveratrol producing genes which can produce resveratrol in free fermentative condition *in-vitro*.

Resveratrol is a billion-dollar molecule with multifarious application; however, it's extremely low fermentative production has largely hindered further research. it is envisaged that fungal resveratrol is novel and promising alternative for large scale production in very adequate and cost-effective way in small period of time, thus an endeavour was made to enhance the production of resveratrol from potent resveratrol producing endophytic fungus *Arcopilus aureus* by optimizing the physiological as well as nutritional parameter components (OVAT) and response surface methodology (RSM). RSM allow to high speeding efficient screening of most significant factor which is responsible for production of any secondary metabolite or enzyme product (Shi et al., 2012; Saran et al., 2015; Yun et al., 2018; Zhou et al., 2018).

In present study among the 12 different production media evaluated, all were producing significant amount of biomass and resveratrol. However, all these media tested potato dextrose broth was found to best amongst all that produced high amount of biomass and resveratrol. In the process of optimization, we found that, biomass and resveratrol production were directly proportional to each other. Different parameters and its relationship to bioactive compounds and its biomass, shows the conformity with earlier studies (Santoset al., 2015; Toghueo et al., 2018; Ben Mefteh et al., 2019). Some other studies have illustrated that PDB is the best medium for the growth and enhancement of fungal secondary metabolite rich in antimicrobial and antioxidant activity (Jain et al., 2011; Goutam et al., 2014; Ganesh Kumar et al., 2019). Potato dextrose broth is also reported best production medium for biomass production of antioxidative and antiproliferative compounds from fungus *Talaromyces purpureogenus* (Kumari et al., 2018; Ganesh Kumar et al., 2019). In order to enhancing resveratrol production, the growth kinetics and incubation period must be optimized in batch fermentation. Generally secondary metabolites are secreted in stationary phase, so it is essential to optimize the stationary phase of fungus to achieve maximum amount of resveratrol. In current study, nine different incubation periods were evaluated, and it is found that *Arcopilus aureus* grew best at 8^{th} days of incubation period with maximum resveratrol and biomass production, which elucidates that 8^{th} day is optimum incubation period found for growth of *Arcopilus aureus* and production of resveratrol. After the culture starts going to death phase resulting in decline in biomass as well as resveratrol that may be due to aggregation of some inhibitory /toxic residue against secreted resveratrol.

Hence, we can conclude that every fungus has its own fermentation potential to produce secondary metabolite at different incubation time (Calvo et al., 2002; Goutam et al., 2014). Incubation period also explains the growth rate of endophytic fungi as well as its secondary metabolite production.

Apart from production medium and incubation time, physiological factors also play a major role in on enhancement of secondary metabolite (Bhagat et al., 2016; Zhang et al., 2017). In present study, among the 10 different temperature evaluated, 30 ºC was found to best temperature for production of resveratrol while on increasing the temperature there was a decrease in resveratrol as well as biomass production. The present study also correlates with the previous study on resveratrol where a resveratrol producing endophytic fungi showed maximum resveratrol production at 28 ºC (Shi et al., 2012) and 30 ºC (Liu et al., 2016). Bhattacharyya et al., (2011) also found similar result, where endophytic *Aspergillus* showed best production of secondary metabolite at 30 ºC. Apart from considering temperature, pH of production media is also a crucial factor which affects the growth and production of secondary metabolite by affecting the enzyme activity, charge of microbial cell membranes and by affecting the dissociation of medium components (Calvo et al., 2002; Goutam et al., 2014; Bhalkar et al., 2016; Qiao et al., 2017; Zhou et al., 2018). In current study 8 different pH ranging from (3-10) were tested and among them neutral pH (7.0) was found to be the optimum pH for production of resveratrol while by increasing the pH, decrease in resveratrol as well as biomass production was observed. This finding also supported by the previous reports (Zhang et al., 2013; Liu et al., 2016; Zhou et al., 2018) whereas maximum resveratrol was also produced by fungus at neutral pH (7.0). PDB media supplemented with 1% glucose was found to enhance the level of resveratrol production of endophytic fungus *Arcopilus aureus*. Earlier reports also corroborate our study where glucose is used as a carbon source and found its ability to significantly enhance the production of resveratrol in free fermentative condition (Shi et al., 2012; Zhang et al., 2013; Goutam et al., 2014; Liu et al., 2016; Zhou et al., 2018), whereas others studies were quite variant with respect to our results (Bhattacharyya et al., 2011; Premalatha et al., 2012; Mathanet al., 2013). Gogoiet al.,(2008) and found that the leaf extract media supplemented with dextrose as carbon source and yeast as nitrogen source resulted in higher production of bioactive

metabolite from endophytic fungus (DF-2) isolated from *Taxus wallichiana*. From the above results we can conclude that endophytic fungus initially exploits primary sugar rather than complex sugar like disaccharides or polysaccharides for its survival and adaptation (Niu et al., 2017; El-Sayed et al., 2019). Resveratrol production and biomass of endophytic fungi was also increased when PDB media was supplemented with 0.5% peptone as a nitrogen source, among the 8 different nitrogen sources which were tested for enhancement of resveratrol. Peptone was found to best nitrogen source for resveratrol production.

Dissolved oxygen (DO) play a major role in both aerobic and anaerobic fermentation to maximize the production as well as improve the product quality (Shi et al., 2012; Zhou et al., 2018). In shake flask fermentation, the level of DO is commonly enhanced by increasing the RPM in incubator shaker, due to absence of air supply equipment as it available in large fermentor (Shi et al., 2012; Zhou et al., 2018). RSM is a best method for optimization of many industrially important products, including many enzymes, food and beverages and pharmaceutical products. Compared with OVAT method, statically designed experiments can answer interaction between the factors in linear and quadratic terms. For instance, Shi et al., (2012) applied RSM approach for medium optimization for resveratrol production by *Alternaria* sp. MG1. Recently, Zhu et al., (2018) reported increases in pinoresinol production by *Phomopsis* sp. XP-8 using RSM approach. In the current study the optimization of resveratrol production by *Arcopilus aureus* was performed with help of response surface methodology. Among the variables tested for OVAT, six variables viz. RPM, temp, incubation days, pH, glucose concentration and peptone concentration were taken for BBD of RSM to assess their impact on resveratrol production by endophytic fungus *Arcopilus aureus*.

It is well known that production of secondary metabolite is depends on physiological and nutritional parameter (Shi et al., 2012; Liu et al., 2016; Zhang et al., 2017; Dwibedi and Saxena 2018; El-Sayed et al., 2019). During RSM it is observed that nitrogen and carbon source was found most important component in resveratrol production. From the study maximum resveratrol production was obtained when the condition was RPM, temp, incubation days, pH, glucose, peptone were 115, 30, 8.5, 7, 1.25 and 0.63 respectively which closely correlates with earlier study by Shi et al., (2012) reported maximum resveratrol 100 rpm and 28° C. Further

increase or decrease in the respective condition further lead to decrease in resveratrol production (Shi et al., 2012). The validation of RSM predicted results was again performed by same optimizing condition for resveratrol production. The predicted response in BBD design for resveratrol production gave a value of 133.53µg/ml while the actual experimental value was found 133.59µg/ml, which suggesting that experimental and predicted value were in good agreement.

In our study resveratrol production by the classical method (OVAT) yielded 109.61^{a} ± 0.74 µg/ml of resveratrol which is 1.22fold increase in resveratrol production. After RSM this finally resulted in production of 133.53 µg/ml (1.49 fold increase) yield of resveratrol. These studies were carried out in 50 ml of resveratrol production medium contained in 250ml Erlenmeyer flasks. Previously, the maximum resveratrol production from *Alternaria sp*. (Shi et al., 2012) was 0.45 mg/L, and by *Aspergillus niger* (Liu et al., 2016) 1.48 mg /L but in this study, the production was 133.53 mg/L after optimization by RSM. Statistical method was found to very efficacious in optimizing the selected variables components evident from positive diagnostic plots and R2 values. The closer the value of R2 is to 1, stronger the model to predict the response (Zhu et al., 2010; Bhalkar et al., 2015; Rajput et al., 2018). The observed R2 value was comparable with the earlier reports (Zhu et al., 2010; Bhalkar et al., 2015; Zhang et al., 2017; Rajput et al., 2018; Zhou et al., 2018). In brief, the successive process optimization strategy acquire with series of non-statistical and statistical methods, found to strongly improve the resveratrol production by the *Arcopilus aureus*.

After optimization of various physiological and nutritional parameters using one variable at a time (OVAT) and response surface methodology (RSM), the optimized conditions were put to test for epigenetic modification considering the increase demand for resveratrol in pharmaceutical, food as well as cosmetic industries.

Recent advancements in the fungal molecular genetics have established that fungi have many cryptic genes (Phenotypically silent or unexpressed gene). These genes can be expressed by epigenetic modifiers like microbial and chemical elicitor, precursor, and the epigenetic treatment which leads to enhancement of novel secondary metabolite that is exploitable in nutraceutical, pharmaceutical as well as agrochemical industries (UL-Hassan et al., 2012; Kumar et al., 2018;

Collemare and Seidl, 2019; Dwibedi et al., 2019; Frank et al., 2019; Pfannenstiel and Keller, 2019). Epigenetic modifiers such as HDAC inhibitors (SAHA) and DMNTs (AZA) inhibitors represent an exciting chemical tool to express a cryptic or silent gene which is unexpressed under standard laboratory condition (Collemare et al., 2019; Frank et al., 2019; Kumar et al., 2018).

HPLC and LC-MS analysis played a crucial character for identification, structure elucidation as well as quantification of bioactive compounds. With the help of HPLC analysis it is found that resveratrol concentration was maximum and enhanced in the case of treatment with 5 µM SAHA (165.55 µg/mL) , 1 µM SAHA (156.51µg/mL) and 10 µM SAHA (155.17µg/mL followed by 10 µM AZA (153.18 µg/ml) and 20 µM AZA (147.92 µg/mL) (Dwibedi et al., 2019). An HPLC and LC-MS spectrum shows an enhancement of 1.85 (165.55 µg/mL) fold in case of SAHA 5 µM which was earlier present in very less amount (89.1µg/mL) in the crude ethyl acetate extract. A similar observation was made by some other like in a study by Lopes et al., (2012) they used a range of epigenetic modifiers on *Nigrospora* sp. SS67; and they found that epigenetic modifiers increase the production of the secondary metabolite. In earlier reports *Colletotrichum gloeosporioides* an endophytic fungus was also reported to have an increase in cryptic compounds as well as their antimicrobial potential by using epigenetic modifiers, grape extract and turmeric extracts (Sharma et al., 2017). Earlier the successful attempt of epigenetic modulation have been made using 5- azacytidine in mycodiesel producing endophytic fungus *Hypoxylon* sp. (CI-4), producing a vast 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). Li et al. (2017) performed the co-culturing experiment of endophytic fungi *Camporesiasambuci* (FT1061) and *Epicoccum sorghinum* (FT1062) and reported a new N-methoxypyridone analog, together with four known compounds. Establishment of a high producing endophytic epigenetic mutant strain for industrial production of resveratrol is a genuine metabolic engineering challenge.

All these studies vigorously advocate that fungi also have many genes like other eukaryotic microbes which remain cryptic under normal lab condition and need some epigenetic modifiers to activate fungal gene transcription, leading to the fungal strain improvement for production of known as well as novel secondary metabolites. Based on these promising results in the present

study is a step forward in proving the potential of endophytic fungi and commercial production of this billion-dollar drugs which are highly demanding commercial drugs in pharmaceutical and nutraceutical industries.

Chapter- 7

Conclusion

7.0. CONCLUSION

1. This is the first study that concurrently examined the resveratrol production of the fungal endophytes isolated from different varieties of *Vitis vinifera* from India. Present study also describes the composition of the endophytic fungal community within the plant tissue of *Vitis vinifera* from India.
2. The results indicate that *Vitis vinifera* harbours rich endophytic fungal community. *Arcopilus aureus, Fusarium solani, Xylaria psidii* and *Fusarium equiseti* were found to promising candidates for resveratrol production. Among them *Arcopilus* aureus was found to be the most potent producer of resveratrol and further taken for process optimization and strain improvement required to achieve fermentation route of resveratrol production.
3. The PCR based molecular screening approach for resveratrol producing endophytic fungi was also carried out using *STS* specific primers and it was found that all the four resveratrol producing isolates harbors the *STS* gene.
4. To the best of our knowledge, this is the first study that concurrently examined the phenolic content, anti-staphylococcal activity and antioxidant potential of the resveratrol producing endophytes isolated from different varieties of *Vitis vinifera* from India. These results indicate that resveratrol producing isolates from *Vitis vinifera* harbour rich, strong anti-oxidant with strong anti-fungal activity as well as anti-staphylococcal activity, therefore exhibits a possibility for exploration of a presumptive role in food preservation apart from being used as health or nutritional supplement.

5. The endophytic isolate #12VVLPM (*Arcopilus aureus*) exhibited the best activity similar to the standard in the entire anti-oxidant assay performed during the present course of investigation. Yet another important result was the anti-mycotic activity against different plant as well as human pathogenic fungi tested. Among the resveratrol producing endophytic isolates again #12VVLPM was the potent resveratrol producing as well as exhibited the best inhibitory activity against all the cultures tested in the panel. Hence, endophytic isolate #12VVLPM (*Arcopilus aureus*) was used for further research.

6. The fungal resveratrol was obtained in pure form by eluting the column with mobile phase of MeOH: DCM :: 1.75:98.25. Fungal resveratrol (25 mg) was observed pure by TLC system 5% MeOH: DCM and concentrated by rotary evaporator. The homogeneity of the purified fungal resveratrol was confirmed by HPLC, which exhibited a single, symmetrical peak at R_t 3.36 min on C_{18} reverse phase column similar to that of standard resveratrol. For further confirmation of the presence of fungal resveratrol purified from the spent broth of *Arcopilus aureus*, ^{1}H-NMR, ^{13}C-NMR. MS and HR-MS, FTIR analysis was carried out which revealed the presence and purity of fungal resveratrol.
7. Optimization of different physic-chemical and nutritional parameters affecting resveratrol production by 'one-variable-at-a-time' approach resulted in the production of $109.61^{a} \pm 0.74$ µg/mL (1.22 fold increase) resveratrol from $89.1^{a} \pm 0.08$ µg/mL from initial yield of resveratrol. The interactions of the most influential factors obtained by one-variable-at-a-time method were further optimized using a statistical approach, response surface methodology (RSM). This finally resulted in production of 133.5 µg/mL (1.49 fold increase) yield of resveratrol. These studies were carried out in 50 mL of resveratrol production medium contained in 250 mL Erlenmeyer flasks.

   After optimization of various physiological and nutritional parameters using one variable at a time and RSM, the optimized conditions were put to test for epigenetic modification Considering the increase demand for resveratrol in pharmaceutical, food as well as cosmetic industries. The enhancement of resveratrol production, using the epigenetic modifiers such as HDAC inhibitors (SAHA) and dMNTs (AZA) inhibitors was found as a potent chemical tool for induction of cryptic gene in the endophytic fungus *Arcopilus aureus* for enhancement of the resveratrol production. This finally resulted in production of 165.0 µg/mL (1.85 fold increase) yield of resveratrol.

This study a step forward to proving that endophytic fungi are able to produce billion dollar drugs which are highly demandable due to its multifarious application and paves a new way for replacement of conventional sources of these molecules by serving as alternative.

Chapter - 8

References

8.0. REFERENCES

1. Aggarwal, B. B., Bhardwaj, A., Aggarwal, R. S., Seeram, N. P., Shishodia, S., & Takada, Y. (2004). Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer research*, *24*(5A), 2783-2840.
2. Aghcheh, R. K., & Kubicek, C. P. (2015). Epigenetics as an emerging tool for improvement of fungal strains used in biotechnology. *Applied microbiology and biotechnology*, *99*(15), 6167-6181.
3. Ahmadi, S., Marino, T., Prejanò, M., Russo, N., & Toscano, M. (2018). Antioxidant properties of the Vam3 derivative of resveratrol. *Molecules*, *23*(10), 2446.
4. Akinwumi, B., Bordun, K. A., & Anderson, H. (2018). Biological activities of stilbenoids. *International journal of molecular sciences*, *19*(3), 792.
5. Alarcon De La Lastra, C., & Villegas, I. (2005). Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. *Molecular nutrition & food research*, *49*(5), 405-430.
6. Al-Jumaily, E. F., Hamid, G. S., & Ali, K. F. (2014). Synthesis and total phenol content of new resveratrol derivative. *American Journal of advanced drug discovery, (2–3)*, 320-329.
7. Aly, A. H., Debbab, A., & Proksch, P. (2011). Fungal endophytes: unique plant inhabitants with great promises. *Applied microbiology and biotechnology*, *90*(6), 1829-1845.
8. Arnold, A. E., & Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots?. *Ecology*, *88*(3), 541-549.
9. Arnold, A. E., Mejía, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., & Herre, E. A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the national academy of sciences*, *100*(26), 15649-15654.
10. Avtanski, D., & Poretsky, L. (2018). Phyto-polyphenols as potential inhibitors of breast cancer metastasis. *Molecular medicine*, *24*(1), 29.
11. Ayinampudi, S., Wang, Y. H., Avula, B., Smillie, T., & Khan, I. (2011). Quantitative analysis of oxyresveratrol in different plant parts of *Morus* species and related genera by HPTLC and HPLC. *JPC-journal of planar chromatography-modern TLC*, *24*(2), 125-129.

12. Babu, S. K., Kumar, K. V., & Subbaraju, G. V. (2005). Estimation of trans-resveratrol in herbal extracts and dosage forms by high-performance thin layer chromatography. *Chemical and pharmaceutical bulletin*, *53(6)*, 691-693.
13. Bacon, C. W., & Hinton, D. M. (2019). Fungal and bacterial maize kernel interactions with the vertically transmitted endophytic state of *Fusarium verticillioides* in seed endophytes. *Springer, Cham*, 191-209.
14. Bacon, C. W., & White, J. F. (2016). Functions, mechanisms and regulation of endophytic and epiphytic microbial communities of plants. *Symbiosis*, *68*(1-3), 87-98.
15. Bagchi, B., & Banerjee, D. (2013). Diversity of fungal endophytes in Bauhinia vahlii (a lianas) from different regions of Paschim Medinipur district of West Bengal. *International journal of science, environment and technology*, *2*, 748-756.
16. Balea, Ş. S., Pârvu, A. E., Pop, N., Marín, F. Z., & Pârvu, M. (2018). Polyphenolic compounds, antioxidant, and cardioprotective effects of pomace extracts from fetească neagră cultivar. *Oxidative medicine and cellular longevity*, *2018*, 1-11.
17. Balik, J., Kyselakova, M., Vrchotová, N., Tříska, J., Kumšta, M., Veverka, J., & Lefnerová, D. (2008). Relations between polyphenols content and antioxidant activity in vine grapes and leaves. *Czech journal of food sciences*, *26*, S25-S32.
18. Barbulova, A., Apone, F., & Colucci, G. (2014). Plant cell cultures as source of cosmetic active ingredients. *Cosmetics*, *1*(2), 94-104.
19. Barnett, H. L., & Hunter, B. B. (1972). Illustrated genera of imperfect fungi. *Illustrated genera of imperfect fungi.*, (3rd ed).
20. Bastianetto, S., Ménard, C., & Quirion, R. (2015). Neuroprotective action of resveratrol. *Biochimicaet Biophysica Acta (BBA)-Molecular basis of disease*, *1852*(6), 1195-1201.
21. Baur, J. A., & Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nature reviews drug discovery*, *5*(6), 493.
22. Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., & Pistell, P. J. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*, *444*(7117), 337.

23. Beekwilder, J., Wolswinkel, R., Jonker, H., Hall, R., de Vos, C. R., & Bovy, A. (2006). Production of resveratrol in recombinant microorganisms. *Applied and environmental microbiology*, *72*(8), 5670-5672.
24. Belchí-Navarro, S., Almagro, L., Lijavetzky, D., Bru, R., & Pedreño, M. A. (2012). Enhanced extracellular production of trans-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and methyljasmonate. *Plant cell reports*, *31*(1), 81-89.
25. Ben Mefteh, F., Frikha, F., Daoud, A., ChenariBouket, A., Luptakova, L., Alenezi, F. N., & Belbahri, L. (2019). Response surface methodology optimization of an acidic protease produced by *Penicillium bilaiae* Isolate TDPEF30, a newly recovered endophytic fungus from healthy roots of date palm trees (Phoenix dactylifera L.). *Microorganisms*, *7*(3), 74.
26. Beňová, B., Adam, M., Onderková, K., Královský, J., & Krajíček, M. (2008). Analysis of selected stilbenes in *Polygonum cuspidatum* by HPLC coupled with CoulArray detection. *Journal of separation science*, *31*(13), 2404-2409.
27. Berman, A. Y., Motechin, R. A., Wiesenfeld, M. Y., & Holz, M. K. (2017). The therapeutic potential of resveratrol: a review of clinical trials. *NPJ precision oncology*, *1*(1), 35.
28. Bhagat, J., Kaur, A., Kaur, R., Yadav, A. K., Sharma, V., & Chadha, B. S. (2016). Cholinesterase inhibitor (Altenuene) from an endophytic fungus *Alternaria alternata*: optimization, purification and characterization. *Journal of applied microbiology*, *121*(4), 1015-1025.
29. Bhalkar, B. N., Bedekar, P. A., Patil, S. M., Patil, S. A., & Govindwar, S. P. (2015). Production of camptothecine using whey by an endophytic fungus: standardization using response surface methodology. *RSC Advances*, *5*(77), 62828-62835.
30. Bhalkar, B. N., Patil, S. M., & Govindwar, S. P. (2016). Camptothecine production by mixed fermentation of two endophytic fungi from *Nothapodytes nimmoniana*. *Fungal biology*, *120*(6-7), 873-883.
31. Bhan, N., Xu, P., Khalidi, O., & Koffas, M. A. (2013). Redirecting carbon flux into malonyl-CoA to improve resveratrol titers: proof of concept for genetic interventions predicted by opt force computational framework. *Chemical engineering science*, *103*, 109-114.
32. Bhat, M. I., & Kapila, R. (2017). Dietary metabolites derived from gut microbiota: critical modulators of epigenetic changes in mammals. *Nutrition reviews*, *75*(5), 374-389.

33. Bhattacharyya, P. N., & Jha, D. K. (2011). Optimization of cultural conditions affecting growth and improved bioactive metabolite production by a subsurface *Aspergillus*. *International journal of applied biology and pharmaceutical technology*, *2*(4), 133-143.
34. Bhullar, K. S., & Hubbard, B. P. (2015). Lifespan and health span extension by resveratrol. *Biochimicaet Biophysica Acta (BBA)-Molecular basis of disease*, *1852*(6), 1209-1218.
35. Bills, G. F., & Polishook, J. D. (1994). Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia*, *86*(2), 187-198.
36. Bonnefont-Rousselot, D. (2016). Resveratrol and cardiovascular diseases. *Nutrients*, *8*(5), 250.
37. Borah, H. J., Singhal, R., & Hazarika, S. (2017). Artocarpus lakoocha roxb.: An untapped bioresource of resveratrol from North East India, its extractive separation and antioxidant activity. *Industrial crops and products*, *95*, 75-82.
38. Boue, S. M., Shih, B. Y., Burow, M. E., Eggleston, G., Lingle, S., Pan, Y. B., & Bhatnagar, D. (2013). Postharvest accumulation of resveratrol and piceatannol in sugarcane with enhanced antioxidant activity. *Journal of agricultural and food chemistry*, *61*(35), 8412-8419.
39. Bradamante, S., Barenghi, L., & Villa, A. (2004). Cardiovascular protective effects of resveratrol. *Cardiovascular drug reviews*, *22*(3), 169-188.
40. Braga, A., Ferreira, P., Oliveira, J., Rocha, I., & Faria, N. (2018). Heterologous production of resveratrol in bacterial hosts: current status and perspectives. *World journal of microbiology and biotechnology*, *34*(8), 122.
41. Brasanac-Vukanovic, S., Mutic, J., Stankovic, D., Arsic, I., Blagojevic, N., Vukasinovic-Pesic, V., & Tadic, V. (2018). Wild bilberry (*Vaccinium myrtillus* L., *Ericaceae*) from montenegro as a source of antioxidants for use in the production of nutraceuticals. *Molecules*, *23*(8), 1864.
42. Brewer, D., Jerram, W.A. & Taylor, A. (1968). The production of cochliodinol and a related metabolite by *Chaetomium* species. *Canadian Journal of microbiology*, *14*, 861-866.
43. Brum, M. C. P., Araújo, W. L., Maki, C. S., & Azevedo, J. L. (2012). Endophytic fungi from *Vitis labrusca* L. (*'Niagara Rosada'*) and its potential for the biological control of *Fusarium oxysporum*. *Genet Mol Res*, *11*(4), 4187-4197.
44. Burnett, C., Valentini, S., Cabreiro, F., Goss, M., Somogyvári, M., Piper, M. D., & Vazquez-

Manrique, R. P. (2011). Absence of effects of Sir2 overexpression on lifespan in *C. elegans* and *Drosophila*. *Nature*, *477*(7365), 482.

45. Burns, J., Yokota, T., Ashihara, H., Lean, M. E., & Crozier, A. (2002). Plant foods and herbal sources of resveratrol. *Journal of agricultural and food chemistry*, *50*(11), 3337-3340.
46. Busch, F., Mobasheri, A., Shayan, P., Lueders, C., Stahlmann, R., & Shakibaei, M. (2012). Resveratrol modulates interleukin-1β-induced phosphatidylinositol 3-kinase and nuclear factor κB signaling pathways in human tenocytes. *Journal of biological chemistry*, *287*(45), 38050-38063.
47. Calvo, A. M., Wilson, R. A., Bok, J. W., & Keller, N. P. (2002). Relationship between secondary metabolism and fungal development. *Microbiology and molecular biology reviews*, *66*(3), 447-459.
48. Casagrande, M., Zanela, J., Pereira, D., de Lima, V. A., Oldoni, T. L. C., & Carpes, S. T. (2019). Optimization of the extraction of antioxidant phenolic compounds from grape pomace using response surface methodology. *Journal of Food Measurement and Characterization*, *13*(2), 1120-1129.
49. Casieri, L., Hofstetter, V., Viret, O., & Gindro, K. (2009). Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. *Phytopathologia Mediterranea*, *48*(1), 73-83.
50. Chagnon, P. L., U'Ren, J. M., Miadlikowska, J., Lutzoni, F., & Arnold, A. E. (2016). Interaction type influences ecological network structure more than local abiotic conditions: evidence from endophytic and endolichenic fungi at a continental scale. *Oecologia*, *180*(1), 181-191.
51. Chan, M. M. Y. (2002). Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. *Biochemical pharmacology*, *63*(2), 99-104.
52. Chan, V., Fenning, A., Iyer, A., Hoey, A., & Brown, L. (2011). Resveratrol improves cardiovascular function in DOCA-salt hypertensive rats. *Current pharmaceutical biotechnology*, *12*(3), 429-436.
53. Chang, H. C., & Guarente, L. (2014). SIRT1 and other sirtuins in metabolism. *Trends in endocrinology & metabolism*, *25*(3), 138-145.
54. Chao, S. C., Chen, Y. J., Huang, K. H., Kuo, K. L., Yang, T. H., Huang, K. Y., & Liu, S. H. (2017).

Induction of sirtuin-1 signaling by resveratrol induces human chondrosarcoma cell apoptosis and exhibits antitumor activity. *Scientific reports*, *7*(1), 3180.

55. Chaplin, A., Carpéné, C., & Mercader, J. (2018). Resveratrol, metabolic syndrome, and gut microbiota. *Nutrients*, *10*(11), 1651.
56. Che, J., Shi, J., Gao, Z., & Zhang, Y. (2016). A new approach to produce resveratrol by enzymatic bioconversion. *Journal of microbiology and biotechnology*, *26*(8), 1348-1357.
57. Che, J., Shi, J., Gao, Z., & Zhang, Y. (2016). A new approach to produce resveratrol by enzymatic bioconversion. *Journal of microbiology and biotechnology*, *26*(8), 1348-1357.
58. Che, J., Shi, J., Gao, Z., & Zhang, Y. (2016). Transcriptome analysis reveals the genetic basis of the resveratrol biosynthesis pathway in an endophytic fungus (*Alternaria* sp. MG1) isolated from *Vitis vinifera*. *Frontiers in microbiology*, *7*, 1257.
59. Chedea, V. S., Vicaş, S. I., Sticozzi, C., Pessina, F., Frosini, M., Maioli, E., & Valacchi, G. (2017). Resveratrol: from diet to topical usage. *Food & function*, *8*(11), 3879-3892.
60. Chen, C., Jiang, X., Lai, Y., Liu, Y., & Zhang, Z. (2015). Resveratrol protects against arsenic trioxide-induced oxidative damage through maintenance of glutathione homeostasis and inhibition of apoptotic progression. *Environmental and molecular mutagenesis*, *56*(3), 333-346.
61. Chen, L. K., Qiang, P. F., Xu, Q. P., Zhao, Y. H., Dai, F., & Zhang, L. (2013). Trans-3, 4, 5, 4′-tetramethoxystilbene, a resveratrol analog, potently inhibits angiogenesis *in vitro* and *in vivo*. *Acta pharmacologica Sinica*, *34*(9), 1174.
62. Chen, Q. W., Dong, K., Qin, H. X., Yang, Y. K., He, J. L., Li, J., & Chen, J. P. (2019). Direct and indirect inhibition effects of resveratrol against toxoplasma gondii tachyzoites *in vitro*. *Antimicrobial agents and chemotherapy*, *63*(3), e01233-18.
63. Cherblanc, F. L., Davidson, R. W., Di Fruscia, P., Srimongkolpithak, N., & Fuchter, M. J. (2013). Perspectives on natural product epigenetic modulators in chemical biology and medicine. *Natural product reports*, *30*(5), 605-624.
64. Chien, M. H., Lee, W. J., Yang, Y. C., Tan, P., Pan, K. F., Tsai, H. C., & Hua, K. T. (2018). Naa10p promotes metastasis by stabilizing matrix metalloproteinase-2 protein in human osteosarcomas. *bioRxiv*, 243592.

65. Choi, O., Lee, J. K., Kang, S. Y., Pandey, R. P., Sohng, J. K., Ahn, J. S., & Hong, Y. S. (2014). Construction of artificial biosynthetic pathways for resveratrol glucoside derivatives. *Journal of nicrobiology and biotechnology*, *24*(5), 614-618.
66. Chong, Z. Z., Wang, S., Shang, Y. C., & Maiese, K. (2012). Targeting cardiovascular disease with novel SIRT1 pathways. *Future cardiology*, *8*(1), 89-100.
67. Christian, N., Sullivan, C., Visser, N. D., & Clay, K. (2016). Plant host and geographic location drive endophyte community composition in the face of perturbation. *Microbial ecology*, *72*(3), 621-632.
68. Chu, M., Almagro, L., Chen, B., Burgos, L., & Pedreño, M. A. (2018). Recent trends and comprehensive appraisal for the biotechnological production of trans-resveratrol and its derivatives. *Phytochemistry reviews*, 1-18.
69. Chung, I. M., Park, M. R., Chun, J. C., & Yun, S. J. (2003). Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic stresses and hormones in peanut plants. *Plant science*, *164*(1), 103-109.
70. Collemare, J., & Seidl, M. F. (2019). Chromatin-dependent regulation of secondary metabolite biosynthesis in fungi: Is the picture complete?. *FEMS Microbiology Reviews*, 1-17.
71. Commodari, F., Khiat, A., Ibrahimi, S., Brizius, A. R., & Kalkstein, N. (2005). Comparison of the phytoestrogen trans-resveratrol (3, 4′, 5-trihydroxystilbene) structures from x-ray diffraction and solution NMR. *Magnetic resonance in chemistry*, *43*(7), 567-572.
72. Crandall, J. P., & Barzilai, N. (2013). Exploring the promise of resveratrol: where do we go from here?. *Diabetes*, *62*(4), 1022-1023.
73. Cunha, L. C., de Morais, S. A., de Aquino, F. J., Chang, R., de Oliveira, A., Martins, M. M., & do Nascimento, E. A. (2017). Bioassay-guided fractionation and antimicrobial and cytotoxic activities of *cassia bakeriana* extracts. *RevistaBrasileira de Farmacognosia*, *27*(1), 91-98.
74. Danagoudar, A., Joshi, C. G., Sunil Kumar, R., Poyya, J., Nivya, T., Hulikere, M. M., & Anu Appaiah, K. A. (2017). Molecular profiling and antioxidant as well as anti-bacterial potential of polyphenol producing endophytic fungus- *Aspergillus austroafricanus* CGJ-B3. *Mycology*, *8*(1), 28-38.

75. Dani, C., Bonatto, D., Salvador, M., Pereira, M. D., Henriques, J. A., & Eleutherio, E. (2008). Antioxidant protection of resveratrol and catechin in *Saccharomyces cerevisiae*. *Journal of agricultural and food chemistry*, *56*(11), 4268-4272.
76. Dasgupta, B., & Milbrandt, J. (2007). Resveratrol stimulates AMP kinase activity in neurons. *Proceedings of the national academy of sciences*, *104*(17), 7217-7222.
77. Dastoor, R., Bakhshi, D., & Aliakbar, A. (2018). Evaluation of total phenolic and flavonoid content, antioxidant capacity and resveratrol of selected medicinal plants of northern iran. *Agriculturae conspectus scientificus*, *83*(2), 175-180.
78. David, A. S., Seabloom, E. W., & May, G. (2016). Plant host species and geographic distance affect the structure of above ground fungal symbiont communities, and environmental filtering affects below ground communities in a coastal dune ecosystem. *Microbial ecology*, *71*(4), 912-926.
79. de Bary, A. (1866). Morphologie und Physiologie Pilze, Flechten, und myxomyceten. Hofmeister's Handbook of Physiological Botany, vol 2. Leipzig: Verlag Von Wilhelm Engelmann.
80. De La Lastra, C. A., & Villegas, I. (2007). Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochemical society transactions*, *35*(5), 1156-1160.
81. De Matos, A. M., de Macedo, M. P., & Rauter, A. P. (2018). Bridging type 2 diabetes and alzheimer's disease: assembling the puzzle pieces in the quest for the molecules with therapeutic and preventive potential. *Medicinal research reviews*, *38*(1), 261-324.
82. Delaunois, B., Cordelier, S., Conreux, A., Clément, C., & Jeandet, P. (2009). Molecular engineering of resveratrol in plants. *Plant biotechnology journal*, *7*(1), 2-12.
83. Deng, N., Liu, C., Chang, E., Ji, J., Yao, X., Yue, J., & Shi, S. (2017). High temperature and UV-C treatments affect stilbenoid accumulation and related gene expression levels in *Gnetum parvifolium*. *Electronic journal of biotechnology*, *25*, 43-49.
84. Dercks, W., & Creasy, L. L. (1989). Influence of fosetyl-Al on phytoalexin accumulation in the *Plasmopara viticola*-grapevine interaction. *Physiological and molecular plant pathology*, *34*(3), 203-213.

85. Deshmukh, S. K., Verekar, S. A., & Bhave, S. V. (2015). Endophytic fungi: a reservoir of antibacterials. *Frontiers in microbiology*, *5*, 715.
86. Deshmukh, S., Gupta, M., Prakash, V., & Saxena, S. (2018). Endophytic fungi: a source of potential antifungal compounds. *Journal of fungi*, *4*(3), 77.
87. Dincheva, I., Badjakov, I., Tsvetkov, I., Dzhambazova, T., Kondakova, V., & Batchvarova, R. (2011). GC-MS determination of secondary metabolites in red wine from Bulgarian wild grape (*Vitis vinifera* ssp. sylvestris. *Journal of Mountain Agriculture on the Balkans*, *14*(6), 1331-1343.
88. Ding, T., & Melcher, U. (2016). Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. *PloS one*, *11*(3), e0150895.
89. Donnez, D., Jeandet, P., Clément, C., & Courot, E. (2009). Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. *Trends in biotechnology*, *27*(12), 706-713.
90. Duan, J., Yue, W., Jian Yu, E., Malhotra, J., Lu, S. E., Gu, J., & Tan, X. L. (2016). In vitro comparative studies of resveratrol and triacetylresveratrol on cell proliferation, apoptosis, and STAT3 and NFκB signaling in pancreatic cancer cells. *Scientific reports*, *6*, 31672.
91. Duncan, K. W., & Campbell, J. E. (2017). Epigenetic Modulators. In *Cancer II*. Springer, Cham, 227.
92. Dwibedi, V., & Saxena, S. (2018). *Arcopilus aureus*, a resveratrol-producing endophyte from *vitis vinifera*. *Applied biochemistry and biotechnology*, 1-20.
93. Dwibedi, V., Kalia, S., & Saxena, S. (2019). Isolation and enhancement of resveratrol production in *Xylaria psidii* by exploring the phenomenon of epigenetics: using DNA methyltransferases and histone deacetylase as epigenetic modifiers. *Molecular biology reports*, 1-15.
94. Ellis, M. B., & Ellis, J. P. (1985). *Microfungi on land plants. An identification handbook*. Croom Helm Ltd..
95. El-Readi, M. Z., Eid, S., Abdelghany, A. A., Al-Amoudi, H. S., Efferth, T., & Wink, M. (2019). Resveratrol mediated cancer cell apoptosis, and modulation of multidrug resistance proteins and metabolic enzymes. *Phytomedicine*, *55*, 269-281.

96. El-Sayed, E. S. R., Ismaiel, A. A., Ahmed, A. S., Hassan, I. A., & El-Din, A. Z. A. K. (2019). Bioprocess optimization using response surface methodology for production of the anticancer drug paclitaxel by *Aspergillus fumigatus* and *Alternaria tenuissima*: Enhanced production by ultraviolet and gamma irradiation. *Biocatalysis and agricultural biotechnology*, *18*, 100996.
97. Erdogan, C., & Vang, O. (2016). Challenges in analyzing the biological effects of resveratrol. *Nutrients*, *8*(6), 353.
98. Esmaeel, Q., Jacquard, C., Clément, C., Sanchez, L., & Barka, E. A. (2019). Genome sequencing and traits analysis of Burkholderia strains reveal a promising biocontrol effect against grey mould disease in grapevine (*Vitis vinifera* L.). *World journal of microbiology and biotechnology*, *35*(3), 40.
99. Estrov, Z., Shishodia, S., Faderl, S., Harris, D., Van, Q., Kantarjian, H. M., & Aggarwal, B. B. (2003). Resveratrol blocks interleukin-1β–induced activation of the nuclear transcription factor NF-κB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood*, *102*(3), 987-995.
100. Eyberger, A. L., Dondapati, R., & Porter, J. R. (2006). Endophyte fungal isolates from *podophyllum peltatum* produce podophyllotoxin. *Journal of natural products*, *69*(8), 1121-1124.
101. Fan, E., Zhang, K., Zhu, M., & Wang, Q. (2010). Obtaining resveratrol: from chemical synthesis to biotechnological production. *Mini-reviews in organic chemistry*, *7*(4), 272-281.
102. Fei, J., Ishii, H., Hoeksema, M. A., Meitinger, F., Kassavetis, G. A., Glass, C. K., & Kadonaga, J. T. (2018). NDF, a nucleosome-destabilizing factor that facilitates transcription through nucleosomes. *Genes & development*, *32*(9-10), 682-694.
103. Felber, A. C., Orlandelli, R. C., Rhoden, S. A., Garcia, A., Costa, A. T., Azevedo, J. L., & Pamphile, J. A. (2016). Bioprospecting foliar endophytic fungi of Vitis labrusca Linnaeus, Bordô and Concord cv. *Annals of Microbiology*, *66*(2), 765-775.
104. Fernández-Pachón, M. S., Villano, D., Troncoso, A. M., & García-Parrilla, M. C. (2006). Determination of the phenolic composition of sherry and table white wines by liquid

chromatography and their relation with antioxidant activity. *Analytica Chimica Acta*, *563*(1-2), 101-108.

105. Fernández-Pachón, M. S., Villano, D., Garcıa-Parrilla, M. C., & Troncoso, A. M. (2004). Antioxidant activity of wines and relation with their polyphenolic composition. *Analytica Chimica Acta*, *513*(1), 113-118.

106. Feurtey, A., & Stukenbrock, E. H. (2018). Interspecific gene exchange as a driver of adaptive evolution in fungi. *Annual review of microbiology*, *72*, 377-398.

107. Fia, G., Gori, C., Bucalossi, G., Borghini, F., & Zanoni, B. (2018). A naturally occurring antioxidant complex from unripe grapes: the case of sangiovese (v. *Vitis vinifera*). *Antioxidants*, *7*(2), 27.

108. Frank, M., Özkaya, F. C., Müller, W. E., Hamacher, A., Kassack, M. U., Lin, W., & Proksch, P. (2019). Cryptic secondary metabolites from the sponge-associated fungus Aspergillus *ochraceus*. *Marine drugs*, *17*(2), 99.

109. Frémont, L., Belguendouz, L., & Delpal, S. (1999). Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life sciences*, *64*(26), 2511-2521.

110. Gabaston, J., Cantos-Villar, E., Biais, B., Waffo-Teguo, P., Renouf, E., Corio-Costet, M. F., & Mérillon, J. M. (2017). Stilbenes from *vitis vinifera* L. waste: a sustainable tool for controlling *plasmopara viticola*. *Journal of agricultural and food chemistry*, *65*(13), 2711-2718.

111. Ganesh Kumar, A., Balamurugan, K., VijayaRaghavan, R., Dharani, G., & Kirubagaran, R. (2019). Studies on the antifungal and serotonin receptor agonist activities of the secondary metabolites from piezo tolerant deep-sea fungus *Ascotricha* sp. *Mycology*, *10*(2), 92-108.

112. Gao, Y., & Tollefsbol, T. (2018). Combinational proanthocyanidins and resveratrol synergistically inhibit human breast cancer cells and impact epigenetic–mediating machinery. *International journal of molecular sciences*, *19*(8), 2204.

113. Garyali, S., Kumar, A., & Reddy, M. S. (2014). Enhancement of taxol production from endophytic fungus *Fusarium redolens*. *Biotechnology and bioprocess engineering*, *19*(5), 908-915.

114. Gerke, J., & Braus, G. H. (2014). Manipulation of fungal development as source of novel

secondary metabolites for biotechnology. *Applied microbiology and biotechnology*, *98*(20), 8443-8455.

115. Ghildiyal, A., & Pandey, A. (2008). Isolation of cold tolerant antifungal strains of *Trichoderma* sp. from glacial sites of Indian Himalayan region. *Research journal of microbiology*, *3*(8), 559-564.

116. Giacomini, E., Rupiani, S., Guidotti, L., Recanatini, M., & Roberti, M. (2016). The use of stilbene scaffold in medicinal chemistry and multi-target drug design. *Current medicinal chemistry*, *23*(23), 2439-2489.

117. Global Resveratrol Market Research Report 2018 (https://www.themarketreports.com).

118. Gluck-Thaler, E., & Slot, J. C. (2018). Specialized plant biochemistry drives gene clustering in fungi. *The ISME journal*, *12*(7), 1694.

119. Glynou, K., Ali, T., Buch, A. K., Haghi Kia, S., Ploch, S., Xia, X., & Maciá-Vicente, J. G. (2016). The local environment determines the assembly of root endophytic fungi at a continental scale. *Environmental microbiology*, *18*(8), 2418-2434.

120. Gogoi, D. K., Boruah, H. P. D., Saikia, R., & Bora, T. C. (2008). Optimization of process parameters for improved production of bioactive metabolite by a novel endophytic fungus *Fusarium* sp. DF2 isolated from *taxus wallichiana* of north east india. *World journal of microbiology and biotechnology*, *24*(1), 79-87.

121. Gond, S. K., Mishra, A., Sharma, V. K., Verma, S. K., Kumar, J., Kharwar, R. N., & Kumar, A. (2012). Diversity and antimicrobial activity of endophytic fungi isolated from Nyctanthes arbor-tristis, a well-known medicinal plant of India. *Mycoscience*, *53*(2), 113-121.

122. Gonzalez, J. B., Fernandez, F. J., & Tomasini, A. (2003). Microbial secondary metabolites production and strain improvement. *Indian journal of Biotechnology*, *2*(3), 322-333.

123. González, V., & Tello, M. L. (2011). The endophytic mycota associated with *Vitis vinifera* in central Spain. *Fungal Diversity*, *47*(1), 29-42.

124. Goutam, J., Sharma, V. K., Verma, S. K., Singh, D. K., Kumar, J., Mishra, A., & Kharwar, R. N. (2014). Optimization of culture conditions for enhanced production of bioactive metabolites rich in antimicrobial and antioxidant activities isolated from *Emericella quadrilineata* an endophyte of *Pteris pellucida*. *Journal of pure and applied microbiology*, *8*, 2059-2073.

125. Griffin, E. A., Harrison, J. G., Kembel, S. W., Carrell, A. A., Joseph Wright, S., & Carson, W. P. (2019). Plant host identity and soil macronutrients explain little variation in sapling endophyte community composition: Is disturbance an alternative explanation?. *Journal of Ecology*.

126. Gu, X., Cai, Z., Cai, M., Liu, K., Liu, D., Zhang, Q., & Ma, Q. (2018). AMPK/SIRT1/p38 MAPK signaling pathway regulates alcohol-induced neurodegeneration by resveratrol. *Molecular medicine reports*, *17*(4), 5402-5408.

127. Gülçin, İ. (2010). Antioxidant properties of resveratrol: a structure–activity insight. *Innovative food science & emerging technologies*, *11*(1), 210-218.

128. Hall, M. E., & Wilcox, W. F. (2019). Identification and frequencies of endophytic microbes within healthy grape berries. *American Journal of Enology and Viticulture*, *70*(2), 212-219.

129. Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., & Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.*, *79*(3), 293-320.

130. Harman, G. E., & Uphoff, N. (2019). Symbiotic root-endophytic soil microbes improve crop productivity and provide environmental benefits. *Scientifica*, *2019*, 1-25.

131. Hasan, M., & Bae, H. (2017). An overview of stress-induced resveratrol synthesis in grapes: perspectives for resveratrol-enriched grape products. *Molecules*, *22*(2), 294.

132. Hata, K., & Futai, K. (1995). Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge, The *codiplosis japonensis*. *Canadian journal of botany*, *73*(3), 384-390.

133. Ho, R., Violette, A., Cressend, D., Raharivelomanana, P., Carrupt, P. A., & Hostettmann, K. (2012). Antioxidant potential and radical-scavenging effects of flavonoids from the leaves of *Psidium cattleianum* grown in french polynesia. *Natural product research*, *26*(3), 274-277.

134. Hong, Y. B., Kang, H. J., Kim, H. J., Rosen, E. M., Dakshanamurthy, S., Rondanin, R., & Bae, I. (2009). Inhibition of cell proliferation by a resveratrol analog in human pancreatic and breast cancer cells. *Experimental & molecular medicine*, *41*(3), 151.

135. Houghton, P. J. (1995). The role of plants in traditional medicine and current therapy. *The journal of alternative and complementary medicine*, *1*(2), 131-143.

136. Houille, B., Papon, N., Boudesocque, L., Bourdeaud, E., Besseau, S., Courdavault, V., & Clastre, M. (2014). Antifungal activity of resveratrol derivatives against *Candida* species. *Journal of natural products*, *77*(7), 1658-1662.

137. Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., & Scherer, B. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature*, *425*(6954), 191.

138. Huang, L. H., Yuan, M. Q., Ao, X. J., Ren, A. Y., Zhang, H. B., & Yang, M. Z. (2018). Endophytic fungi specifically introduce novel metabolites into grape flesh cells in vitro. *PloS one*, *13*(5), e0196996.

139. Hubbard, B. P., & Sinclair, D. A. (2014). Small molecule SIRT1 activators for the treatment of aging and age-related diseases. *Trends in pharmacological sciences*, *35*(3), 146-154.

140. Hwang, D., & Lim, Y. H. (2015). Resveratrol antibacterial activity against *Escherichia coli* is mediated by Z-ring formation inhibition via suppression of FtsZ expression. *Scientific reports*, *5*, 10029.

141. Ismaiel, A. A., Ahmed, A. S., Hassan, I. A., El-Sayed, E. S. R., & El-Din, A. Z. A. K. (2017). Production of paclitaxel with anticancer activity by two local fungal endophytes, *Aspergillus fumigatus* and *Alternaria tenuissima*. *Applied microbiology and biotechnology*, *101*(14), 5831-5846.

142. Jain, P., & Pundir, R. K. (2011). Effect of fermentation medium, pH and temperature variations on antibacterial soil fungal metabolite production. *Journal of agricultural and technoloy*, *7*(2), 247-69

143. Jeandet, P., Clément, C., & Courot, E. (2014). Resveratrol production at large scale using plant cell suspensions. *Engineering in life sciences*, *14*(6), 622-632.

144. Jeandet, P., Delaunois, B., Aziz, A., Donnez, D., Vasserot, Y., Cordelier, S., & Courot, E. (2012). Metabolic engineering of yeast and plants for the production of the biologically active hydroxystilbene, resveratrol. *Journal of biomed research*, *2012*.

145. Jerkovic, V., Bröhan, M., Monnart, E., Nguyen, F., Nizet, S., & Collin, S. (2010). Stilbenic profile of cocoa liquors from different origins determined by RP-HPLC-APCI (+)-MS/MS. Detection of a new resveratrol hexoside. *Journal of agricultural and food chemistry*, *58*(11), 7067-7074.

146. Jerkovic, V., Callemien, D., & Collin, S. (2005). Determination of stilbenes in hop pellets from different cultivars. *Journal of agricultural and food chemistry*, *53*(10), 4202-4206.

147. Jia, M., Chen, L., Xin, H. L., Zheng, C. J., Rahman, K., Han, T., & Qin, L. P. (2016). A friendly relationship between endophytic fungi and medicinal plants: a systematic review. *Frontiers in microbiology*, *7*, 906.

148. Jia, R., Li, Y., Cao, L., Du, J., Zheng, T., Qian, H., & Yin, G. (2019). Antioxidative, anti-inflammatory and hepatoprotective effects of resveratrol on oxidative stress-induced liver damage in tilapia (Oreochromisniloticus). *Comparative biochemistry and physiology part c: toxicology & pharmacology*, *215*, 56-66.

149. Jia, Y., Wang, N., & Liu, X. (2017). Resveratrol and amyloid-beta: mechanistic insights. *Nutrients*, *9*(10), 1122.

150. Jin, Z., Li, D., Liu, T., Yu, F., Zhang, Z., Su, C., & Liu, Z. (2017). Cultural endophytic fungi associated with Dendrobium officinale: identification, diversity estimation and their antimicrobial potential. *Current Science*, *112*(8), 1690.

151. Kanokmedhakul, S., Kanokmedhakul, K., Phonkerd, N., Soytong, K., Kongsaeree, P. & Suksamrarn, A. (2001). Anti mycobacterial anthraquinone-chromanone compound and diketopiperazine alkaloid from the fungus *Chaetomium globosum* KMITLN0802. *Plantamedica*, *68(9)*, 834-836.

152. Kaur, G., Roberti, M., Raul, F., & Pendurthi, U. R. (2007). Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol. *Thrombosis research*, *119*(2), 247-256.

153. Kayashima, Y., Katayanagi, Y., Tanaka, K., Fukutomi, R., Hiramoto, S., & Imai, S. (2017). Alkyl resorcinols activate SIRT1 and delay ageing in drosophila melanogaster. *Scientific reports*, *7*, 43679.

154. Kernaghan, G., Mayerhofer, M., & Griffin, A. (2017). Fungal endophytes of wild and hybrid *Vitis* leaves and their potential for vineyard biocontrol. *Canadian journal of microbiology*, *63*(7), 583-595.

155. Khan, M. A., Nabi, S. G., Prakash, S., & Zaman, A. (1986). Pallidol, a resveratrol dimer from cissus pallida. *Phytochemistry*, *25*(8), 1945-1948.

156. Khanduja, K. L., & Bhardwaj, A. (2003) Stable free radical scavenging and anti-peroxidative properties of resveratrol compared *in vitro* with some other bioflavonoids. *Indian journal of Biochemical and Biophysics*, *40*(6),416-22

157. Kim, J. K., Kim, M., Cho, S. G., Kim, M. K., Kim, S. W., & Lim, Y. H. (2010). Biotransformation of mulberroside A from *Morus alba* results in enhancement of tyrosinase inhibition. *Journal of industrial microbiology & biotechnology*, *37*(6), 631-637.

158. Kiselev, K. V. (2011). Perspectives for production and application of resveratrol. *Applied microbiology and biotechnology*, *90*(2), 417-425.

159. Klinge, C. M., Wickramasinghe, N. S., Ivanova, M. M., & Dougherty, S. M. (2008). Resveratrol stimulates nitric oxide production by increasing estrogen receptor α-Src-caveolin-1 interaction and phosphorylation in human umbilical vein endothelial cells. *The FASEB Journal*, *22*(7), 2185-2197.

160. Koeduka, T., Hatada, M., Suzuki, H., Suzuki, S., & Matsui, K. (2019). Molecular cloning and functional characterization of an O-methyltransferase catalyzing 4′-O-methylation of resveratrol in *Acorus calamus*. *Journal of bioscience and bioengineering*, *127*(5), 539-543.

161. Kornreich, D. A., & Saif, M. W. (2012). Epistaxis is secondary to panitumumab in a patient with colon cancer. *Anticancer research*, *32*(11), 4983-4985.

162. Kou, X., & Chen, N. (2017). Resveratrol as a natural autophagy regulator for prevention and treatment of Alzheimer's disease. *Nutrients*, *9*(9), 927.

163. Kukreja, A., Wadhwa, N., & Tiwari, A. (2014). Therapeutic role of resveratrol and piceatannol in disease prevention. *Journal of blood disorders and transfusion*, *5*(9), 10-4172.

164. Kumar, J., Sharma, V. K., Singh, D. K., Mishra, A., Gond, S. K., Verma, S. K., ... & Kharwar, R. N. (2016). Epigenetic activation of antibacterial property of an endophytic Streptomyces coelicolor strain AZRA 37 and identification of the induced protein using MALDI TOF

MS/MS. *PloS one*, *11*(2), e0147876.

165. Kumar, A., D'silva, M., Dholakia, K., & Levenson, A. (2018). *In vitro* anticancer properties of table grape powder extract (gpe) in prostate cancer. *Nutrients*, *10*(11), 1804.

166. Kumar, S., Pagar, A. D., Ahmad, F., Dwibedi, V., Wani, A., Bharatam, P. V., & Singh, I. P. (2019). Xanthine oxidase inhibitors from an endophytic fungus Lasiodiplodia pseudotheobromae. *Bioorganic chemistry*, *87*, 851-856.

167. Kumari, M., Taritla, S., Sharma, A., & Jayabaskaran, C. (2018). Antiproliferative and antioxidative bioactive compounds in extracts of marine-derived endophytic fungus *Talaromyces purpureo* genus. *Frontiers in microbiology*, *9*, 1777.

168. Kuo, H. P., Wang, R., Lin, Y. S., Lai, J. T., Lo, Y. C., & Huang, S. T. (2017). Pilot scale repeated fed-batch fermentation processes of the wine yeast *Dekkera bruxellensis* for mass production of resveratrol from *polygonum cuspidatum*. *Bioresource technology*, *243*, 986-993.

169. Kusari, S., Hertweck, C., & Spiteller, M. (2012). Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chemistry & biology*, *19*(7), 792-798.

170. Kusari, S., Lamshöft, M., Zühlke, S., & Spiteller, M. (2008). An endophytic fungus from *Hypericum perforatum* that produces hypericin. *Journal of natural products*, *71*(2), 159-162.

171. Labinskyy, N., Csiszar, A., Veress, G., Stef, G., Pacher, P., Oroszi, G., & Ungvari, Z. (2006). Vascular dysfunction in aging: potential effects of resveratrol, an anti-inflammatory phytoestrogen. *Current medicinal chemistry*, *13*(9), 989-996.

172. Lambert, C., Richard, T., Renouf, E., Bisson, J., Waffo-Téguo, P., Bordenave, L., & Cluzet, S. (2013). Comparative analyses of stilbenoids in canes of major *Vitis vinifera* L. cultivars. *Journal of agricultural and food chemistry*, *61*(47), 11392-11399.

173. Lançon, A., Frazzi, R., & Latruffe, N. (2016). Anti-oxidant, anti-inflammatory and anti-angiogenic properties of resveratrol in ocular diseases. *Molecules*, *21*(3), 304.

174. Lange, B. M. (2018). Commercial-scale tissue culture for the production of plant natural products: successes, failures and outlook. *In biotechnology of natural products*. Springer, Cham, 189-218.

175. Lange, K. W., & Li, S. (2018). Resveratrol, pterostilbene, and dementia. *Biofactors*, *44*(1), 83-90.

176. Lelono, R. A., & Tachibana, S. (2013). Bioassay-guided isolation and identification of antioxidative compounds from the bark of *eugenia polyantha*. *Pakistan journal of biological sciences*, *16*(16), 812-818.

177. Leong, X. F., Ng, C. Y., & Jaarin, K. (2015). Animal models in cardiovascular research: hypertension and atherosclerosis. *BioMed research international*, *2015*.

178. Li, C., Sarotti, A. M., Yang, B., Turkson, J., & Cao, S. (2017) A new n-methoxypyridone from the co-cultivation of hawaiian endophytic fungi *Camporesiasambuci* FT1061 and *Epicoccumsorghinum* FT1062. *Molecules*, 22(7): 1166.

179. Li, J., Zhang, C. X., Liu, Y. M., Chen, K. L., & Chen, G. (2017). A comparative study of anti-aging properties and mechanism: resveratrol and caloric restriction. *Oncotarget*, *8*(39), 65717.

180. Li, M., Schneider, K., Kristensen, M., Borodina, I., & Nielsen, J. (2016). Engineering yeast for high-level production of stilbenoid antioxidants. *Scientific reports*, *6*, 36827.

181. Liang, Z., Zhang, J., Zhang, X., Li, J., Zhang, X., & Zhao, C. (2015). Endophytic fungus from *Sinopodo phyllumemodi* (Wall.) Ying that produces Podophyllotoxin. *Journal of chromatographic science*, *54*(2), 175-178.

182. Lim, C. G., Fowler, Z. L., Hueller, T., Schaffer, S., & Koffas, M. A. (2011). High-yield resveratrol production in engineered Escherichia coli. *Applied and environmental microbiology*.

183. Lin, C., Crawford, D. R., Lin, S., Hwang, J., Sebuyira, A., Meng, R., & Davis, P. J. (2011). Inducible COX-2-dependent apoptosis in human ovarian cancer cells. *Carcinogenesis*, *32*(1), 19-26.

184. Lin, J. Q., Zhao, X. X., Wang, C. C., Xie, Y., Li, G. H., & He, Z. M. (2013). 5-Azacytidine inhibits aflatoxin biosynthesis in *Aspergillus flavus*. *Annals of microbiology*, *63*(2), 763-769.

185. Liu, D., Li, B., Liu, H., Guo, X., & Yuan, Y. (2017).Profiling influences of gene overexpression on heterologous resveratrol production in Saccharomyces cerevisiae. *Frontiers of Chemical Science and Engineering*, *11*(1), 117-125.

186. Liu, X., Wu, X., Ma, Y., Zhang, W., Hu, L., Feng, X., & Tang, X. (2017). Endophytic fungi from mangrove inhibit lung cancer cell growth and angiogenesis in vitro. *Oncology reports*, *37*(3), 1793-1803.

187. Liu, Y., Nan, L., Liu, J., Yan, H., Zhang, D., & Han, X. (2016). Isolation and identification of resveratrol-producing endophytes from wine grape cabernet sauvignon. *Springerplus*, *5*(1), 1029.

188. Liu, Y., Zhou, J., Qu, Y., Yang, X., Shi, G., Wang, X., & Zhao, X. (2016). Resveratrol antagonizes antimicrobial lethality and stimulates recovery of bacterial mutants. *PloS one*, *11*(4), e0153023.

189. Lopes, A. A., da Silva, D. B., Lopes, N. P., & Pupo, M. T. (2012). Epigenetic modulation changed the secondary metabolite profile in the endophyte *Nigrospora sphaerica* SS67. *Planta Medica*, *78*(14), PL38.

190. Lu, Y., Shao, D., Shi, J., Huang, Q., Yang, H., & Jin, M. (2016). Strategies for enhancing resveratrol production and the expression of pathway enzymes. *Applied microbiology and biotechnology*, *100*(17), 7407-7421.

191. Ly, Q. T., & Dao, T. T. A. (2018). Is Resveratrol a cancer immunomodulatory molecule?. *Frontiers in pharmacology*, *9*, 1255.

192. Ma, D. S., Tan, L. T. H., Chan, K. G., Yap, W. H., Pusparajah, P., Chuah, L. H., & Goh, B. H. (2018). Resveratrol—potential antibacterial agent against foodborne pathogens. *Frontiers in pharmacology*, *9*, 102.

193. Ma, H., Johnson, S. L., Liu, W., DaSilva, N. A., Meschwitz, S., Dain, J. A., & Seeram, N. P. (2018). Evaluation of polyphenol anthocyanin-enriched extracts of blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry for free radical scavenging, reactive carbonyl species trapping, anti-glycation, anti-β-amyloid aggregation, and microglial neuroprotective effects. *International journal of molecular sciences*, *19*(2), 461.

194. Márquez, S. S., Bills, G. F., Acuña, L. D., & Zabalgogeazcoa, I. (2010). Endophytic mycobiota of leaves and roots of the grass Holcus lanatus. *Fungal Diversity*, *41*(1), 115-123..

195. Martínez, L., Durán, M., Malovini, E., De Rosas, M. I., Deis, L., & Cavagnaro, J. B. (2019). A very promising molecule: resveratrol, induced synthesis, and health benefits. In *psychiatry and neuroscience update*. Springer, Cham, 153-164

196. Mathan, S., Subramanian, V., & Nagamony, S. (2013). Optimization and antimicrobial metabolite production from endophytic fungi *Aspergillus terreus* KC 582297. *Eur J ExpBiol*, *3*(4), 138-144.

197. Medina, I., Alcántara, D., González, M. J., Torres, P., Lucas, R., Roque, J., & Morales, J. C. (2010). Antioxidant activity of resveratrol in several fish lipid matrices: Effect of acylation and glucosylation. *Journal of agricultural and food chemistry*, *58*(17), 9778-9786.

198. Mei, Y. Z., Liu, R. X., Wang, D. P., Wang, X., & Dai, C. C. (2015). Biocatalysis and biotransformation of resveratrol in microorganisms. *Biotechnology letters*, *37*(1), 9-18.

199. Milke, L., Aschenbrenner, J., Marienhagen, J., &Kallscheuer, N. (2018). Production of plant-derived polyphenols in microorganisms: current state and perspectives. *Applied microbiology and biotechnology*, *102*(4), 1575-1585.

200. Moes, A., Severs, D., Verdonk, K., van der Lubbe, N., Zietse, R., Danser, J., & Hoorn, E. J. (2018). Mycophenolate mofetil attenuates DOCA-salt hypertension: effects on vascular tone. *Frontiers in physiology*, *9*, 578.

201. Mohammad, M. A., Tisserant, L. P., & Boitel-Conti, M. (2016). Development of a liquid-liquid extraction method of resveratrol from cell culture media using solubility parameters. *Separation and purification technology*, *170*, 138-145.

202. Mokni, M., Hamlaoui, S., Karkouch, I., Amri, M., Marzouki, L., Limam, F., & Aouani, E. (2013). Resveratrol provides cardioprotection after ischemia/reperfusion injury via modulation of antioxidant enzyme activities. *Iranian journal of pharmaceutical research: IJPR*, *12*(4), 867.

203. Moraga, J., Gomes, W., Pinedo, C., Cantoral, J. M., Hanson, J. R., Carbú, M., & Collado, I. G. (2019). The current status on secondary metabolites produced by plant pathogenic *Colletotrichum* species. *Phytochemistry reviews*, *18*(1), 215-239.

204. Mora-Pale, M., Bhan, N., Masuko, S., James, P., Wood, J., McCallum, S., & Koffas, M. A. (2015). Antimicrobial mechanism of resveratrol-trans-dihydrodimer produced from

peroxidase-catalyzed oxidation of resveratrol. *Biotechnology and bioengineering*, *112*(12), 2417-2428.

205. Mostert, L., Crous, P. W., & Petrini, O. (2000). Endophytic fungi associated with shoots and leaves of Vitis vinifera, with specific reference to the *Phomopsis viticola* complex. *Sydowia*, *52*(1), 46-58.

206. Moussa, C., Hebron, M., Huang, X., Ahn, J., Rissman, R. A., Aisen, P. S., & Turner, R. S. (2017). Resveratrol regulates neuro-inflammation and induces adaptive immunity in alzheimer's disease. *Journal of neuroinflammation*, *14*(1), 1.

207. Movahed, A., Nabipour, I., Lieben Louis, X., Thandapilly, S. J., Yu, L., Kalantarhormozi, M., & Netticadan, T. (2013). Antihyperglycemic effects of short term resveratrol supplementation in type 2 diabetic patients. *Evidence-based complementary and alternative medicine*, *2013*.

208. Muñoz, O., Muñoz, R., & Bustamante, S. (2015). Pharmacological properties of resveratrol. a pre-clinical and clinical review. *BiochemPharmacol (Los Angel)*, *4*(184), 2167-0501.

209. Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., Marzani, C., & Pertot, I. (2006). Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus Alternaria alternata. *Phytopathology*, *96*(7), 689-698.

210. Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (2016). *Response surface methodology: process and product optimization using designed experiments*. John Wiley & Sons.

211. Nagamani, A., Kunwar, I. K., & Manoharachary, C. (2006). *Handbook of soil fungi*. IK international.

212. Nalli, Y., Arora, P., Khan, S., Malik, F., Riyaz-Ul-Hassan, S., Gupta, V., & Ali, A. (2019). Isolation, structural modification of macrophin from endophytic fungus phomamacrostoma and their cytotoxic potential. *Medicinal chemistry research*, 1-7.

213. Nalli, Y., Arora, P., Khan, S., Malik, F., Riyaz-Ul-Hassan, S., Gupta, V., & Ali, A. (2019).Isolation, structural modification of macrophin from endophytic fungus *Phoma macrostoma* and their cytotoxic potential. *Medicinal Chemistry Research*, *28*(3), 260-266.

214. Nandagopal, K., Halder, M., Dash, B., Nayak, S., & Jha, S. (2018). Biotechnological approaches for production of anti-cancerous compounds resveratrol, podophyllotoxin and zerumbone. *Current medicinal chemistry*, *25*(36), 4693-4717.

215. Nanjan, M. J., & Betz, J. (2014). Resveratrol for the Management of diabetes and its downstream pathologies. *European endocrinology*, *10*(1), 31.

216. Nawaz, W., Zhou, Z., Deng, S., Ma, X., Ma, X., Li, C., & Shu, X. (2017). Therapeutic versatility of resveratrol derivatives. *Nutrients*, *9*(11), 1188.

217. Netzker, T., Fischer, J., Weber, J., Mattern, D. J., König, C. C., Valiante, V., & Brakhage, A. A. (2015). Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Frontiers in microbiology*, *6*, 299.

218. Ngo, Y. L., Lau, C. H., & Chua, L. S. (2018). Review on rosmarinic acid extraction, fractionation and its anti-diabetic potential. *Food and chemical toxicology*, *121*, 687-700.

219. Niesen, D. B., Hessler, C., & Seeram, N. P. (2013). Beyond resveratrol: A review of natural stilbenoids identified from 2009–2013. *Journal of berry research*, *3*(4), 181-196.

220. Niu, Y., Fan, L., Gu, D., Wu, J., & Chen, Q. (2017). Characterization, enhancement and modelling of mannosylerythritol lipid production by fungal endophyte *Ceriporia lacerate* CHZJU. *Food chemistry*, *228*, 610-617.

221. Novelle, M. G., Wahl, D., Dieguez, C., Bernier, M., & de Cabo, R. (2015). Resveratrol supplementation: where are we now and where should we go?. *Ageing research reviews*, *21*, 1-15.

222. Oh, W. Y., & Shahidi, F. (2017). Lipophilization of resveratrol and effects on antioxidant activities. *Journal of agricultural and food chemistry*, *65*(39), 8617-8625.

223. Osono, T., & Mori, A. (2004). Distribution of phyllosphere fungi within the canopy of giant dogwood. *Mycoscience*, *45*(3), 161-168.

224. Oyenihi, O. R., Oyenihi, A. B., Adeyanju, A. A., & Oguntibeju, O. O. (2016). Antidiabetic effects of resveratrol: the way forward in its clinical utility. *Journal of diabetes research*, *2016*.

225. Palem, P. P., Kuriakose, G. C., & Jayabaskaran, C. (2015). An endophytic fungus, *Talaromyces radicus*, isolated from *Catharanthus roseus*, produces vincristine and vinblastine, which induce apoptotic cell death. *PloS one*, *10*(12), e0144476.

226. Pan, D., Kobayashi, A., Jiang, P., de Andrade, L. F., Tay, R. E., Luoma, A. M., & Long, H. W. (2018). A major chromatin regulator determines resistance of tumor cells to T cell–mediated killing. *Science*, *359*(6377), 770-775.

227. Parage, C., Tavares, R., Réty, S., Baltenweck-Guyot, R., Poutaraud, A., Renault, L., & Hugueney, P. (2012). Structural, functional and evolutionary analysis of the unusually large stilbene synthase gene family in grapevine (*Vitis vinifera*). *Plant Physiology*, pp-112.

228. Park, J., & Boo, Y. C. (2013). Isolation of resveratrol from *Vitis viniferae* caulis and its potent inhibition of human tyrosinase. *Evidence-Based Complementary and Alternative Medicine*.

229. Park, S. J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., & Kim, M. K. (2012). Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell*, *148*(3), 421-433.

230. Parker, J. A., Arango, M., Abderrahmane, S., Lambert, E., Tourette, C., Catoire, H., & Néri, C. (2005). Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. *Nature genetics*, *37*(4), 349.

231. Pasinetti, G. M., Wang, J., Ho, L., Zhao, W., & Dubner, L. (2015). Roles of resveratrol and other grape-derived polyphenols in Alzheimer's disease prevention and treatment. *BiochimicaetBiophysicaActa (BBA)-Molecular basis of disease*, *1852*(6), 1202-1208.

232. Patel, A., Patel, A., Patel, A., & Patel, N. M. (2010). Determination of polyphenols and free radical scavenging activity of *Tephrosia purpurealinn* leaves (Leguminosae). *Pharmacognosy research*, *2*(3), 152.

233. Paul, B., Masih, I., Deopujari, J., & Charpentier, C. (1999). The occurrence of resveratrol and pterostilbene in age-old darakchasava, an ayurvedic medicine from India. *Journal of ethnopharmacology*, *68*(1-3), 71-76.

234. Paulo, L., Ferreira, S., Gallardo, E., Queiroz, J. A., & Domingues, F. (2010). Antimicrobial activity and effects of resveratrol on human pathogenic bacteria. *World journal of microbiology and biotechnology*, *26*(8), 1533-1538.

235. Pearson, K. J., Baur, J. A., Lewis, K. N., Peshkin, L., Price, N. L., Labinskyy, N., & Jamieson, H. A. (2008). Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. *Cell metabolism*, *8*(2), 157-168.

236. Petrovski, G., Gurusamy, N., & Das, D. K. (2011). Resveratrol in cardiovascular health and disease. *Annals of the New York Academy of Sciences*, *1215*(1), 22-33.

237. Peuler, J. D. (2018). Resveratrol's potential in the adjunctive management of cardiovascular disease, obesity, diabetes, alzheimer disease, and cancer. *J Am Osteopath Assoc*, *118*(9), 596-605.

238. Pezzuto, J. M. (2008). Resveratrol as an inhibitor of carcinogenesis. *Pharmaceutical Biology*, *46*(7-8), 443-573.

239. Pfannenstiel, B. T., & Keller, N. P. (2019). On top of biosynthetic gene clusters: how epigenetic machinery influences secondary metabolism in fungi. *Biotechnology advances*.

240. Premalatha, N., Gopal, N. O., Jose, P. A., Anandham, R., & Kwon, S. W. (2015). Optimization of cellulase production by *Enhydrobacter* sp. ACCA2 and its application in biomass saccharification. *Frontiers in microbiology*, *6*, 1046.

241. Puri, S. C., Nazir, A., Chawla, R., Arora, R., Riyaz-ul-Hasan, S., Amna, T., & Sharma, A. (2006). The endophytic fungus *Trameteshirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralinlignans. *Journal of biotechnology*, *122*(4), 494-510.

242. Qiao, W., Ling, F., Yu, L., Huang, Y., & Wang, T. (2017). Enhancing taxol production in a novel endophytic fungus, *Aspergillus aculeatinus* Tax-6, isolated from *Taxus chinensis* var. mairei. *Fungal biology*, *121*(12), 1037-1044.

243. Rajput, H., Dhir, A., & kumarSangal, V. (2018). GO mediated TiO_2 nanotube electrode for the photoelectrocatalytic degradation of pentachlorophenol. *Journal of the electrochemical society*, *165*(2), H16-H26.

244. Ramirez-Lopez, L. M., & DeWitt, C. A. (2014). Analysis of phenolic compounds in commercial dried grape pomace by high-performance liquid chromatography electrospray ionization mass spectrometry. *Food science & nutrition*, *2*(5), 470-477.

245. Ran, X., Zhang, G., Li, S., & Wang, J. (2017). Characterization and antitumor activity of camptothecin from endophytic fungus *Fusarium solani* isolated from *Camptotheca acuminate*. *African health sciences*, *17*(2), 566-574.

246. Rascón, B., Hubbard, B. P., Sinclair, D. A., & Amdam, G. V. (2012). The lifespan extension effects of resveratrol are conserved in the honey bee and may be driven by a mechanism related to caloric restriction. *Aging (Albany NY)*, *4*(7), 499.

247. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*, *26*(9-10), 1231-1237.

248. Reinisalo, M., Kårlund, A., Koskela, A., Kaarniranta, K., & Karjalainen, R. O. (2015). Polyphenol stilbenes: molecular mechanisms of defence against oxidative stress and aging-related diseases. *Oxidative medicine and cellular longevity*.

249. Renaud, S. D., & de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet*, *339*(8808), 1523-1526.

250. Rimando, A. M., & Cody, R. (2005). Determination of stilbenes in blueberries. *LCGC North America*, *23*(11), 1192-1200.

251. Rodriguez, R. J., White Jr, J. F., Arnold, A. E., & Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New phytologist*, *182*(2), 314-330.

252. Rokaya, M. B., Maršík, P., & Münzbergová, Z. (2012). Active constituents in *Rheum acuminatum* and *Rheum australe* (*Polygonaceae*) roots: A variation between cultivated and naturally growing plants. *Biochemical systematics and ecology*, *41*, 83-90.

253. Rozpądek, P., Domka, A. M., & Turnau, K. (2019). Are fungal endophytes merely mycorrhizal copycats? The role of fungal endophytes in the adaptation of plants to metal toxicity. *Frontiers in microbiology*, *10*, 371.

254. Ruch, R. J., Cheng, S. J., & Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from chinese green tea. *Carcinogenesis*, *10*(6), 1003-1008.

255. Ruderman, N. B., Julia Xu, X., Nelson, L., Cacicedo, J. M., Saha, A. K., Lan, F., & Ido, Y. (2010). AMPK and SIRT1: a long-standing partnership?. *American journal of physiology-*

endocrinology and metabolism, *298*(4), E751-E760.

256. Rühmann, S., Pfeiffer, J., Brunner, P., Szankowski, I., Fischer, T. C., Forkmann, G., & Treutter, D. (2013). Induction of stilbene phytoalexins in grapevine (*Vitis vinifera*) and transgenic stilbene synthase-apple plants (Malus domestica) by a culture filtrate of *Aureobasidium pullulans*. *Plant physiology and biochemistry*, *72*, 62-71.

257. Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, *4*(4), 406-425.

258. Salehi, B., Mishra, A., Nigam, M., Sener, B., Kilic, M., Sharifi-Rad, M., & Sharifi-Rad, J. (2018). Resveratrol: a double-edged sword in health benefits. *Biomedicines*, *6*(3), 91.

259. Samaradivakara, S. P., Samarasekera, R., Handunnetti, S. M., Weerasena, O. J., Al-Hamashi, A. A., Slama, J. T., & Tillekeratne, L. V. (2018). A bioactive resveratrol trimer from the stem bark of the sri lankan endemic plant vateria copallifera. *Journal of natural products*, *81*(8), 1693-1700.

260. Sánchez-Moreno, C. (2002). Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food science and technology international*, *8*(3), 121-137.

261. Santos, I. P. D., Silva, L. C. N. D., Silva, M. V. D., Araújo, J. M. D., Cavalcanti, M. D. S., & Lima, V. L. D. M. (2015). Antibacterial activity of endophytic fungi from leaves of Indigo *ferasuffruticosa* Miller (Fabaceae). *Frontiers in microbiology*, *6*, 350.

262. Santos-Buelga, C., Gonzalez-Manzano, S., Dueñas, M., & Gonzalez-Paramas, A. M. (2012). Extraction and isolation of phenolic compounds. In *Natural products isolation*. Humana Press, 427-464.

263. Saran, S., Mukherjee, S., Dalal, J., & Saxena, R. K. (2015). High production of erythritol from *Candida sorbosivorans* SSE-24 and its inhibitory effect on biofilm formation of *Streptococcus* mutans. *Bioresource technology*, *198*, 31-38.

264. Sasikumar, P., Lekshmy, K., Sini, S., Prabha, B., Kumar, N. A., Sivan, V. V., & Radhakrishnan, K. V. (2019). Isolation and characterization of resveratrol oligomers from the stem bark of Hopeaponga (Dennst.) Mabb. And their antidiabetic effect by modulation of digestive

enzymes, protein glycation and glucose uptake in L6 myocytes. *Journal of ethnopharmacology*, *236*, 196-204.

265. Saxena, S., & Srivastava, A. (2014). Resveratrol: biological activities and therapeutic potential.

266. Schulz, B., Wanke, U., Draeger, S., & Aust, H. J. (1993). Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological research*, *97*(12), 1447-1450.

267. Sergent, T., Kohnen, S., Jourez, B., Beauve, C., Schneider, Y. J., & Vincke, C. (2014). Characterization of black locust (Robinia pseudoacacia L.) heartwood extractives: identification of resveratrol and piceatannol. *Wood science and technology*, *48*(5), 1005-1017.

268. Setati, M. E., Jacobson, D., & Bauer, F. F. (2015). Sequence-based analysis of the Vitis vinifera L. cv Cabernet Sauvignon grape must mycobiome in three South African vineyards employing distinct agronomic systems. *Frontiers in microbiology*, *6*, 1358.

269. Shannon, C. E., Weaver, W., & Burks, A. W. (1951). The mathematical theory of communication.

270. Sharma, V. K., Kumar, J., Singh, D. K., Mishra, A., Verma, S. K., Gond, S. K., & Kharwar, R. N. (2017). Induction of Cryptic and Bioactive Metabolites through Natural Dietary Components in an Endophytic Fungus *Colletotrichum gloeosporioides* (Penz.) Sacc. *Frontiers in microbiology*, *8*, 1126.

271. Shen, T., Wang, X. N., & Lou, H. X. (2009). Natural stilbenes: an overview. *Natural product reports*, *26*(7), 916-935.

272. Shen, X., Du, Q., Xu, Y., & Lu, Y. (2012). Stimulation of trans-resveratrol biosynthesis in Vitis vinifera cv. Kyoho cell suspension cultures by 2, 3-dihydroxypropyl jasmonate elicitation. *Electronic journal of biotechnology*, *15*(5), 3-3.

273. Shi, D. D., Dong, C. M., Ho, L. C., Lam, C. T. W., Zhou, X. D., Wu, E. X., & Zhang, Z. J. (2018). Resveratrol, a natural polyphenol, prevents chemotherapy-induced cognitive impairment: Involvement of cytokine modulation and neuroprotection. *Neurobiology of disease*, *114*, 164-173.

274. Shi, J., Zeng, Q., Liu, Y., & Pan, Z. (2012). *Alternaria* sp. MG1, a resveratrol-producing fungus: isolation, identification, and optimal cultivation conditions for resveratrol production. *Applied microbiology and biotechnology*, *95*(2), 369-379.

275. Shrikanta, A., Kumar, A., & Govindaswamy, V. (2015) Resveratrol content and antioxidant properties of underutilized fruits. *Journal of food science and technology, 52*(1), 383-390

276. Sidhoum, W., & Fortas, Z. (2019). The beneficial role of indigenous arbuscular mycorrhizal fungi in phytoremediation of wetland plants and tolerance to metal stress. *Archives of environmental protection*, *45*(1).

277. Signorelli, P., Munoz-Olaya, J. M., Gagliostro, V., Casas, J., Ghidoni, R., & Fabriàs, G. (2009). Dihydroceramide intracellular increase in response to resveratrol treatment mediates autophagy in gastric cancer cells. *Cancer letters*, *282*(2), 238-243.

278. Simioni, C., Zauli, G., Martelli, A. M., Vitale, M., Sacchetti, G., Gonelli, A., & Neri, L. M. (2018). Oxidative stress: role of physical exercise and antioxidant nutraceuticals in adulthood and aging. *Oncotarget*, *9*(24), 17181.

279. Simpson, E. H. (1951). The interpretation of interaction in contingency tables. *Journal of the royal statistical society: series b (Methodological)*, *13*(2), 238-241.

280. Singh, D. K., Sharma, V. K., Kumar, J., Mishra, A., Verma, S. K., Sieber, T. N., & Kharwar, R. N. (2017). Diversity of endophytic mycobiota of tropical tree *Tectonagrandis Linn*. f.: Spatiotemporal and tissue type effects. *Scientific reports*, *7*(1), 3745.

281. Singh, D., Mendonsa, R., Koli, M., Subramanian, M., & Nayak, S. K. (2019). Antibacterial activity of resveratrol structural analogues: A mechanistic evaluation of the structure-activity relationship. *Toxicology and applied pharmacology*.

282. Sinha, K., Chaudhary, G., & Gupta, Y. K. (2002). Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. *Life sciences*, *71*(6), 655-665.

283. Sivakumar, B., Murugan, R., Baskaran, A., Khadangale, B., Murugan, S., & Senthilkumar, U. (2013). Identification and characterization of process-related impurities of trans-resveratrol. *Scientia pharmaceutica*, *81*(3), 683-696.

284. Snopek, L., Mlcek, J., Sochorova, L., Baron, M., Hlavacova, I., Jurikova, T., & Sochor, J. (2018). The contribution of red wine consumption to human health protection. *Molecules (Basel, Switzerland)*, *23*(7).

285. Sobolev, V. S., Khan, S. I., Tabanca, N., Wedge, D. E., Manly, S. P., Cutler, S. J., & Gloer, J. B. (2011). Biological activity of peanut (Arachis hypogaea) phytoalexins and selected natural and synthetic stilbenoids. *Journal of agricultural and food chemistry*, *59*(5), 1673-1682.

286. Soleas, G. J., Diamandis, E. P., & Goldberg, D. M. (1997). Resveratrol: a molecule whose time has come? And gone?. *Clinical biochemistry*, *30*(2), 91-113.

287. Somrithipol, S. (2004). Coprophilous fungi. In: Thai fungal diversity (eds. E.B.G. Jones, M. Tanticharoen and K.D. Hyde). BIOTEC, Thailand: 119-128

288. Soural, I., Vrchotová, N., Tříska, J., Balík, J., Horník, Š.,Cuřínová, P., & Sýkora, J. (2015). Various extraction methods for obtaining stilbenes from grape cane of *Vitis vinifera* L. *Molecules*, *20*(4), 6093-6112.

289. Soytong, K. & Quimio, T.H. (1989). A taxonomic study on the philippine species of *Chaetomium*. *The Philippine agriculturist*, *72(1)*, 59-72.

290. 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.

291. Stone, J. K., Bacon, C. W., & White Jr, J. F. (2000). An overview of endophytic microbes: endophytism defined. In *Microbial endophytes*, 17-44).

292. Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and molecular biology reviews*, *67*(4), 491-502.

293. Strobel, G., Daisy, B., Castillo, U., & Harper, J. (2004). Natural products from endophytic microorganisms. *Journal of natural products*, *67*(2), 257-268.

294. Su, D., Cheng, Y., Liu, M., Liu, D., Cui, H., Zhang, B., & Mei, Q. (2013). Comparision of piceid and resveratrol in antioxidation and antiproliferation activities in vitro. *PloS one*, *8*(1), e54505.

295. Sung, M. M., Das, S. K., Levasseur, J., Byrne, N. J., Fung, D., Kim, T. T., & Dyck, J. R. (2015). Resveratrol treatment of mice with pressure-overload–induced heart failure improves diastolic function and cardiac energy metabolism. *Circulation: Heart Failure*, *8*(1), 128-137.

296. Suryanarayanan, T. S., Thirunavukkarasu, N., Govindarajulu, M. B., Sasse, F., Jansen, R., & Murali, T. S. (2009). Fungal endophytes and bioprospecting. *Fungal biology reviews*, *23*(1-2), 9-19.

297. Szkudelski, T., & Szkudelska, K. (2015). Resveratrol and diabetes: from animal to human studies. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1852*(6), 1145-1154.

298. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, *28*(10), 2731-2739.

299. Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729.

300. Tang, G. Y., Zhao, C. N., Liu, Q., Feng, X. L., Xu, X. Y., Cao, S. Y., & Li, H. B. (2018). Potential of grape wastes as a natural source of bioactive compounds. *Molecules*, *23*(10), 2598.

301. Tassoni, A., Fornale, S., Franceschetti, M., Musiani, F., Michael, A. J., Perry, B., & Bagni, N. (2005). Jasmonates and Na-orthovanadate promote resveratrol production in *Vitis vinifera* cv. Barbera cell cultures. *New Phytol*, *166*, 895–905.

302. Tastekin, B., Pelit, A., Polat, S., Tuli, A., Sencar, L., Alparslan, M. M., & Daglioglu, Y. K. (2018). Therapeutic potential of pterostilbene and resveratrol on biomechanic, biochemical, and histological parameters in streptozotocin-induced diabetic rats. *Evidence-based complementary and alternative medicine*, *2018*.

303. Tavares, S., Vesentini, D., Fernandes, J. C., Ferreira, R. B., Laureano, O., Ricardo-Da-Silva, J. M., & Amâncio, S. (2013). *Vitis vinifera* secondary metabolism as affected by sulfate depletion: diagnosis through phenyl propanoid pathway genes and metabolites. *Plant physiology and biochemistry*, *66*, 118-126.

304. Tedersoo, L., Nilsson, R. H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., & Kõljalg, U. (2010). 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. *New phytologist*, *188*(1), 291-301.

305. Tejesvi, M. V., Kajula, M., Mattila, S., & Pirttilä, A. M. (2011). Bioactivity and genetic diversity of endophytic fungi in *Rhododendron tomentosum* Harmaja. *Fungal diversity*, *47*(1), 97-107.

306. Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., & Byrne, D. H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of food composition and analysis*, *19*(6-7), 669-675.

307. Tian, Y., Wang, Y., Ma, Y., Zhu, P., He, J., & Lei, J. (2017). Optimization of subcritical water extraction of resveratrol from grape seeds by response surface methodology. *Applied Sciences*, *7*(4), 321.

308. Timperio, A. M., d'Alessandro, A., Fagioni, M., Magro, P., & Zolla, L. (2012). Production of the phytoalexins trans-resveratrol and delta-viniferin in two economy-relevant grape cultivars upon infection with Botrytis cinerea in field conditions. *Plant physiology and biochemistry*, *50*, 65-71.

309. Toghueo, R. M. K., Sahal, D., Zabalgogeazcoa, Í., Baker, B., & Boyom, F. F. (2018). Conditioned media and organic elicitors underpin the production of potent antiplasmodial metabolites by endophytic fungi from Cameroonian medicinal plants. *Parasitology research*, 1-13.

310. Treviño-Saldaña, N., & García-Rivas, G. (2017). Regulation of sirtuin-mediated protein deacetylation by cardioprotective phytochemicals. *Oxidative medicine and cellular longevity*, *2017*.

311. Tsai, H. Y., Ho, C. T., & Chen, Y. K. (2017). Biological actions and molecular effects of resveratrol, pterostilbene, and 3′-hydroxypterostilbene. *Journal of food and drug analysis*, *25*(1), 134-147.

312. Tsai, T. F., Lin, J. F., Chou, K. Y., Lin, Y. C., Chen, H. E., & Hwang, T. I. S. (2018). miR-99a-5p acts as tumor suppressor via targeting to mTOR and enhances RAD001-induced apoptosis in human urinary bladder urothelial carcinoma cells. *OncoTargets and therapy*, *11*, 239.

313. Turner, R. S., Thomas, R. G., Craft, S., Van Dyck, C. H., Mintzer, J., Reynolds, B. A., & Mintzer, J. (2015). A randomized, double-blind, placebo-controlled trial of resveratrol for alzheimer disease. *Neurology*, *85*(16), 1383-1391.

314. Turrini, E., Calcabrini, C., Tacchini, M., Efferth, T., Sacchetti, G., Guerrini, A., & Fimognari, C. (2018). In Vitro Study of the Cytotoxic, Cytostatic, and Antigenotoxic Profile of Hemidesmus indicus (L.) R. Br. (Apocynaceae) Crude Drug Extract on T Lymphoblastic Cells. *Toxins*, *10*(2), 70.

315. Ul-Hassan, S. R., Strobel, G. A., Booth, E., Knighton, B., Floerchinger, C., & Sears, J. (2012). Modulation of volatile organic compound formation in the mycodiesel-producing endophyte *Hypoxylon* sp. CI-4. *Microbiology*, *158*(2), 465-473.

316. Unterseher, M., Gazis, R., Chaverri, P., Guarniz, C. F. G., & Tenorio, D. H. Z. (2013). Endophytic fungi from Peruvian highland and lowland habitats form distinctive and host plant-specific assemblages. *Biodiversity and conservation*, *22*(4), 999-1016.

317. VanderMolen, K. M., Darveaux, B. A., Chen, W. L., Swanson, S. M., Pearce, C. J., & Oberlies, N. H. (2014). Epigenetic manipulation of a filamentous fungus by the proteasome-inhibitor bortezomib induces the production of an additional secondary metabolite. *RSC advances*, *4*(35), 18329-18335.

318. Vannozzi, A., Dry, I. B., Fasoli, M., Zenoni, S., & Lucchin, M. (2012). Genome-wide analysis of the grapevine stilbene synthase multigenic family: genomic organization and expression profiles upon biotic and abiotic stresses. *BMC plant biology*, *12*(1), 130.

319. Varanda, C. M. R., Oliveira, M., Materatski, P., Landum, M., Clara, M. I. E., & do Rosário Félix, M. (2016). Fungal endophytic communities associated to the phyllosphere of grapevine cultivars under different types of management. *Fungal biology*, *120*(12), 1525-1536.

320. Varoni, E. M., Lo Faro, A. F., Sharifi-Rad, J., & Iriti, M. (2016). Anticancer molecular mechanisms of resveratrol. *Frontiers in nutrition*, *3*, 8.

321. Vastano, B. C., Chen, Y., Zhu, N., Ho, C. T., Zhou, Z., & Rosen, R. T. (2000). Isolation and Identification of Stilbenes in Two Varieties of *Polygonum cuspidatum*. *Journal of agricultural and food chemistry*, *48*(2), 253-256.

322. Verma, V. C., Gond, S. K., Kumar, A., Kharwar, R. N., & Strobel, G. (2007). The endophytic mycoflora of bark, leaf, and stem tissues of *azadirachta indica* A. Juss (Neem) from Varanasi (India). *Microbial Ecology*, *54*(1), 119-125.

323. Vogt, T. (2010). Phenylpropanoid biosynthesis. *Molecular plant*, *3*(1), 2-20.

324. Vrchotová, N., & Sera, B. (2008). Allelopathic properties of knotweed rhizome extracts. *Plant soil environment*, *54*(7), 301-303.

325. Vuong, T. V., Franco, C., & Zhang, W. (2014). Treatment strategies for high reseveratrol induction in Vitis vinifera L. cell suspension culture. *Biotechnology reports*, *1*, 15-21.

326. Waddington, C. H. (1942). The epigenotype. *Endeavour*, *1*, 18-20.

327. Walle, T., Hsieh, F., DeLegge, M. H., Oatis, J. E., & Walle, U. K. (2004). High absorption but very low bioavailability of oral resveratrol in humans. *Drug metabolism and disposition*, *32*(12), 1377-1382.

328. Wang, D. G., Liu, W. Y., & Chen, G. T. (2013). A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*. *Journal of pharmaceutical analysis*, *3*(4), 241-247.

329. Wang, J., Cox, D. G., Ding, W., Huang, G., Lin, Y., & Li, C. (2014). Three new resveratrol derivatives from the mangrove endophytic fungus *Alternaria* sp. *Marine drugs*, *12*(5), 2840-2850.

330. Wang, J., Wang, S., Liu, G., Edwards, E. J., Duan, W., Li, S., & Wang, L. (2016). The synthesis and accumulation of resveratrol are associated with veraison and abscisic acid concentration in Beihong (*Vitis vinifera*× *Vitis amurensis*) Berry Skin. *Frontiers in plant science*, *7*, 1605.

331. Wang, Q., Xu, J., Rottinghaus, G. E., Simonyi, A., Lubahn, D., Sun, G. Y., & Sun, A. Y. (2002). Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain research*, *958*(2), 439-447.

332. Wang, X. W., Houbraken, J., Groenewald, J. Z., Meijer, M., Andersen, B., Nielsen, K. F., & Samson, R. A. (2016). Diversity and taxonomy of *Chaetomium* and *chaetomium*-like fungi from indoor environments. *Studies in Mycology*, *84*, 145-224.

333. Wang, Y., Chen, S., & Yu, O. (2011). Metabolic engineering of flavonoids in plants and microorganisms. *Applied microbiology and biotechnology*, *91*(4), 949.

334. Wang, Y., Zeng, Q. G., Zhang, Z. B., Yan, R. M., Wang, L. Y., & Zhu, D. (2011). Isolation and characterization of endophytic huperzine A-producing fungi from *Huperzia serrata*. *Journal of industrial microbiology & biotechnology*, *38*(9), 1267-1278.

335. Wani, Z. A., Ashraf, N., Mohiuddin, T., & Riyaz-Ul-Hassan, S. (2015). Plant-endophyte symbiosis, an ecological perspective. *Applied microbiology and biotechnology*, *99*(7), 2955-2965.

336. Wei, Y., Li, P., Ma, L., & Li, J. (2014). Separation and purification of four stilbenes from *Vitis vinifera* L. cv. cabernet sauvignon roots through high-speed counter-current chromatography. *South african journal of enology and viticulture*, *35*(2), 226-233.

337. White Jr, J. F., & Torres, M. S. (2010). Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection?. *Physiologia plantarum*, *138*(4), 440-446.

338. White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. L. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, *18*(1), 315-322.

339. Whittaker, R. H. (1972). Evolution and measurement of species diversity. *Taxon*, 213-251.

340. Williams, R. B., Henrikson, J. C., Hoover, A. R., Lee, A. E., & Cichewicz, R. H. (2008). Epigenetic remodeling of the fungal secondary metabolome. *Organic & biomolecular chemistry*, *6*(11), 1895-1897.

341. Wilson, S. A., & Roberts, S. C. (2012). Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant biotechnology journal*, *10*(3), 249-268.

342. Wong, R., & Howe, P. (2018). Resveratrol Counteracts Insulin Resistance—Potential Role of the Circulation. *Nutrients*, *10*(9), 1160.

343. Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M., & Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay aging in metazoans. *Nature*, *430*(7000), 686.

344. Xu, D., Yu, G., Xi, P., Kong, X., Wang, Q., Gao, L., & Jiang, Z. (2018). Synergistic effects of resveratrol and pyrimethanil against *Botrytis cinerea* on grape. *Molecules*, *23*(6), 1455.

345. Xu, F., Wang, S., Li, Y., Zheng, M., Xi, X., Cao, H., & Han, C. (2018). Yield enhancement strategies of rare pharmaceutical metabolites from endophytes. *Biotechnology letters*, 1-11.

346. Xu, W., Yu, Y., Zhou, Q., Ding, J., Dai, L., Xie, X., & Wang, Y. (2011). Expression pattern, genomic structure, and promoter analysis of the gene encoding stilbene synthase from Chinese wild Vitis pseudoreticulata. *Journal of experimental botany*, *62*(8), 2745-2761.

347. Yang, M. Z., Ma, M. D., Yuan, M. Q., Huang, Z. Y., Yang, W. X., Zhang, H. B., & Shan, H. (2016). Fungal endophytes as a metabolic fine-tuning regulator for wine grape. *PloS one*, *11*(9), e0163186.

348. Yazir, Y., Şahin, T. D., Rençber, S. F., Gacar, G., Halbutoğulları, Z. S., Utkan, T., & Aricioglu, F. (2018). Restorative effect of resveratrol on expression of endothelial and neuronal nitric oxide synthase in cavernous tissues of chronic unpredictable mild stress-exposed rats: an impact of inflammation. *International journal of impotence research*, 1.

349. Yu, S., & Li, G. (2010). MicroRNA expression and function in cardiac ischemic injury. *Journal of cardiovascular translational research*, *3*(3), 241-245.

350. Yuan, H., Ma, Q., Ye, L., & Piao, G. (2016).The traditional medicine and modern medicine from natural products. *Molecules*, *21*(5), 559.

351. Yun, T. Y., Feng, R. J., Zhou, D. B., Pan, Y. Y., Chen, Y. F., Wang, F., & Xie, J. H. (2018). Optimization of fermentation conditions through response surface methodology for enhanced antibacterial metabolite production by *Streptomyces* sp. 1-14 from cassava rhizosphere. *PloS one*, *13*(11), e0206497.

352. Zenthoefer, M., Geisen, U., Hofmann-Peiker, K., Fuhrmann, M., Kerber, J., Kirchhöfer, R., & Kalthoff, H. (2017). Isolation of polyphenols with anticancer activity from the Baltic Sea brown seaweed *Fucus vesiculosus* using bioassay-guided fractionation. *Journal of applied phycology*, *29*(4), 2021-2037.

353. Zgoda-Pols, J. R., Freyer, A. J., Killmer, L. B., & Porter, J. R. (2002). Antimicrobial Resveratrol Tetramers from the Stem Bark of *Vatica o blongifolia* ssp. o blongifolia. *Journal of natural products*, *65*(11), 1554-1559.

354. Zhang, D., Li, X., Hao, D., Li, G., Xu, B., Ma, G., & Su, Z. (2009). Systematic purification of polydatin, resveratrol and anthraglycoside B from *Polygonum cuspidatum* Sieb. etZucc. *Separation and purification technology*, *66*(2), 329-339.

355. Zhang, H. W., Song, Y. C., & Tan, R. X. (2006). Biology and chemistry of endophytes. *Natural product reports*, *23*(5), 753-771.

356. Zhang, J., Shi, J., & Liu, Y. (2013). Bioconversion of resveratrol using resting cells of non–genetically modified Alternaria sp. *Biotechnology and applied biochemistry*, *60*(2), 236-243.

357. Zhang, Q., Li, Y., Xu, F., Zheng, M., Xi, X., Zhang, X., & Han, C. (2017).Optimization of Submerged Fermentation Medium for Matrine Production by *Aspergillus terreus*, an Endophytic Fungus Harboring Seeds of Sophoraflavescens, Using Response Surface Methodology. *Mycobiology*, *45*(2), 90-96.

358. Zhang, T., Chi, Y., Ren, Y., Du, C., Shi, Y., & Li, Y. (2019). Resveratrol reduces oxidative stress and apoptosis in podocytes via sir2-related enzymes, sirtuins1 (sirt1)/peroxisome proliferator-activated receptor γ co-activator 1α (pgc-1α) axis. *medical science monitor: international medical journal of experimental and clinical research*, *25*, 1220.

359. Zhao, J., Fu, Y., Luo, M., Zu, Y., Wang, W., Zhao, C., & Gu, C. (2012). Endophytic fungi from pigeon pea [*Cajanus cajan* (L.) Millsp.] produce antioxidant cajaninstilbene acid. *Journal of agricultural and food chemistry*, *60*(17), 4314-4319.

360. Zheng, X., Shi, J., Yu, Y., Shen, Y., Tan, B., Ye, X., & Feng, J. (2017). Exploration of Elite Stilbene Synthase Alleles for Resveratrol Concentration in Wild Chinese *Vitis* spp. and *Vitis* Cultivars. *Frontiers in plant science*, *8*, 487.

361. Zhou, Y., Zhou, X., Yu, D. L., Sang, B., Feng, J. T., Han, L. R., & Zhang, X. (2018). Optimization of fermentation conditions and bench-scale for improvement of a novel glycoprotein gp-1 production by *Streptomyces kanasenisi* ZX01. *Molecules*, *23*(1), 137.

362. Zhou, Z., Wang, M., Guo, Z., & Zhang, X. (2016). Pharmacokinetic evaluation of the interaction between oral kaempferol and ethanol in rats. *ActaPharmaceutica*, *66*(4), 563-568.

363. Zhu, T., Heo, H. J., & Row, K. H. (2010). Optimization of crude polysaccharides extraction from Hizikia fusiformis using response surface methodology. *Carbohydrate polymers*, *82*(1), 106-110.

364. Zordoky, B. N., Robertson, I. M., & Dyck, J. R. (2015). Preclinical and clinical evidence for the role of resveratrol in the treatment of cardiovascular diseases. *BiochimicaetBiophysicaActa (BBA)-molecular basis of disease*, *1852*(6), 1155-1177.

365. Zutz, C., Bandian, D., Neumayer, B., Speringer, F., Gorfer, M., Wagner, M., & Rychli, K. (2014). Fungi treated with small chemicals exhibit increased antimicrobial activity against facultative bacterial and yeast pathogens. *BioMed research international*, *2014*.

EXECUTIVE SUMMARY

The present study focusses on the diversity of resveratrol producing fungal endophytes associated with different wine grape varieties grown in India. A total of 145 endophytic isolates were recovered of which 55 were from Pune region, 54 from Nashik while 26 were from Lucknow and 9 from Bengaluru. Only 30% isolates exhibited extracellular resveratrol production in the preliminary screening. However only 14 fungal endophytes exhibited significant resveratrol production in the range of 4.4g/L-89.1g/L in liquid cultures which was evaluated by HPLC analysis. Four potent resveratrol producing endophytes isolated were #12VVLPM; #4(P)VVLNM; #22(P) VVLPM and #19VVLPM. #12 VVLPM was identified to be *Arcopilus* aureus, while #22(P) VVLPM was identified to be *Xylaria psidii*. Both *Arcopilus aureus* and *Xylaria psidii* have so far not been reported to be resveratrol producers. The other two isolates viz. #4(P)VVLNM and #19VVLPM are generalists identified as *Fusarium solani* and Fusarium *equiseti*. Highest resveratrol production, 89.1 g/l was recorded in #12VVLPM identified as *Arcopilus aureus*.

The fungal resveratrol exhibited a strong anti-oxidant activity in all the tested assays.The endophytic isolate #12VVLPM exhibited the best activity similar to the standards in the entire anti-oxidant assay performed during the present course of investigations. Yet another important result was the anti-mycotic activity against different plant as well as human pathogenic fungi tested. The highest inhibition was observed against *B. cinerea* (57.8± 0.8%) followed by *Cercospora beticola* (ATCC24888) by extracts of #12VVLPM while the least inhibition was observed in #22(P) VVLPM and #4(P) VVLNM against same pathogen. Among the resveratrol producing endophytic isolates #12VVLPM exhibited the best inhibitory activity against all the cultures tested in the panel.

The fungal resveratrol was obtained in pure form ethnolic extract by eluting it in a column with mobile phase of MeOH: DCM :: 1.75:98.25. Fungal resveratrol (25 mg) was observed pure by TLC system 5% MeOH: DCM and concentrated by rotary evaporator. The homogeneity of the purified fungal resveratrol was confirmed by HPLC, which exhibited a single, symmetrical peak at R_t 3.36 min on C_{18} reverse phase column similar to that of standard resveratrol. For further confirmation of the presence of fungal resveratrol purified from the spent broth of *Arcopilus aureus*, ^{1}H-NMR, ^{13}C-NMR. MS and HR-MS, FTIR analysis was carried out which revealed the presence and purity of fungal resveratrol.

Optimization of different physic-chemical and nutritional parameters affecting resveratrol production by 'one-variable-at-a-time' approach resulted in the production 1.22 fold increases of (109.61[a] ± 0.7 µg/ml resveratrol from 89.1[a] ± 0.08 µg/ml from initial yield of resveratrol. The interactions of the most influential factors obtained by one-variable-at-a-time method were further optimized using a statistical approach, response surface methodology (RSM). This finally resulted in 1.49 fold increase production of resveratrol (133.5 µg/ml) yield of resveratrol. After optimization of various physiological and nutritional parameters using one variable at a time and RSM using shake flasks, the optimized conditions were put to test for epigenetic modification. This finally resulted in 1.85 fold increase yield of resveratrol i.e. 165.0 µg/ml. The present study provides prime information on a novel source for the fermentative production of the billion dollar drug resveratrol by the endophytic fungus *Arcopilus aureus*. Fungal resveratrol was found to be as efficient as that of standard resveratrol which paves new way for exploitation of these bio actively potent endophytic fungi for industrial bioprocess. This study a step forward to proving that endophytic fungi are able to produce billion dollar drugs which are highly demandable due to its multifarious application and paves a new way for replacement of conventional sources of these molecules by serving as alternative.

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ORIGINAL ARTICLE

Diversity and phylogeny of resveratrol-producing culturable endophytic fungi from *Vitis* species in India

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Abstract

In the present study, resveratrol producing culturable endophytes were recovered from the Indian vineyards. Of the 145 endophytic fungi recovered, only 30.3% were positive for resveratrol production in the preliminary screening. Culture broth of only 13 isolates exhibited the actual presence of resveratrol in the range of 4.4–52.3 µg/ml by HPLC quantification. The most potent endophytic isolate producing the highest extracellular resveratrol was #19VVLPM isolated from the Pinot Noir variety. Further LC–MS analysis confirmed the similarity of fungal resveratrol with standard resveratrol. The Merlot variety exhibited the highest colonization frequency of the resveratrol-producing fungal endophytes. Using morphological methods as well as molecular phylogeny #19VVLPM was identified as *Fusarium equiseti*. It is a promising candidate for further development into a commercial strain.

Keywords *Vitis vinifera* · Resveratrol · Molecular phylogeny · Fungi · Colonization frequency

Introduction

Endophytic fungi generally colonize within the living tissues of the host plants by latent infection to obtain shelter and nutrition and therefore do not exhibit any obvious signatures of their existence. The signal molecules so produced by them to overcome inherent plant defence mechanisms purportedly play a role in aiding the host plant to combat different abiotic and biotic stresses (Busby et al. 2017; Kaul et al. 2017).

The concept of exploiting these signal molecules for crop protection or development into drug molecules has been the basis of endophytism which emerged two decades back with the discovery of *Taxomyces andreanae*, the taxol producing fungus from *Taxus brevifolia*. The production of the host metabolite by a fungus was attributed to the phenomena of horizontal gene transfer (Schulz et al. 1993; Strobel and Daisy 2003) which can now be traced using molecular tools.

Resveratrol (RESV) (3,4,5-trihydroxy-*trans*-stilbene) was initially isolated as phytoalexin and later as a phytochemical constituent of grapevines, mulberry and Japanese Knotweed (Langcake and Pryce 1977). The medicinal properties of RESV came into the limelight with the phenomena of the "French Paradox", which suggested that the consumption of red wine by French people prevented them from coronary heart disease despite their food habits. Alternative sources of production of resveratrol other than phytochemical extraction from *Polygonum cuspidatum* is extensively being explored to meet the global demand of this molecule.

One possible mechanisms of enhancing the production of RESV is through exploration of culturable endophytic fungi capable of producing extracellular resveratrol. The biosynthetic capabilities of the RESV-producing endophytes can be further confirmed by screening the genes of phenylpropanoid pathway, which is generally used for the production of RESV in the plants. One such gene is STS gene which converts the p-coumaryl Co A into 4-coumaryl CoA molecule, which along with 3 malonyl CoA molecules forms RESV (Schijlen et al. 2006; Beekwilder et al. 2006; Schwekendiek et al. 2007).

The majority of the studies from India primarily focus on the characterization and epidemiology of pathogenic

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fungi associated with *Vitis vinifera* or explains the method of crop protection. However, the present study is the very first of its kind in exploring the culturable endophytes of different varieties of *V. vinifera* growing in vineyards for their potential to produce resveratrol by the fermentation route.

Materials and methods

Plant sample collection and isolation of endophytes

The plant samples were collected from vineyards in Pune, Nashik, Bangalore and Lucknow during the month of July–August 2016/2017. Briefly, the plant samples comprised leaves and stem pieces from different varieties of *V. vinifera* viz. Merlot, Shiraz, Pinot Noir, Muscat and Wild. Three samples each of leaves and stems were collected for a variety from different selected locations, which were subsequently stored in sterile zip pouches at 4 °C till further use.

For isolation of the fungal endophytes of the plant samples, a standard procedure was adopted which comprised surface sterilization of the sample by 0.1% solution of sodium hypochlorite for 1 min followed by 70% ethanol for 45 s, then by dipping in 30% ethanol for 30 s and finally drying them aseptically. Leaf segments of 3×3 mm and 2 mm transverse sections of stems were subsequently prepared under aseptic conditions and placed on pre-made water agar plates (Schulz et al. 1993). The plates were then incubated at 26 ± 1 °C for 10 days with 12 h photoperiod in a cycle. Individual colonies after 10 days incubation at 26 ± 1 °C were picked from the edge of the advancing colony with a fine needle under the stereo-zoom microscope (Nikon) and transferred on to potato dextrose agar (PDA). Pure cultures of the endophytic fungi so obtained were preserved on PDA supplemented with 10% glycerol.

Culture filtrate production

Culture filtrate production was done by inoculating presterilized PDB with a 5 mm mycelial plug of 1-week-old culture of the endophytic fungus and incubated at 26 ± 1 °C, 130 rpm for 10 days. After the incubation period was over, the fungal biomass was separated from the broth via filtration, followed by centrifugation at 10,000 rpm (Hitachi RXII series, Japan) for 10 min at room temperature. The supernatant so collected was passed through 0.22 µm nitrocellulose membrane (GE Health care and life Sciences, Merck, Millipore, USA) to make it cell free.

Phytochemical screening for the presence of polyphenols (resveratrol)

Three phytochemical tests, viz., chromogenic reaction, acetic anhydride test and Liebermann test were carried out to detect the presence of polyphenols (RESV).

Chromogenic reaction

In this assay, the fungal extract was subjected to ferric chloride–potassium ferricyanide colour reaction. Briefly the chromogenic agent was prepared by using 0.1% $FeCl_3$:0.1% K_3 [Fe $(CN)_6$] in the ratio of 1:1 (v/v). The reaction was started by the addition of 2 ml of sample, followed by addition of 2 ml of MeOH to which subsequently three to four drops of chromogenic agent were added. The presences of polyphenols (resveratrol) gives a characteristic blue colour. In this assay, RESV served as positive control, while uninoculated PDB was used as the negative control (Liu et al. 2016).

Acetic anhydride test

This test was used to confirm the presence of the hydroxyl group of the phenolic compound present in the culture filtrate. The reaction was initiated by adding 100 µl of acetic anhydride to 100 µl of culture filtrate, followed by the addition of 500 µl of concentrated sulphuric acid. The formation of purple or pink precipitate confirmed the presence of the –OH group of the phenolic compound in the culture filtrate in the test sample (Al-Jumaily et al. 2014).

Liebermann test

In this test, the culture filtrate was mixed with an equal amount of sulphuric acid, followed by the addition of two to three drops of 1% sodium nitrate. The appearance of a blue-violet colour in the reaction mixture confirmed the presence of phenolic compound in the culture filtrate (Al-Jumaily et al. 2014). In this test, uninoculated PDB was used as a negative control, while RESV (1 mg/ml; Sigma-Aldrich, USA) served as the positive control. The blue-violet colour of the reaction mixture indicates the presence of a free para-position in phenolic compounds.

Liquid–liquid extraction

On the basis of phytochemical screening, ethyl acetate (Merck, GR, USA) was used for recovering resveratrol from the lyophilized cell-free culture filtrates of endophytic fungi in a ratio of 3:1. The organic layers so obtained were pooled

and dehydrated over anhydrous sodium sulphate (HiMedia, Mumbai, India) and subsequently concentrated in vacuo to obtain the crude fraction. The fraction so obtained was reconstituted in methanol and was re-assayed for the presence of RESV using previously described phytochemical tests (Shi et al. 2012). Crude fractions which exhibited positive results in the preliminary test were subjected to HPLC analysis for further confirmation as well as quantification.

Quantification and confirmation of RESV

The identification and concentration of resveratrol in crude bioactive fractions was determined via HPLC (PerkinElmer-200, USA) using C18 reverse phase discovery column (Sigma-Aldrich, USA) equipped with series-200 pump, dual wavelength detector and TotalChrom Workstation software. 20 µl of the sample of pre-defined concentration was injected into the HPLC column and eluted using orthophosphoric acid (0.1%, pH 2.3 Sigma Aldrich) and acetonitrile (Sigma Aldrich), in a ratio of 45–55% as mobile phase with a flow rate of 1 ml/min. The yield of fungal RESV was determined against a standard curve of standard RESV (0.1–1 mg/ml) under similar conditions. The peak area vs. concentration of the standard as well as number of peaks and peak height was used to estimate the concentration of polyphenols (RESV) (Shi et al. 2012). The residues of fungal resveratrol from #19VVLPM, #22(P) VVLPM and standard resveratrol were analysed by LC–ESI-MS/MS (Waters, Micromass Q–TOF micro using Waters Alliance 2795 separation module) to further confirm the presence of resveratrol. The confirmation was done on the basis of molecular ion and fragmentation pattern comparison between standard and fungal resveratrol (Dincheva et al. 2011; Ramirez-Lopez and DeWitt 2014).

Taxonomic identification of potent RESV-producing endophytic isolate

Preliminary identification of endophytic fungus was done using classical morphotaxonomic tools.

Morphotaxonomic studies

For morphotaxonomic studies, the endophytic fungi were grown over different media, i.e. water agar (WA), Czapek Dox Agar (CDA), grape leaf agar (GLA), potato dextrose agar (PDA), corn meal agar (CMA), Fusarium minimal media (FMA) and synthetic nutrient deficient agar (SNA) for 15 days at 26 ± 2 °C and 98% relative humidity (with 12 h of photoperiod). Colony growth rate, appearance, along with its microscopic features like hyphae characteristics, conidia formation and other cellular bodies were critically observed under Nikon stereo zoom microscope (Nikon, Japan). All the micrometric observations were carried out using Nikon NIS element software with at least 30 observations per structure (Ellis Martin and Ellis Pamela 1997; Barnett and Hunter 1998; Nagamani et al. 2006).

ITS-rDNA-based molecular taxonomy of selected resveratrol-producing endophytic fungus

For genomic DNA extraction, the mycelia of 3–4 days old culture (approx. 0.1–0.2 g) was scrapped off with sterile inoculation loop and crushed to very fine powder in a sterile mortar and pestle under liquid nitrogen. Further DNA extraction was done by using the Wizard Genomic DNA purification kit (Promega, Madison, WI) as per manufacturer's instructions. The internal transcribed spacer (ITS) region 1, 5.8S and 2 was amplified using ITS 1 and ITS 4 primers. The reaction mixture (25 µl) comprised 1.5U of Taq DNA polymerase in 10× Taq buffer containing 25 mM MgCl2 (Bangalore GeNei, Bengaluru, India), 2.5 mM of dNTP, 1 µl of extracted genomic DNA and 10 mM of each primer. The conditions for the polymerase chain reaction were 96 °C for 5 min, followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s followed by final extension at 72 °C for 10 min (White et al. 1990). The ITS amplicons so obtained were examined using a 1.5% agarose gel under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software (Bio-Rad, Hercules, CA). The PCR products were purified with Wizard SV gel and PCR cleanup system (Promega, Madison, WI) as per manufacturer's instructions. The obtained PCR products were sequenced with Eurofins (Bangalore, India).

Colonization frequency of RESV-producing endophytes

Further, their diversity was calculated by finding the colonization frequency. The percentage colonization frequency (% CF) and isolation rate (IR) were calculated by the method adopted by Hata and Futai (1995) and Gond et al. (2012). Samples were incubated and growth was examined daily during 6 weeks and colonization frequency was calculated by the following formula:

$$\text{Colonization frequency (\%CF)} = \frac{\text{no. of individual fungi recorded}}{\text{total number of segments screened}}.$$

Statistical analysis

All the assays were performed in triplicate and the data were represented in terms of mean ± standard deviation. The results were analysed by ANOVA followed by Tukey's

post hoc test ($p < 0.05$). Statistical analysis was performed using GraphPad Prism 7 software and $p < 0.05$ was considered significant.

Results

Isolation of endophytic fungi

A total of 145 isolates were isolated from 525 segments of leaf and stem from all the plant samples collected. Among 145 isolates, 44 isolates were recovered from Merlot variety of *V. vinifera*, 27 from wild variety, 22 from Pinot Noir, 33 from Shiraz and 19 from Muscat varieties of *V. vinifera* (Table 1).

Preliminary screening of resveratrol-producing endophytic fungi

Based on the three phytochemical tests for the detection of polyphenolic compounds, viz., Liebermann, acetic anhydride and chromogenic reaction carried out on ethyl acetate extracts of all endophytic fungi, only 44 endophytic fungal isolates exhibited the presence of polyphenolics presumably resveratrol. However, it was expected that high RESV content would be present in isolates, viz., #20(b) VVLPM, #101VVBLK, #53VVSTBK and #18VVLPM, exhibiting the highest presence in one of the three phytochemical tests. However, the moderate presence of polyphenols (resveratrol) was exhibited by isolates #6 VVBLK, #19VVLPM and #193 VVSTPM. For further details about the preliminary screening for the presence of RESV, kindly refer to Supplementary File 1.

Quantification and confirmation of RESV

The fungal RESV gave a peak at Rt 3.36 min on reverse phase C-18 column which was similar to that of standard resveratrol by HPLC. The peak area versus concentration obtained by using standard RESV was used to quantify the fungal RESV. Out of 44 isolates, only 13 endophytic fungi produced RESV in the range of 4.4–52.3 µg/ml in liquid culture which was confirmed by HPLC analysis. Out of 13 isolates, we chose the top potent RESV producer, i.e. #19VVLPM which exhibited a concentration of 52.3 µg/ml (Table 2). Based on one-way ANOVA and Tukey's post hoc analysis ($p < 0.05$), #109VVLNM and #53VVSTBK did not give any significant difference in their mean RESV

Table 1 Colonization frequency of the endophytic fungi in different varieties of *V. vinifera*

Vitis vinifera variety	No. of segments of plant sample used for isolation	No. of endophytic fungal isolates recovered	Colonization frequency (%)
Merlot	115	44	38.3
Wild type	125	27	21.6
Pinot Noir	95	22	23.2
Shiraz	95	33	34.74
Muscat	95	19	20.0

Table 2 Quantification of RESV content in culture filtrate of endophytic fungi isolated from different varieties of *V. vinifera* by HPLC

S. no.	Culture code	*V. vinifera* variety	Tentative identification*	RESV production (µg/ml)*
01	#20(b) VVLPM	Pinot noir	*Botryosphaeria* sp.	15.3 ± 0.10
02	#101VVLBK	Merlot	*Aspergillus* sp.	22.4 ± 0.16
03	#53VVSTBK	Wild	*Aspergillus* sp.	23.9 ± 0.10
04	#18VVLPM	Pinot noir	*Botryosphaeria* sp.	37.3 ± 0.15
05	#6(b)VVLLK	Merlot	Unidentified	11.9 ± 0.15
06	##19VVLPM	Pinot noir	*Fusarium* sp.	52.3 ± 0.10
07	#193VVSTPM	Merlot	*Nigrospora* sp.	25.2 ± 0.10
08	#8VVGSTL	Wild	Unidentified	13.2 ± 0.06
09	#98VVSTPM	Merlot	*Penicillium* sp.	21.9 ± 0.06
10	#22(P)VVLPM	Shiraz	*Xylaria* sp.	35.4 ± 0.10
11	#4(p)VVLNM	Shiraz	*Fusarium* sp.	31.3 ± 0.11
12	#83VVLNM	Merlot	*Aspergillus* sp.	4.4 ± 0.20
13	#109VVLNM	Merlot	*Alternaria* sp.	24.1 ± 0.10

*Tentative identification is based on the morphotaxonomy of the fungus

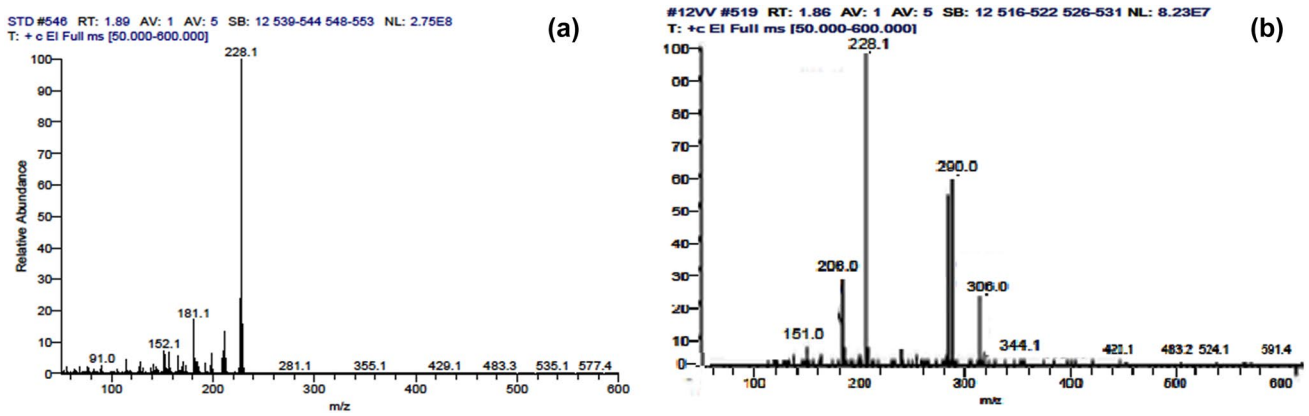

Fig. 1 Fragmentation pattern by LC–MS analysis of **a** standard resveratrol, **b** fungal resveratrol from bioactive extract of #19VVLPM

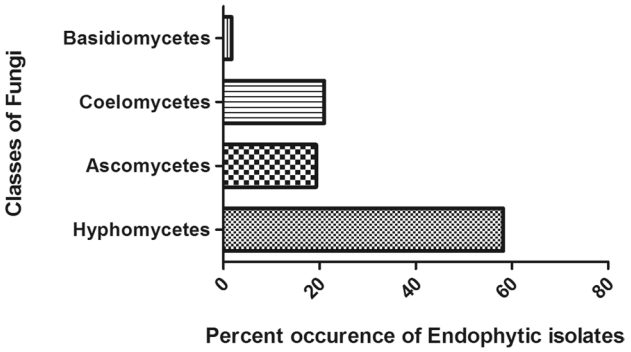

Fig. 2 Maximum likelihood tree using Tamura and Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) #19VVLPM—*F. equiseti*

concentration among the different RESV-producing endophytic isolates (Table 2).

For further confirmation of the presence of fungal resveratrol in the bioactive ethyl acetate residue obtained from the spent broth of #19VVLMP, LC–MS/MS was carried out which revealed the presence of resveratrol apart from other constituents present such as piceatannol, stilbene, quercetin, etc., which had different retention times. The retention time of resveratrol was 1.86. Further MS analysis revealed that the fungal resveratrol from #19VVLMP and standard resveratrol exhibited the same base peak at 228.1 *m/z* (Fig. 1).

Taxonomic identification of potent RESV-producing endophytic isolate

The morphological as well as molecular taxonomic studies were under taken to identify the fungal isolates till species level. The morphological studies are presented in Supplementary file no. 2. The ITS sequence of #19VVLPM was submitted to GenBank with accession no. MH181157. Subsequently, homologue ITS sequences of #19VVLPM were identified using BLAST and were used for assessing the phylogenetic association, which was analysed by preparing the maximum likelihood tree using Tamura and Nei model (Tamura et al. 2011). In this tree, #19VVLPM clustered with *Fusarium equisetum* isolate TMC as well as *F. equisetum* (Fig. 2). Based on the phylogenetic clustering of #19VVLPM and morphotaxonomic data, it was assigned the name of *Fusarium equiseti.* #19VVLPM exhibited the presence of STS gene which is responsible for the production of RESV.

Colonization frequency of RESV-producing endophytes

A total of 144 isolates belonging to 18 fungal genera were isolated from 525 segments of leaf and stem collectively. Among all the endophyte isolates recovered from *V. vinifera* in the present study, 58.2% were Hyphomycetes, 19.3% Ascomycetes, 21% Coelomycetes and 1.7% Basidiomycetes (Fig. 3). Maximum endophytic cultures were isolated from samples collected from Merlot variety, i.e. 38.3%, while the minimum were recovered from Muscat variety of *V. vinifera* (20%). The dominant fungi observed were *Aspergillus* sp., *Fusarium* sp., *Alternaria* sp., however *Botryosphaeria* sp. and *Xylaria* sp. and were reported for the first time from *V. vinifera* as endophytes.

Discussion

Endophytic fungi since the last two decades have attained remarkable recognition in the field of drug discovery and development as a sustainable source of unique and diverse bioactive compounds which can be directly or indirectly be used as drug candidates for combating major global diseases (Strobel and Daisy 2003; Zhang et al. 2006). Endophytic

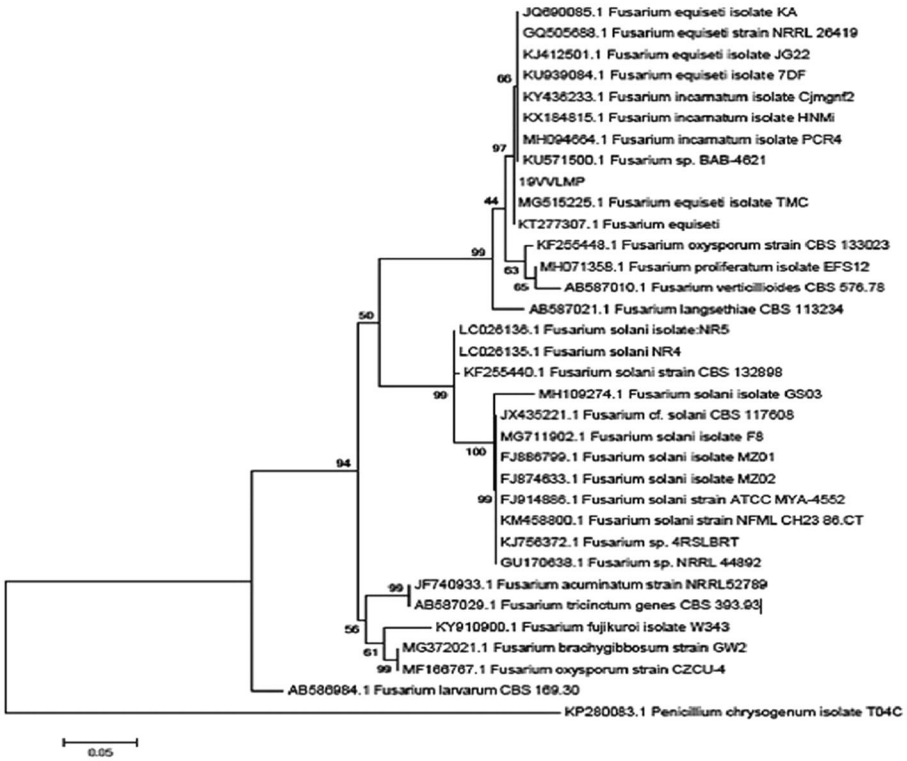

Fig. 3 The relative recovery of different endophytic groups from the different locations in India

fungi generally co-exist within the plant tissues and play a crucial role in promoting host growth and adaptation to different biotic and abiotic stresses. It has been well documented that endophytic fungi co-evolve with the host plant, thereby attaining the host biosynthetic capabilities and producing phytomedicinals which are commercially used such as paclitaxel (Stierle et al. 1993), vinblastine (Zhao et al. 2011) and camptothecin (Puri et al. 2005; Kusari et al. 2009). Fungal endophytes produce a variety of compounds which belong to different classes such as alkaloids, polyphenols, terpenoids, quinones and steroids.

Exploration of endophytic fungi capable of producing resveratrol is a relatively new aspect, after its therapeutic properties came into limelight. Resveratrol has been associated with various health benefits, since it has different properties such as anti-inflammatory and anti-oxidant activity, is an anti-diabetic, can prevent cardiovascular diseases and cancer and is an anti-ageing moiety (Saxena and Srivastava 2014, Wang et al. 2013). There exist very limited data on exploration of endophytic fungi for the production of RESV. Powell et al. (1994) for the first time isolated RESV from endophytic *Acremonium* sp., and further endophytic fungi including *Alternaria* sp., *Aspergillus niger*, *Penicillium* sp. and *Botryosphaeria* sp. are amongst the recently reported producers of RESV (Wang et al. 2013; Shi et al. 2012; Liu et al. 2016).

Thus, this study was oriented towards exploring endophytic fungi associated with different *V. vinifera* varieties in India, understanding diversity in terms of colonization frequency and finally screening them for the production of resveratrol. In this study, we reported 18 fungal genera from 525 segments, whereas González and Tello (2011) reported 51 genera from six locations in Spain, while Brum et al. (2012) reported the isolation of 550 fungal endophytes from *Vitis labrusca*. There was difference in the composition of fungal endophytes from *V. vinifera* samples collected from different geographic locations. It varies according to host species and characteristics of site, viz., vegetation, elevation, tissue type, exposure (Bills and Polishook 1994; Petrini and Fisher 1986) and age of tissue (Fisher et al. 1986; Roll-Hansen and Roll-Hansen 1979). In the preliminary screening for the presence of polyphenols, only 29.7% cultures gave a positive test. HPLC-based quantification was further helpful in identifying the resveratrol-producing fungi. Only 9.7% isolates were found to produce resveratrol which was less than that observed by Liu et al. (2016) and Shi et al. (2012) with 19.4% and 32.3%, respectively. HPLC-based quantification has been extensively used in determining the resveratrol concentration in organic extracts in other studies also (Casieri et al. 2009; Kim et al. 2008).

By using LC–MS/MS, the endophytic isolate exhibiting the highest extracellular RESV was found in isolate #19VVLPM. LC–MS/MS has been a preferred tool for quantification and confirmation of resveratrol in complex matrices such as grape juice, cranberry juice, red wine, grape concentrates and by-products of grapes (Wang et al.

2002; Careri et al. 2004). Further morphotaxonomic studies revealed that the isolate #19VVLPM and #22(P) VVLPM belonged to the genus *Fusarium*.

It has been proven that among the regions of ribosomal cistron, internal transcribed spacers (ITS) exhibit the highest probability of successful identification of the broadest range of fungi, successfully distinguishing between intraspecific and interspecific variation and therefore are used as DNA barcode marker for fungi (Schoch et al. 2012). Based on the molecular phylogeny, #19VVLPM was identified as *F. equiseti*. The RESV productions of this isolate were stable over several passages. The activity of these isolates did not decrease as reported in *Alternaria* sp. MG2 and *Botryosphaeria* sp. YG3. However, RESV production decreased severely on passaging in four cultures, viz., #83VVLNM, #6(b) VVLLK, # 8VVGSTL and # 20(b) VVLPM (Liu et al. 2016; Shi et al. 2012). This probably is attributed to the missing *STS* gene in these isolates as compared to #19VVLMP.

The endophytic mycobiota associated with *V. vinifera* in this study is dominated by hyphomycetes, which constitute about 58.8% of the total endophytes isolated. However, the dominance of Ascomycetes was reported in the study conducted on several cultivars of *V. vinifera* from the Madrid region (González and Tello 2011). The low proportion of basidiomycetes taxa found in our study is consistent with other studies (González and Tello 2011; Márquez et al. 2010). The most frequently occurring isolates belonged to the genera *Aspergillus*, *Fusarium*, *Alternaria* and *Botryosphaeria.* In previous research studies, most of these fungi have been identified in grapevines. Pancher et al. (2012) and Mostert et al. (2000) reported *Alternaria* as a dominant genus and also reported the presence of *Phoma* sp. González and Tello (2011) and Musetti et al. (2006) found that genera *Alternaria*, *Fusarium* and *Phoma* are the most frequently occurring isolates of endophytic fungi of *V. vinifera*. In another study, *Alternaria* sp. and *Fusarium* sp. were reported as dominant along with the less frequently occurring *Phoma* sp., *Diaporthe* sp. and *Phomopsis* sp. in the grapevine cultivars (Casieri et al. 2009).

Further, our focus was the exploration of potent isolate producing resveratrol under extracellular fermenting condition for possible large-scale production. In the present study, one incidental isolate #19VVLPM, *F. equiseti*, was found to exhibit the production of resveratrol under free fermenting conditions.

Conclusion

This is the first study that concurrently examined the resveratrol production of the fungal endophytes isolated from different varieties of *V. vinifera* from India. These results indicate that *V. vinifera* harbours rich endophytic fungal community. *F. equiseti* is a promising candidate for further strain improvement to achieve resveratrol production through the fermentation route.

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Author contribution VD collected all the samples from different geographical locations between July and August 2016/2017 and then isolations were carried out under the guidance of SS. SS conceived and designed the present study, while VD performed all experiments and analysed the data. The publication was jointly written by SS and VD.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

References

Al-Jumaily EF, Hamid GS, Ali KF (2014) Synthesis and total phenol content of new resveratrol derivative. Open J Adv Drug Deliv 2(3):320–329

Barnett HL, Hunter BB (1998) Illustrated genera of imperfect fungi, 4th edn. APS Press, St. Paul

Beekwilder J, Wolswinkel R, Jonker H, Hall R, de Vos CR, Bovy A (2006) Production of resveratrol in recombinant microorganisms. Appl Environ Microbiol 72(8):5670–5672

Bills GF, Polishook JD (1994) Abundance and diversity of microfungi in the leaf litter of a lowland rain forest in Costa Rica. Mycologia 86(2):187–198

Brum MCPD, Araujo WLD, Maki CS, Azevedo JLD (2012) Endophytic fungi from *Vitis labrusca* L. ('Niagara Rosada') and its potential for the biological control of *Fusarium oxysporum*. Genet Mol Res 11(4):4187–4197

Busby PE, Soman C, Wagner MR, Friesen ML, Kramer J, Bennett A, Morsy M, Eison JA, Leach JE, Denyl JL (2017) Research priorities for harnessing plant microbiome in sustainable agriculture. PLoS Biol 15(3):e2001793. https://doi.org/10.1371/journal.pbio.2001793

Careri M, Corradini C, Elviri L, Nicoletti I, Zagnoni I (2004) Liquid chromatography—electrospray tandem mass spectrometry of *cis*-resveratrol and *trans*-resveratrol: development, validation, and application of the method to red wine, grapes and winemaking products. J Agric Food Chem 52:6868–6874

Casieri L, Valérie Hofstetter, Olivier Viret, Katia Gindro (2009) Fungal communities are living in the wood of different cultivars of young *Vitis vinifera* plants. Phytopathol Mediterr 48(1):73–83

Dincheva I, Badjakov I, Tsvetkov I, Dzhambazova T, Kondakova V, Batchvarova R (2011) GC-MS determination of secondary metabolites in red wine from Bulgarian wild grape (*Vitis vinifera*). J Mt Agric Balk 14:1331–1343

Ellis Martin B, Ellis Pamela J (1997) Microfungi on land plants, an identification handbook. Hardback, New England

Fisher PJ, Anson AE, Petrini O (1986) Fungal endophytes in *Ulexeuro paeus* and *Ulexgallii*. Trans Br Mycol Soc 86(1):153–156

Gond SK, Mishra A, Sharma VK, Verma SK, Kumar J, Kharwar RN, Kumar A (2012) Diversity and antimicrobial activity of endophytic fungi isolated from Nyctanthes arbor-tristis a well known medicinal plant of India. Mycoscience 53(2):113–121

González V, Tello ML (2011) The endophytic mycota associated with *Vitis vinifera* in central Spain. Fungal Divers 47(1):29–42

Hata K, Futai K (1995) Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge, *Thecodiplosis japonensis*. Can J Bot 73(3):384–390

Kaul S, Choudhury M, Sharma T, Dhar MK (2017) Harnessing the plant microbiome: a key towards sustainable agriculture. In: Singh D, Singh H, Prabha R (eds) Plant-microbe interactions in agro-ecological perspectives. Springer, Singapore

Kim JS, Lee SY, Park SU (2008) Resveratrol production in hairy root culture of peanut, *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains. African Journal of Biotechnology 7(20):3788–3790

Kusari S, Zühlke S, Spiteller M (2009) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogs. J Nat Prod 72(1):2–7

Langcake P, Pryce RJ (1977) A new class of phytoalexins from grapevines. Cell Mol Life Sci 33(2):151–152. https://doi.org/10.1007/BF02124034

Liu Y, Nan L, Liu J, Yan H, Zhang D, Han X (2016) Isolation and identification of resveratrol producing endophytes from wine grape Cabernet Sauvignon. Springer Plus 5:1029

Márquez SS, Bills GF, Acuña LD, Zabalgogeazcoa I (2010) Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. Fungal Divers 41(1):115–123

Mostert L, Crous PW, Petrini O (2000) Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. Sydowia 52(1):46–58

Musetti R, Vecchione A, Stringher L, Borselli S, Zulini L, Marzani C, Pertot I (2006) Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. Phytopathology 96(7):689–698

Nagamani A, Kunwar IK, Manoharachary C (2006) Handbook of soil fungi. I.K. International Pvt. Ltd., New Delhi

Pancher M, Ceol M, Corneo PE, Longa CMO, Yousaf S, Pertot I, Campisano A (2012) Fungal endophytic communities in grapevines (*Vitis vinifera* L) respond to crop management. Appl Environ Microbiol 78(12):4308–4317

Petrini O, Fisher PJ (1986) Fungal endophytes in *Salicornia perennis*. Trans Br Mycol Soc 87(4):647–651

Powell RG, TePaske MR, Plattner RD, White JF, Clement SL (1994) Isolation of resveratrol from *Festuca versuta* and evidence for the widespread occurrence of this stilbene in the Poaceae. Phytochemistry 35(2):335–338

Puri SC, Verma V, Amna T, Qazi GN, Spitteler M (2005) An endophytic fungus from *Nothapodytes foetida* that produces Camptothecin. J Nat Prod 68(12):1717–1719

Ramirez-Lopez LM, DeWitt CA (2014) Analysis of phenolic compounds in commercially dried grape pomace by high-performance liquid chromatography electrospray ionization mass spectrometry. Food Sci Nutr 2(5):470–477

Roll-Hansen F, Roll-Hansen H (1979) *Ascocoryne* species in living stems of *Picea* species: a literature review. For Pathol 9(5):275–280

Saxena S, Srivastava A (2014) Resveratrol: biological activities and therapeutic potential. J Pharm Technol Res Manag 2(2):145–157

Schijlen E, Ric de Vos CH, Jonker H, Van Den Broeck H, Molthoff J, Van Tunen A, Bovy A (2006) Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruit. Plant Biotechnol J 4(4):433–444

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS 109(16):6241–6246

Schulz B, Wanke U, Draeger S, Aust HJ (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol Res 97:1447–1450

Schwekendiek A, Spring O, Heyerick A, Pickel B, Pitsch NT, Peschke F, Weber G (2007) Constitutive expression of a grapevine stilbene synthase gene in transgenic hop (*Humulus lupulus* L.) yields resveratrol and its derivatives in substantial quantities. J Agric Food Chem 55(17):7002–7009

Shi J, Zeng Q, Liu Y, Pan Z (2012) *Alternaria* sp. MG1, a resveratrol-producing fungus: isolation, identification, and optimal cultivation conditions for resveratrol production. Appl Microbiol Biotechnol 95(2):369–379

Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260:214

Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67(4):491–502

Tamura K, Peterson D, Peterson N, StecherG Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Wang Y, Catana F, Yang Y, Roderick R, van Breeman RB (2002) An LC-MS method for analysing total resveratrol in grape juice, cranberry juice and in wine. J Agric Food Chem 50:431–435

Wang DG, Liu WY, Chen GT (2013) A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*. J Pharm Anal 3(4):241–247

White TJ, Bruns T, Lee SJWT, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc Guide Methods Appl 18:315–322

Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. Nat Prod Rep 23(5):753–771

Zhao J, Shan T, Mou Y, Zhou L (2011) Plant-derived bioactive compounds produced by endophytic fungi. Mini Rev Med Chem 11(2):159–168

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Arcopilus aureus, a Resveratrol-Producing Endophyte from *Vitis vinifera*

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Abstract Resveratrol is extensively being used as a therapeutic moiety, as well as a pharmacophore for development of new drugs due to its multifarious beneficial effects. The objective of the present study was to isolate and screen the resveratrol-producing endophytic fungi from different varieties of *Vitis vinifera*. A total of 53 endophytic fungi belonging to different fungal genera were isolated from the stem and leaf tissues of *Vitis vinifera* (merlot, wild, pinot noir, Shiraz, muscat) from different grape-producing locations of India. Only 29 endophytic fungal isolates exhibited a positive test for phenolics by phytochemical methods. The resveratrol obtained after ethyl acetate extraction was confirmed using standard molecule on thin layer chromatography (TLC) with a retention factor (R_f) of 0.69. The purified and standard resveratrol were visualized under UV light as a violet-colored spot. In HPLC analysis of the ethyl acetate extract of culture broth of 11 endophytic isolates, the highest resveratrol content was found in #12VVLPM (89.1 μg/ml) followed by #18VVLPM (37.3 μg/ml) and 193VVSTPM (25.2 μg/ml) exhibiting a retention time of 3.36 min which corresponded to the standard resveratrol. The resveratrol-producing isolates belong to seven genera viz. *Aspergillus*, *Botryosphaeria*, *Penicillium*, *Fusarium*, *Alternaria*, *Arcopilus*, and *Lasiodiplodia*, and using morphological and molecular methods, #12VVLPM was identified as *Arcopilus aureus*.

Keywords Endophyte · Fungi · Grapes · *Arcopilus* sp. · Resveratrol

Introduction

Resveratrol (RESV) or trans-3, 4, 5-trihydroxy stilbene (trans-RESV) is a polyphenolic flavonoid, which has garnered much attention due to its myriad beneficial effects on human health which is mediated via different enzymatic or biosynthetic pathways and cellular signaling, as well as modification of gene expression. It has been reported to have beneficial effects in reducing the progression of neurological diseases like Alzheimer's, dementia, and Parkinson's diseases;

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alleviating cardiovascular problems; treating ischemic conditions; overcoming stress; and delaying the process of aging in organisms from yeasts to vertebrates [1–4].

It was initially identified from a poisonous plant *Veratrum album* var. *grandifolium* as a resorcinol derivative and hence named resveratrol. This moiety is also produced as a phytoalexin in response to attack or as a defense molecule to prevent attack by the fungus *Botrytis cinerea* in grapes (*Vitis vinifera*). The nutraceutical and therapeutic investigations on this molecule began after it was identified as a constituent of red wine. It was attributed to lower incidence of cardiovascular diseases despite the dietary habits of French people due to red wine being an integral component of their diet [5]. The plant sources of trans-RESV comprise primarily of *Polygonum cuspidatum* (Japanese knotweed), *Vitis vinifera* (grapes), *Arachis hypogea* (groundnuts), and cranberries [4, 6, 7]. However, the richest source of trans-RESV is Japanese knotweed which is being exploited commercially for the extraction of this compound for use as a nutraceutical supplement or therapeutic supplement [4, 8].

The report presented by Frost and Sullivan (2013) indicates that the global resveratrol supply market was valued at $50.2 million with 90% going to the food supplement market in the USA. Increasingly, resveratrol is also finding use in the cosmeceutical market. Microbial fermentation has been used for the production of a variety of high-value products such as organic acids, antibiotics, amino acids, and vitamins commercially. The advantage of using these microbes for large-scale production is the efficiency and cost effectiveness, as well as ease of operation. The plant-based extraction of resveratrol has been found to be cost intensive leading to a higher cost of the nutraceutical and therapeutic supplements developed [9, 10].

Hence, researchers are exploring methods of mimicking the resveratrol biosynthetic pathways in microorganisms or optimizing plant cell cultures. Another promising strategy is exploration of the microorganisms which may mimic the production of resveratrol under free-fermenting conditions. It has been already proved that due to the close association and co-evolution of the endophytic fungi with their host plant, it induces the capability of producing the host secondary metabolites or phytochemicals [11, 12]. Hence, these could be commercially exploited for their cost-effective large-scale production in the future. Thus, based on this premise, it was hypothesized that *Vitis vinifera* also holds endophytic fungi capable of producing resveratrol under free-fermenting conditions. Thus, in the present work, we report an endophytic fungus #12VVLPM as a promising candidate for the fermentative production of resveratrol.

Materials and Methods

Plant Sample Collection

Vitis vinifera samples were collected from Pune (12.07° N, 77.03° E), Nasik (20.000° N, 73.78° E), Bengaluru (12.9667° N, 77.567° E), Shimla (31.103° N, 77.1722° E), Chandigarh (30.7500° N, 76.780° E), Patiala (30.3400° N, 76.380° E), and Lucknow (26.800° N, 80.9000 °E) during the months of July–August 2015. Each plant sample was tagged and then placed in sterile bags and stored at 4 °C till further use.

Isolation of the Endophytic Fungi

The plant samples (2 cm approx., predominantly leaves and stems) were thoroughly washed under running tap water for 10–15 min and then surface sterilized with 2% sodium

hypochlorite (Hi-Media, India; AS102) for 10 min. Then, sterilization was done using 70% ethanol for 2 min and 30% ethanol (Hi-Media, India; MB106) for 1 min and subsequently allowed to surface dry under aseptic conditions [13].

Surface-sterilized samples (leaves and stems) were then cut into 2–3-mm segments with the help of a sterile blade and placed on water agar with the ventral side facing the medium. These were then incubated at 26 ± 1 °C for 10 days with 12 h light/dark cycles. Individual colonies were picked up from the edge of advancing colony with a sterile fine needle using stereo zoom microscope (Nikon, Japan; SMZ1270i) and then transferred on potato dextrose agar (PDA) medium (Hi-Media, India (MH096)). After sufficient growth, the endophytic fungi were also transferred on PDA slants supplemented with 10% glycerol (Hi-Media, India; GRM1027) for long-term storage.

Calculation of Isolation Frequency and Isolation Rate

The isolation frequency (IF) or the colonization frequency (CF) and isolation rate (IR) of fungi, location wise and variety wise, were calculated by the following formulas [14, 15];

$$\text{Isolation frequency}(\text{IF}\%) = \frac{\text{No.of individual fungi recorded}}{\text{Total no.of segments screened}} \times 100$$

$$\text{Isolation rate}(\text{IR}) = \frac{\text{Total no.of endophytic fungi recorded}}{\text{Total no.of segments screened}}$$

Culture Filtrate Production and Screening of Fungal Endophytes

All the endophytic cultures were subjected to culture filtrate production by inoculating 5-mm mycelial disc of 7–10-day-old fungal culture to 50 ml pre-sterilized Czapek Dox broth (CDB, Hi-Media, India; M076) medium in 250-ml Erlenmeyer flasks (Borosil, India; 4980021). The flasks were then incubated at 26 ± 2 °C, 120 rpm for 7 days. After the incubation, the spent broth was separated from mycelia by filtration through Whatman filter paper 4 (Sigma Aldrich, USA; Z240567) followed by centrifugation at 12,000 rpm for 10 min and then passing it through 0.2-μm nitrocellulose membrane (Merck Millipore, USA; PR04066) to get cell-free filtrate [4, 16, 17]. Culture broth and organic fraction were examined for resveratrol production using biochemical assays namely (i) Liebermann test and (ii) acetic anhydride test.

Resveratrol is a type of polyphenolic compound belonging to a flavonoid class possessing a stilbene-type structure and chemically has a free para position like phenols and stilbene which gives green/blue-violet color by addition of sodium nitrite and greenish-brown precipitate after reaction with acetic anhydride. Therefore, these chemical characteristic reactions could be employed as a screening method for the presence of polyphenols [17].

Liebermann Test

This test is known to detect the presence of free para position in phenolic compounds. Briefly, culture filtrate and concentrated sulfuric acid (Sigma Aldrich, USA; 339741) were mixed in equal ratios followed by addition of two to three drops of 1% sodium nitrate (Hi-Media, India; GRM1722). The appearance of a green/blue-violet color indicates the presence of phenolic

compound [18]. Resveratrol (1 mg/ml; Sigma Aldrich; R5010) served as the positive control, while uninoculated CDB and methanol (Hi-Media; AS061) were used as negative control.

Acetic Anhydride Test

This test detects the free –OH group in the phenolic compounds. Briefly, the reaction was initiated by addition of 100 μl of acetic anhydride (Sigma Aldrich; 320102) and 500 μl of concentrated sulfuric acid to 100 μl of test compound and then observed for appearance of greenish brown precipitate formation indicating the presence of free –OH phenolic compounds in the test sample [18]. Resveratrol (1 mg/ml) served as the positive control, while uninoculated CDB and methanol were used as negative control.

Liquid-Liquid Extraction and TLC Analysis

The cultures exhibiting positive Liebermann and acetic anhydride tests were subjected to liquid-liquid extraction using ethyl acetate (Sigma Aldrich; 650528). The culture filtrate and ethyl acetate (EA) were taken up in a ratio of 1:3 and extracted three times. The organic layers were pooled and dehydrated by the addition of anhydrous sodium sulfate (Sigma Aldrich; 13462). Subsequently, the solvent fraction was evaporated in a rotary evaporator (DLAB RE 100-Pro, China) at 30 °C to dryness to get the crude fraction. The fraction so obtained was weighed, reconstituted in methanol, and again screened for the presence of phenolic/resveratrol by Liebermann and acetic acid assays as described previously. Crude fractions which exhibited positive Liebermann test and acetic acid tests were subjected to TLC separation to confirm the presence of resveratrol.

PTLC Separation and Analysis

The crude fractions of the cultures exhibiting positive Liebermann and acetic acid assays were further fractionated by being subjected to preparative thin layer chromatography (PTLC). The TLC plate of 0.5-mm thickness was prepared by coating silica gel (Sigma Aldrich; 381276) onto 20 × 15 × 5 mm clean glass plates (Merck Millipore; RP-8F254S) which was activated by incubating at 100 °C for 2 h prior to use. Subsequently, the sample was spotted on to activated TLC plate just 1 cm above the edge of plate with the help of a capillary tube (Sigma Aldrich; Z114960). Simultaneously, the TLC chamber was saturated with different solvent systems (binary/tertiary) for 30 min. The TLC plate with sample was then placed inside the saturated TLC chamber and allowed to run for 65 min when the solvent front reached the desired level. Subsequently, the TLC plate was taken out and allowed to air dry. The chromatogram was developed by visualizing the TLC plate under UV light (Thermo Fisher Scientific; UVGL-58). Resveratrol (1 mg/ml) was used as a standard for the comparison of retention factor (R_f) value. The R_f value of each band was obtained as the ratio of distance moved by solute to that of solvent [19, 20].

Quantification of Resveratrol Through HPLC

Resveratrol concentration in crude fraction of the selected positive isolate was determined using high-performance liquid chromatography (HPLC) (Perkin Elmer, USA) equipped with series 200 pump, dual wavelength detector, and total chrome workstation software. Briefly, 50 μg of crude

bioactive fraction was dissolved in methanol and injected into the HPLC column. Isocratic elution was carried out during quantification of RESV by HPLC using a mobile phase comprising of ACN (acetonitrile, Sigma Aldrich; 34851) and H_2O (containing 0.1% orthophosphoric acid, pH 2.3, Sigma Aldrich; V800287) used in a ratio of 45:55 as mobile phase with a flow rate of 1 ml/min. To determine the concentration of fungal resveratrol, different dilutions of standard resveratrol (1 mg/ml) ranging from 0.1–1.0 mg/ml were prepared in HPLC-grade methanol. Fifteen microliters of each dilution was injected into C18 (5 μm, 25 cm × 4.6 mm) reverse-phase Discovery column (Sigma Aldrich; 58,298), column oven temperature 34 °C. The data of the peak area vs. concentration of the standard RESVl obtained were used to estimate the quantity of fungal resveratrol in crude EA fraction [4, 17].

Morphotaxonomy of Resveratrol-Producing Endophytic Fungus

For morphotaxonomic studies, the endophytic fungus producing resveratrol was cultivated on different nutrient media viz. corn meal agar (CMA, Hi-Media; M146), CDA (Hi-Media; M075), grape leaf agar (GLA), PDA, and water agar (WA), for 10 days at 26 ± 2 °C with 12 h of photoperiod. Colony growth rate and appearance, along with its microscopic features like hypha characteristics, conidium formation, and other cellular bodies such as fruiting bodies (asexual or sexual spores) or structures, were critically observed under a Nikon stereo zoom microscope. All the micrometric observations were carried out using the Nikon NIS element software with at least 30 observations per structure [17, 21].

Molecular Identification of the Resveratrol-Producing Endophytic Fungus

For the genomic DNA extraction, about 0.1–0.2 g of cultured mycelia was scrapped off from the 3–4-day-old culture with sterile inoculation loop and crushed to very fine powder with pestle and mortar using liquid nitrogen. Further DNA extraction was done by using the Wizard® Genomic DNA purification kit (Promega; A1120) following manufacturer instructions. The internal transcribed spacer (ITS) region 1, 5.8S, 2 was amplified using ITS 1 and ITS 4 primers (Eurofins Labs, India) [22]. Twenty-five microliters of the reaction mixture consists of 1 μl of extracted genomic DNA,10 μM of each primer, 2.5 mM of dNTP (Thermo Fisher Scientific; R0192), 1.5 U of Taq DNA Polymerase in 10X Taq buffer containing 25 mM $MgCl_2$ (GeNei, Bangalore; 105926). The thermal cycling conditions consisted of initial denaturation at 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s followed by final extension at 72 °C for 5 min [22]. The ITS amplicons were examined using a 1.5% agarose (Hi-Media; MB080) gel under UV light in Bio-Rad Gel documentation system (USA) using Quantity-1-D analysis software. The PCR products were purified with Wizard® SV gel and PCR cleanup system (Promega; A9281) following manufacturer's protocol. PCR products (500–600 bp) were sequenced at Eurofins Labs (Bangalore, India).

The final sequence was obtained by assembling the obtained sequences using Sequencher ver. 5 (www.genecodes.com) and was submitted to GenBank under the accession number MF597785. Sequence similarity search of the ITS sequences of #12VVLPM was performed using the BLAST algorithm software at the NCBI website. The ITS sequences were aligned with selected sequences of reference taxa obtained from BLAST using the Clustal W option in MEGA 6 [23]. The alignment file involved 20 sequences which comprised of 1 sequence

under study. *Achaetomium strumarium* (accession no. CBS333.67) served as outgroup to root the tree. After the exclusion of non-overlapping leading/trailing gaps, the length of the alignment was 462 bp. The evolutionary relationship was inferred by using the maximum likelihood method based on the Tamura-Nei model using MEGA6. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites in five categories (+G, parameter = 0.1389). The analysis involved 20 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding.

Results

Isolation of Endophytic Fungi

Endophytic fungi were isolated from healthy and symptomless tissues (leaves and stem) of *Vitis vinifera* to calculate their diversity and phylogeny, as well as bioactive potential. A total of 53 isolates belonging to different genera were isolated from the stem and leaf tissues of *Vitis vinifera* (merlot, pinot noir, muscat, Shiraz) collected from different locations in India (Fig. 1, Table 1).

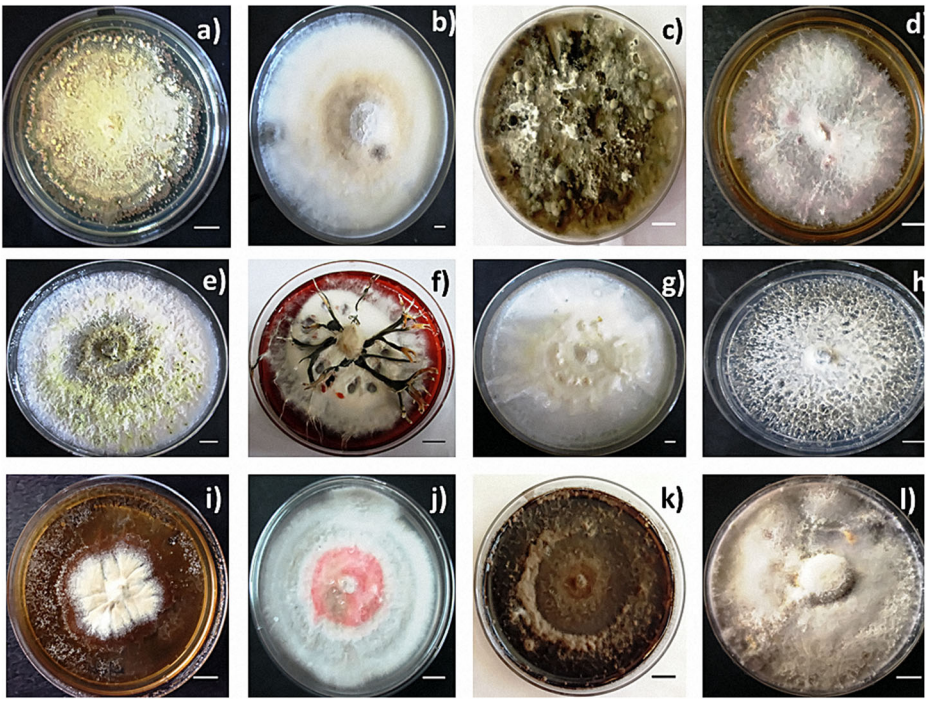

Fig. 1 Endophytic fungal isolates from different *Vitis* sp. used in the study. **a** *Nigrospora* sp., **b** *Fusarium* sp., **c** *Botryosphaeria* sp., **d** *Pestalotiopsis* sp., **e** unidentified, **f** unidentified, **g** *Lasiodiplodia* sp., **h** *Fusarium* sp., **i** *Arcopilus* sp., **j** *Fusarium* sp. **k** Alternaria sp., **l** unidentified (bar = 10 mm), Unidentified (Bar: 10mm)

Table 1 Tentative identification and isolation frequency (IF) of fungal endophytes of diverse varieties of *Vitis vinifera* collected from different parts of India

Location	Plant sample	No. of segments	No. of isolates	Culture code	Morphological identification[a]	IF	
						Grape species wise	Location wise
Alambagh, Lucknow	*Vitis vinifera* (merlot)	25	3	#3VVLLK	*Penicillium* sp.	12.00%	13.33%
				#6(b)VVLLK	Unidentified		
				#9VVLLK	*Aspergillus* sp.		
	Vitis vinifera (pinot noir)	25	1	#5(b)VVLLK	*Aspergillus* sp.	4.00%	
	Vitis vinifera (wild)	25	6	#1VVRLLK	Unidentified	24.00%	
				#1VVRSTL	*Lasiodiplodia* sp.		
				#4VVLLK	*Botryosphaeria sp.*		
				#8VVLLK	*Pestalotiopsis* sp.		
				#8VVGSTL	Unidentified		
				#21VVGSTL	*Aspergillus* sp.		
Pune, Maharashtra	*Vitis vinifera* (Shiraz)	40	4	#10VVLPM	*Botryosphaeria* sp.	10.00%	13.13%
				#10(b)VVLPM	*Fusarium* sp.		
				#25VVLPM	*Fusarium* sp.		
				#27VVLPM	Unidentified		
	Vitis vinifera (muscat)	40	5	#11VVSPM	*Fusarium* sp.	12.50%	
				#12(b)VVLPM	*Aspergillus* sp.		
				##12VVLPM	Unidentified		
				#16(b)VVLPM	*Aspergillus* sp.		
				#17(b)VVLPM	Unidentified		
	Vitis vinifera (merlot)	40	6	#12VVLPM	*Arcopilus* sp.	15.00%	
				#37VVLPM	*Penicillium* sp.		
				#66VVLPM	*Penicillium* sp.		
				#98VVSTPM	*Penicillium* sp.		
				#184VVSTPM	*Lasiodiplodia* sp.		
				#193VVSTPM	*Nigrospora* sp.		
	Vitis vinifera (pinot noir)	40	6	#18VVLPM	*Botryosphaeria* sp.	15.00%	
				#19VVLPM	*Penicillium* sp.		
				#20(b)VVLPM	*Penicillium* sp.		
				#21VVLPM	Unidentified		
				#23VVLPM	*Alternaria* sp.		

Table 1 (continued)

Location	Plant sample	No. of segments	No. of isolates	Culture code	Morphological identification[a]	IF	
						Grape species wise	Location wise
				#24VVLPM	*Alternaria* sp.		
Nasik, Maharashtra	*Vitis vinifera* (merlot)	64	9	#29VVLNM	Unidentified	14.10%	14.10%
				#29(b)VVLNM	*Aspergillus* sp.		
				#30(b)VVLNM	Unidentified		
				#31VVLNM	Unidentified		
				#33VVLNM	Unidentified		
				#35VVLNM	*Alternaria* sp.		
				#83VVLNM	Unidentified		
				#105VVLNM	*Aspergillus* sp.		
				#109VVLNM	*Aspergillus* sp.		
Bangalore, Karnataka	*Vitis vinifera* (merlot)	20	4	#20VVLBK	*Fusarium* sp.	20.00%	18%
				#20(a)VVLBK	*Botryosphaeria* sp.		
				#49(b)VVLBK	*Aspergillus* sp.		
				#101VVLBK	*Aspergillus* sp.		
	Vitis vinifera (wild)	30	5	#13VVLBK	*Lasiodiplodia* sp.	16.70%	
				#23VVSTBK	*Aspergillus* sp.		
				#53VVSTBK	*Aspergillus* sp.		
				#56VVLBK	Unidentified		
				#101VVSTBK	*Alternaria* sp.		
Shimla, HP	*Vitis vinifera* (wild)	29	4	#1VVGLS	*Fusarium* sp.	13.80%	13.80%
				#3VVGLS	*Aspergillus* sp.		
				#7VVGLS	*Fusarium* sp.		
				#11VVGLS	*Lasiodiplodia* sp.		

[a] Tentative identification only based on morphological characters (plate and microscopic characteristics)

The highest isolation frequency of 24% was observed in samples of *Vitis vinifera* (wild) from Alambagh, Lucknow, which was followed by 20% in *Vitis vinifera* (merlot) from Bangalore. The least isolation frequency (4%) was observed in *Vitis vinifera* (pinot noir) sampled from Alambagh, Lucknow (Table 1). Area wise, the highest isolation frequency of 18% was found in plants sampled from Bangalore, Karnataka, which was followed by those in Nasik, with 14% isolation frequency (Table 1). Alambagh, Lucknow, Pune, and Shimla exhibited nearly the same isolation frequency approximating to an average of 13.4%. Isolated endophytic fungi belonged to nine different genera viz. *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Lasiodiplodia*, *Botryosphaeria*, *Nigrospora*, *Pestalotiopsis*, and *Arcopilus*. *Aspergillus* sp. was the most dominant genus, as it exhibited the highest average colonization frequency of 4.28%, followed by *Fusarium* sp. with 2.16%. *Lasiodiplodia* sp. exhibited an average colonization frequency of 1.47% while *Arcopilus* sp. and *Nigrospora* sp. exhibited the least colonization of 0.13% (Table 2). The maximum number of endophytes was recovered from the leaves (43) followed by the stem (10) (Table 3). All the endophytic fungi belonged to the phylum Ascomycota; out of these, 47.5% belonged to class Eurotiomycetes, followed by Dothiodeomycetes (30%) and Sordariomycetes (22.5%). Endophytes isolated from leaf tissues were *Penicillium* sp., *Aspergillus* sp., *Botryosphaeria* sp., *Pestalotiopsis* sp., *Fusarium* sp., *Arcopilus* sp., *Alternaria* sp., and *Lasiodiplodia* sp.; whereas, stem tissues harbor only *Penicillium* sp., *Aspergillus* sp., *Alternaria* sp., *Lasiodiplodia* sp., *Fusarium* sp., and *Nigrospora* sp. (Table 3).

Preliminary Screening for Resveratrol-Producing Endophytic Fungi

Out of 53 fungal strains, only 29 strains were found to be positive for the production of polyphenols. Formation of a red precipitate and dark greenish brown coloration by Liebermann and acetic anhydride tests respectively confirmed the presence of phenolic which was considered as a marker of resveratrol, since resveratrol is also a polyphenol. However, #12VVLPM exhibited the highest content of phenolic and hence could be considered as a potential producer of fungal resveratrol (Table 3).

Table 2 Colonization frequency (in percentage) of different endophytic fungi isolated from *Vitis vinifera* samples in different regions of India

Fungi	Alambagh, Lucknow (UP) (%)	Pune, Maharashtra (%)	Nasik, Maharashtra (%)	Bengaluru, Karnataka (%)	Shimla, Himachal Pradesh (%)	Average colonization frequency (%)
Penicillium sp.	1.30	3.13	0.00	0	0	0.89
Aspergillus sp.	4.00	1.25	4.70	8	3.44	4.28
Lasiodiplodia sp.	1.30	0.63	0.00	2	3.44	1.47
Botryosphaeria sp.	1.30	1.25	0.00	2	0	0.91
Pestalotiopsis sp.	1.30	0.00	0.00	0	0	0.26
Fusarium sp.	0	1.88	0.00	2	6.90	2.16
Arcopilus sp.	0	0.63	0.00	0	0	0.13
Nigrospora sp.	0	0.63	0.00	0	0	0.13
Alternaria sp.	0	1.25	1.56	2	0	0.96

Table 3 Qualitative and quantitative estimation of resveratrol production by endophytic fungi of *Vitis vinifera*

Culture ID	Plant part	Morphological identity	Liebermann test (test 1)	Acetic anhydride test (test 2)	HPLC quantification (μg/ml)
#3VVLLK	Leaf	*Penicillium* sp.	–	–	
#6(b)VVLLK	Leaf	Unidentified	++	++	11.9
#9VVLLK	Leaf	*Aspergillus* sp.	+	+	
#5(b)VVLLK	Leaf	*Aspergillus* sp.	–	–	
#1VVRLLK	Leaf	Unidentified	+	+	
#1VVRSTL	Stem	*Lasiodiplodia* sp.	+	+	
#4VVLLK	Leaf	*Botryosphaeria* sp.	–	–	
#8VVLLK	Leaf	*Pestalotiopsis* sp.	–	–	
#8VVGSTL	Stem	Unidentified	++	++	13.2
#21VVGSTL	Stem	*Aspergillus* sp.	–	–	
#10VVLPM	Leaf	*Botryosphaeria* sp.	–	–	
#10(b)VVLPM	Leaf	*Fusarium* sp.	–	–	
#25VVLPM	Leaf	*Fusarium* sp.	+	+	
#27VVLPM	Leaf	Unidentified	+	+	
#11VVSPM	Stem	*Fusarium* sp.	–	–	
#12(b)VVLPM	Leaf	*Aspergillus* sp.	+	+	
##12VVLPM	Leaf	Unidentified	+	+	
#16(b)VVLPM	Leaf	*Aspergillus* sp.	+	+	
#17(b)VVLPM	Leaf	Unidentified	–	–	
#12VVLPM	Leaf	*Arcopilus* sp.	+++	+++	89.1
#37VVLPM	Leaf	*Penicillium* sp.	–	–	
#66VVLPM	Leaf	*Penicillium* sp.	+	+	
#98VVSTPM	Stem	*Penicillium* sp.	++	++	21.9
#184VVSTPM	Stem	*Lasiodiplodia* sp.	+	+	
#193VVSTPM	Stem	*Nigrospora* sp.	++	++	25.2
#18VVLPM	Leaf	*Botryosphaeria* sp.	++	++	37.3
#19VVLPM	Leaf	*Penicillium* sp.	+	+	
#20(b)VVLPM	Leaf	*Penicillium* sp.	+++	++	15.3
#21VVLPM	Leaf	Unidentified	–	–	
#23VVLPM	Leaf	*Alternaria* sp.	–	–	
#24VVLPM	Leaf	*Alternaria* sp.	–	–	
#29VVLNM	Leaf	Unidentified	+	+	
#29(b)VVLNM	Leaf	*Aspergillus* sp.	–	–	
#30(b)VVLNM	Leaf	Unidentified	+	+	
#31VVLNM	Leaf	Unidentified	–	–	
#33VVLNM	Leaf	Unidentified	–	–	
#35VVLNM	Leaf	Unidentified	–	–	
#83VVLNM	Leaf	*Aspergillus* sp.	++	++	4.4
#105VVLNM	Leaf	*Aspergillus* sp.	+	+	
#109VVLNM	Leaf	*Alternaria* sp.	++	++	24.1
#20VVLBK	Leaf	*Fusarium* sp.	–	–	
#20(a)VVLBK	Leaf	*Botryosphaeria* sp.	+	+	
#49(b)VVLBK	Leaf	*Aspergillus* sp.	–	–	
#101VVLBK	Leaf	*Aspergillus* sp.	+++	++	22.4
#13VVLBK	Leaf	*Lasiodiplodia* sp.	+	+	
#23VVSTBK	Stem	*Aspergillus* sp.	+	+	
#53VVSTBK	Stem	*Aspergillus* sp.	+++	++	23.9
#56VVLBK	Leaf	Unidentified	–	–	
#101VVSTBK	Stem	*Alternaria* sp.	–	–	
#1VVGLS	Leaf	*Fusarium* sp.	–	–	

Table 3 (continued)

Culture ID	Plant part	Morphological identity	Liebermann test (test 1)	Acetic anhydride test (test 2)	HPLC quantification (μg/ml)
#3VVGLS	Leaf	*Aspergillus* sp.	–	–	
#7VVGLS	Leaf	*Fusarium* sp.	–	–	
#11VVGLS	Leaf	*Lasiodiplodia* sp.	+	+	

–, indicates no phenolic content; +, indicates moderate phenolic content (50–65%); ++, indicates good phenolic content (80–90%); +++, indicates very high phenolic content (> 90%)

Extraction and TLC of Resveratrol from Endophytic Fungus

The cell-free filtrate of #12VVLPM and 10 other isolates exhibiting a good phenolic content or higher was subjected to liquid-liquid extraction by ethyl acetate (EA) and subsequently the residue was reconstituted in methanol. The ethyl acetate fraction of #12VVLPM was subjected to different combinations of solvents as mobile phase to achieve good separation in the TLC.

The best separation of EA extract of #12VVLPM was observed in dichloromethane:chloroform:formic acid solvent system, in the ratio of 3.5:1.5:0.1 which resolved the extract into five different bands. The R_f value of band 1 to band 5 was 0.14, 0.27, 0.51, 0.69, and 0.78, respectively. When the UV light was exposed (long wave, 365 nm), the resveratrol in the EA residue showed similar violet color as that of standard resveratrol (Fig. 2). The standard resveratrol exhibited R_f value of 0.69 which was same as that of R_f value of band 4 of crude EA extract of #12VVLPM.

Quantification of Resveratrol Through HPLC

The confirmation and concentration of the fungal resveratrol was confirmed by HPLC analysis, which showed a symmetrical peak at RT 3.36 min similar to that of standard resveratrol (Fig. 3). The UV absorption analysis showed a peak representing absorption at 302 nm. The peak area vs. resveratrol concentration, obtained in the case of the standard resveratrol ($y = 5E + 07 \times - 2E + 06$; $R^2 = 0.9986$), was used to estimate the quantity of fungal resveratrol (Fig. 4). Out of 11 isolates, #12VVLPM showed maximum resveratrol production of 89.1 μg/ml, followed by #18VVLPM producing 37.3 μg/ml of resveratrol whereas #83VVLNM exhibited the least resveratrol production of 4.4 μg/ml (Table 3).

Identification of the Endophytic Fungi

The isolate #12VVLPM was identified on the basis of both classical morphotaxonomy and molecular taxonomy tools.

Morphotaxonomy of the Resveratrol-Producing Endophytic Fungi

The identification of resveratrol-producing endophytic strain (#12VVLPM) was done on the basis of its specific morphological and reproductive characteristics. The endophytic fungus #12VVLPM produced red wine color and orange over PDA and MEA respectively (Fig. 5a).

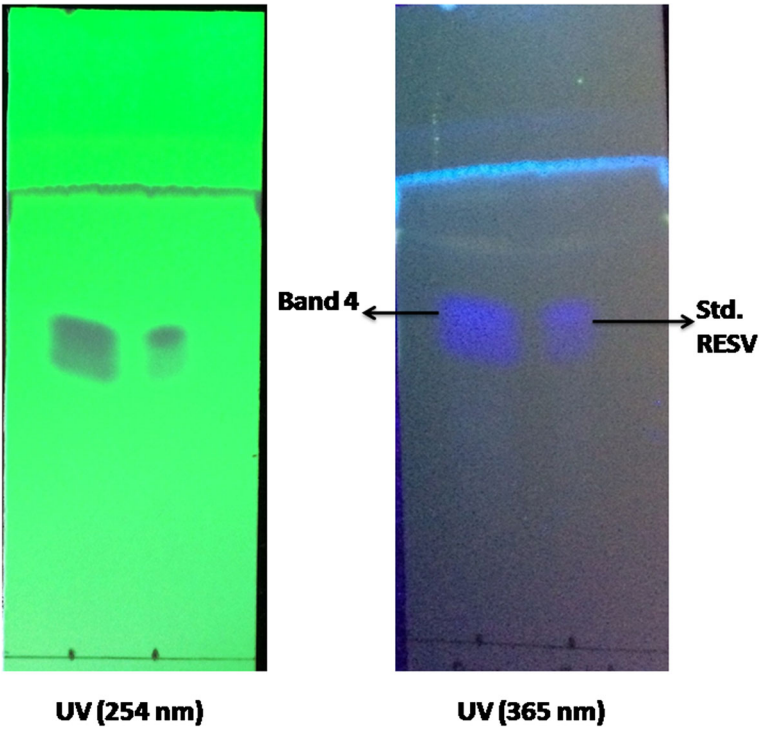

Fig. 2 TLC profile of crude extract of #12VVLPM using dichloromethane:chloroform:formic acid solvent system, in the ratio of 3.5: 1.5: 0.1 as mobile phase. The R_f values of bands 1–5 were ranging between 0.78 and 0.14

Colonies over PDA and MEA are rapidly growing (90 ± 0); young colonies are usually white by aerial mycelium becoming red or orange due to its ascomata and red pigment exudation (Fig. 5a). Over CMA colonies are golden in color from the front and reverse side with flat margin. The fungus produced golden color soluble pigment with fruity smell (Fig. 5b). Among microscopic characteristics, hyphae were septate, thick, branched, and brown in color. Ascomata are olivaceous to brown, maturing within 15–20 days, dark gray green when old, ovate in shape with 130.9–160.2 × 154.3–190.6 μm diameter (Fig. 6). Ascomatal hairs are apically circinate or curved, septate (Fig. 6). Asci are fasciculate, clavate in shape with 6–8 irregularly arranged ascospores per ascus (Fig. 6i). Ascospores are ovate, lunate, and reniform in shape with 8.6–10.0 × 11.4–13.5 μm diameter (Fig. 6).

ITS-rDNA-Based Molecular Taxonomy of #12VVLPM

The phylogenetic placement of the ITS sequence of #12VVLPM with type and non-type sequences recovered from BLAST analysis clearly indicated alignment with two isolates of *Arcopilus aureus* strain WW05 and *Arcopilus aureus* ATT 218 and one type strain *A. aureus* CBS 538.73 in the first clade (Fig. 7). Thus, based on the phylogenetic tree of ITS sequence as well as on the basis of morphological characteristics, #12VVLPM probably can be assigned the name *Arcopilus aureus*. The strain has been deposited at the National Fungal Culture Collection of India (NFFCI-4191) and its rDNA in GenBank with accession no. MF597785.

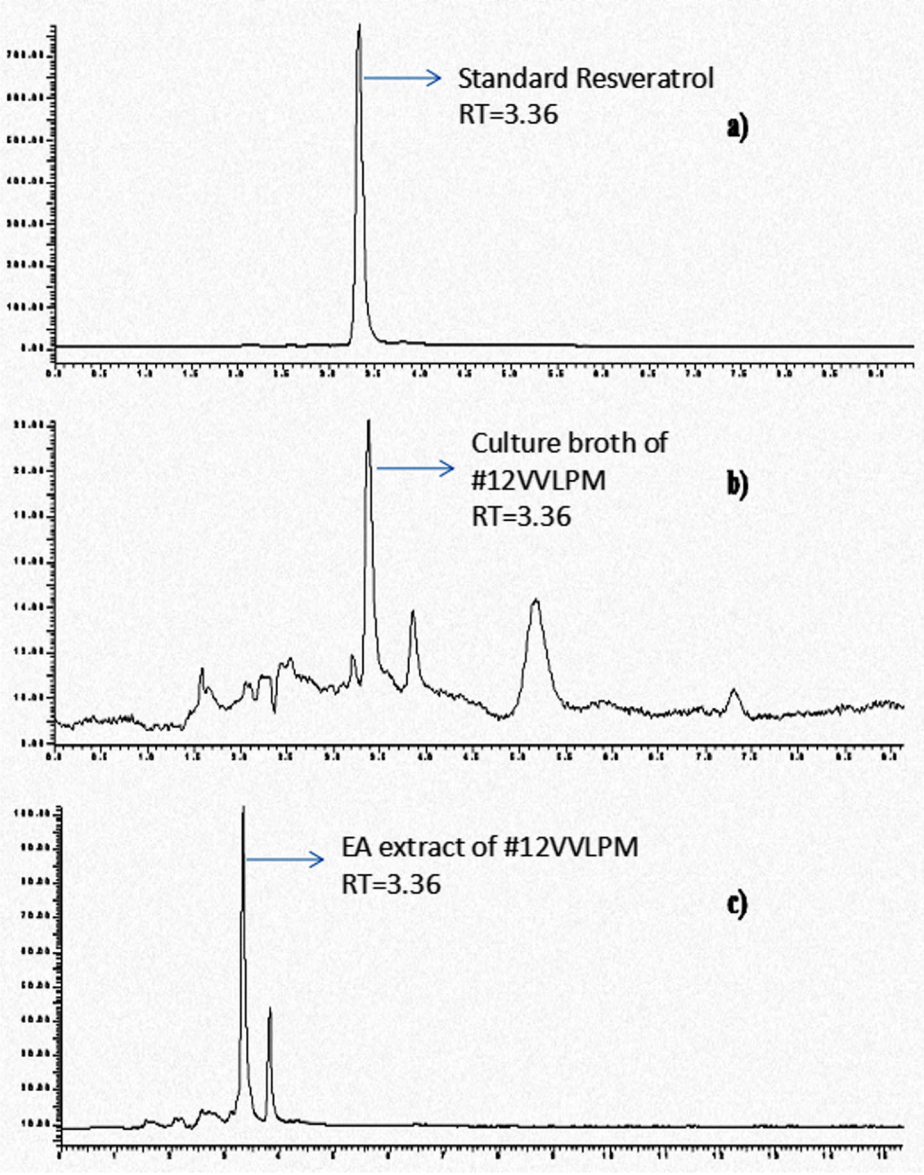

Fig. 3 HPLC spectra of **a** standard resveratrol, **b** culture broth of #12VVLPM, and **c** EA extract fungal resveratrol from #12VVLPM

Discussion

Endophytic fungi from the last two decades have attained remarkable recognition in the field of drug discovery and development as producer as well as sustainable source of unique and diverse bioactive compounds which can serve as pharmaceutical agents and leads of bioactive molecules for drug development to combat major global diseases [8, 11, 24]. Endophytic fungi are in mutualistic or symbiotic relationship with plant tissues and play a crucial role in

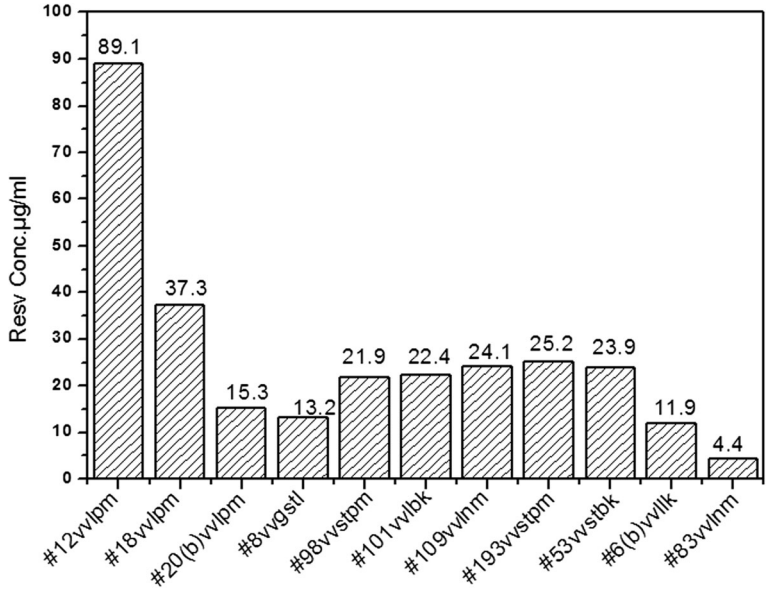

Fig. 4 Quantification of resveratrol-producing fungi

promoting host growth and resistance to environmental stress. These symbiotic microbes contribute toward the host fitness. A number of endophytes have been isolated and are being

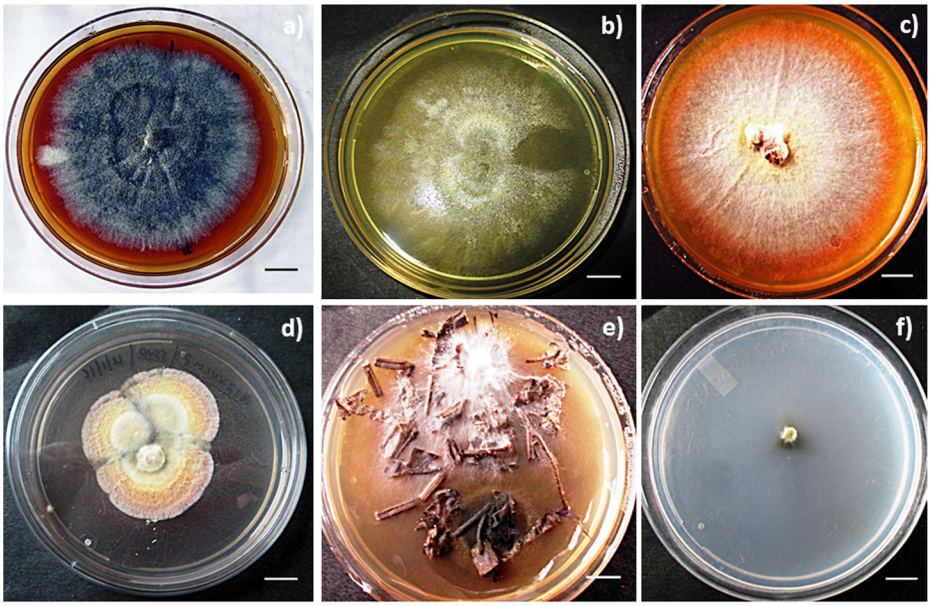

Fig. 5 Colony morphology of #12VVLPM over different media. **a** PDA (pH 7) colonies with red wine color pigmentation. **b** Colony over CMA with golden pigment formation. **c** Colony over CDA. **d** PDA (pH 5), no red wine color pigment formation. **e**, **f** Colony over GLA and WA media respectively. Bar, 10 mm

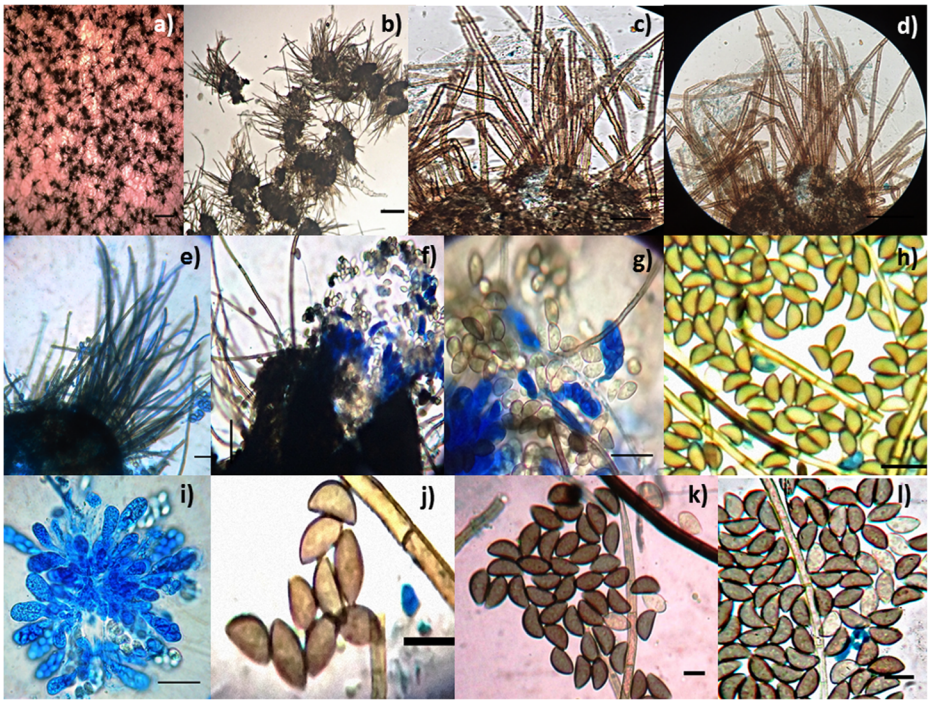

Fig. 6 **a** Microscopic features of #12VVLPM over different media: over PDA. **a** Colony photo of #12VVLPM. **b**, **c** Large, dark brown to black, mature ascomata on PDA. **d** Ascomatal hair. **e** Mature ascomata on CMA. **f** Close-up view of ascomata which demonstrates ostioles and contained asci. **g** 6–8 ascospore arranged in an irregular manner in an ascus. **h** Brown-colored septate hyphae and ovate-shaped ascospores. **i** Young asci. **j, k** Lunate, reniform-shaped ascospores on PDA. **l** Lunate, reniform-shaped ascospores on CMA. Bar, 10 μm

explored for their potential to produce bioactive natural products with medicinal properties [25–28].

Exploration of endophytic fungi capable of producing resveratrol is relatively new ever since it was recognized as a therapeutic moiety with several beneficial effects on human health. Resveratrol has been linked to various benefits like anti-inflammatory and anti-oxidant activity; preventing diabetes, cardiovascular diseases, and cancer; and as an anti-aging substance [3, 4, 8]. Interest in the resveratrol, a polyphenol often touted as the potent compound in red wine and grapes, came into limelight in 2003 when David Sinclair and his co-researchers reported that resveratrol was able to enhance the life span of a yeast cell, by activating a gene known as Sirtuin 1 which in turn induced calorie restriction in various species, including monkeys [29].

Bioprospecting endophytic fungi for production of RESV is a nascent area with very scant preliminary data. The first attempt to harness endophytic fungi for RESV was done by Powell in 1994 when he was successful in isolating RESV from endophytic *Acremonium* sp. Further, endophytes including *Alternaria* sp., *Aspergillus niger*, *Penicillium* sp., and *Botryosphaeria* sp. are among the very few that are recently reported to produce RESV [17, 21, 30]. To the best of our knowledge, the present study for the first time reports *Arcopilus aureus* as a resveratrol-producing endophytic fungus from *Vitis vinifera*.

RESV is produced by the grape plant under stress conditions when it is being attacked by fungi or bacteria [31]. As endophytes are known to produce putative phytochemicals of their

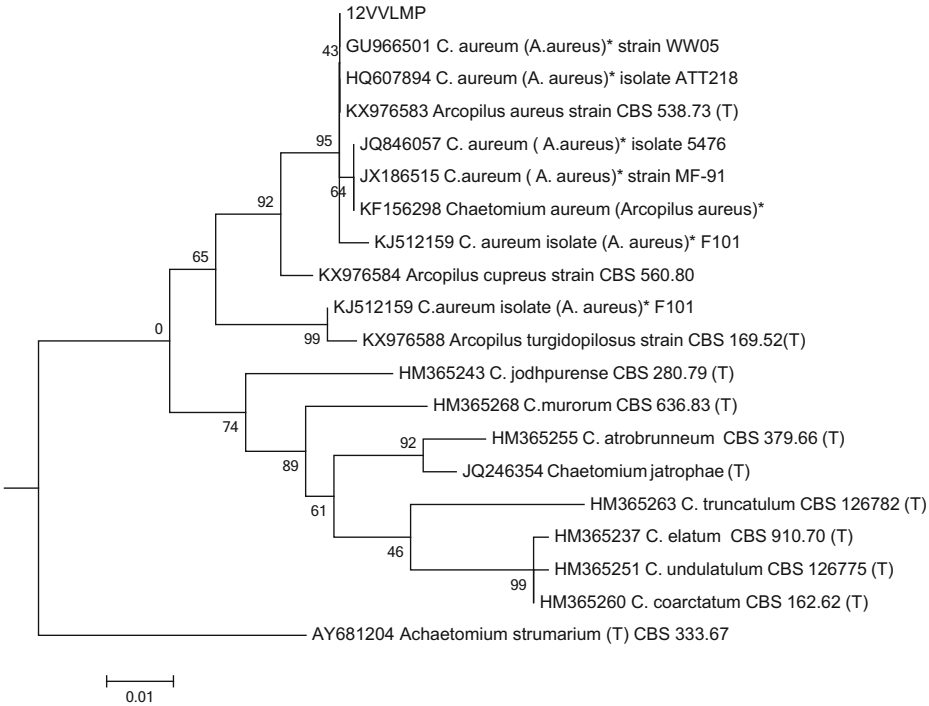

Fig. 7 Maximum likelihood tree based on the ITS1-5.8S-ITS2 region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)

host plants, therefore we attempted the isolation and screening of endophytic fungi as a RESV producer from different *Vitis vinifera* cultivars viz. merlot, wild, pinot noir, muscat, and Shiraz. González et al. [32] reported isolation of endophytic fungi from *Vitis vinifera* belonging to 51 fungal genera from six different locations in Spain, and Brum et al. [33] isolated from *Vitis labrusca* L. 550 fungal endophytes. There was a difference in the endophytes isolated from different locations in the study. The composition of endophytes and the frequency of infection varied according to host species, as did characteristics of site viz. vegetation, elevation, tissue type, exposure [34, 35], and age of tissue [34, 36]. Most fungi isolated in the course of the present study are previously isolated from grapevine [17, 30, 37] which is in agreement with that of the previous report. To the best of our knowledge, this is the first time when a *Chaetomium*-like fungus was found to be the potent producer of resveratrol, though *Chaetomium* spp. have been previously reported from *Vitis vinifera* [38].

The dominant fungal species isolated in the present study was *Aspergillus* sp. followed by *Fusarium* sp. In earlier reports, Liu et al. (2016) [30] isolated 24 strains of endophytic fungi from grapevines; while Shi et al. (2012) [17] isolated 57 endophytic fungi from merlot wine grapes, wild, *Vitis*, and Japanese knotweed. Several endophytes including *Alternaria alternata*, *Aureobasidium pullulans*, *Botryospheria* sp., *Epicoccum nigrum*, *Fusarium* sp., *Nigrospora* sp., *Phomopsis viticola*, and *Penicillium* sp. have been earlier reported from leaves and shoots of *Vitis vinifera* [32, 39]. The endophytic fungal isolates in the present study were dominated by Hyphomycetes followed by Ascomycetes; this corroborates with the previous study carried out by Gond et al. [40] and Kharwar et al. [41] where Hyphomycetes were dominantly colonizing as compared to Ascomycetes. In another study, the endophytic fungi isolated from

several cultivars of *Vitis vinifera* from the Madrid region (central Spain) were dominated by Ascomycetes [32]. The low proportion of Basidiomycetes taxa found in our study is consistent with other studies [32, 42].

The location Alambagh, Lucknow, was dominated by *Aspergillus* sp. and *Penicillium* sp.; Pune, Maharashtra, was dominated by *Aspergillus* sp.; whereas, the locations Nashik, Maharashtra, and Bengaluru, Karnataka, were dominated by *Aspergillus* sp. followed by *Fusarium* sp. and *Botryosphaeria* sp.; and Shimla, Himachal Pradesh, was dominated by *Aspergillus* sp. followed by *Lasiodiplodia* sp. In previous research, most of these fungi have been identified in grapevines. Pancher et al. [37] and Mostert et al. [38] reported *Alternaria* as the dominant genus and also the presence of *Phoma* sp. González et al. [32] and Musetti et al. [43] found the genera *Alternaria*, *Fusarium*, and *Phoma* as the most frequently occurring isolates of endophytic fungi of *Vitis vinifera*. In another study, *Alternaria* sp. and *Fusarium* sp. were reported as dominant along with less frequently occurring *Phoma* sp., *Diaporthe* sp., and *Phomopsis* sp. in the grapevine cultivars [44]. The variation in endophytes from a same host plant from different locations is attributed usually to the retrieval of incidental and rare species with more disseverance distribution; otherwise, relatively a constant proportion of fungal taxa is recovered from all samples [45].

HPLC analysis played an important role in identification of potent RESV-producing endophytic fungi. Out of the 11 cultures, filtrates which displayed good to very good phenolic content by Liebermann test and acetic acid test were confirmed for their RESV-producing potential using HPLC analysis. The RESV-producing isolates in the study (16.67%) were less than that observed by Liu et al. [30] (19.44%) and Shi et al. [17] (32.3%). The main keystone for evaluating RESV in the organic extracts has been HPLC which has also been used in earlier studies [17, 21, 30]. The isolate #12VVLPM was the potent RESV producer out of the 11 isolates as it produced the highest level of RESV and became the main focus of our study. The resveratrol production of the strain #12VVLPM was higher (89.1 μg/ml) than the earlier reports from endophytic fungi [17, 30]. Resveratrol production by #12VVLPM was stable over several passages as compared to other isolates which were also RESV producers. However, the RESV production decreased drastically on passaging in four cultures viz. #83VVLNM, #6(b)VVLLK, #8VVGSTL, and #20(b)VVLPM. The activity of the isolate #12VVLPM did not decrease as previously reported in *Alternaria* sp. MG2 and *Botryosphaeria* sp. YG3 [17, 21, 30] and also reports the similar decrease and loss of the capability of other compounds in other metabolite-producing endophytes. The decrease in resveratrol production in several isolates could possibly be due to unstable expression of resveratrol-related genes and the transformation of metabolic pathway within the cells. Research on the biosynthesis pathway and the key genes encoding these pathways would provide essential information for the exact mechanism of loss in production.

The RESV-producing isolate was identified using morphological and ITS-based molecular taxonomy. On the basis of its morphological characters and the ability to produce red wine at neutral pH, the isolate #12VVLPM was identified as *Arcopilus aureus*. Further, for species-level delineation of the fungus, ITS-based molecular taxonomy was used which corroborates with the findings of morphotaxonomy. ITS-based taxonomy is the most globally accepted approach for species-level identification of the fungi [22, 23, 46]. RESV-producing fungi including *Alternaria* sp. MG 1 and *Aspergillus niger* have been identified using ITS-based molecular taxonomy [17, 33].

Chaetomium-like species predominantly are non-pathogenic to plants generally reported to possess allergenic properties; however, some of them are coprophilous, while others are found

in seed and soil [47, 48] apart from their presence in inorganic compost [48]. They degrade cellulose and other organic materials and act as antagonist against plant fungal pathogens [48]. *Chaetomium*-like fungi are also known for production of secondary metabolites possessing different biological activities. Several compounds have been isolated from the genus *Chaetomium* such as benzoquinone derivatives [49], chaetomanone—new anthraquinone-chromanone compounds and several known compounds such as ergosterol, ergosterylpalmitate, chrysophanol, chaetoglobosin C, alternariol momethyl ether, echinuline, and isochaetoglobosin D. Echinulin and chaetomanone have been reported to exhibit anti-tubercular potential, as they have been found to inhibit *Mycobacterium tuberculosis* [50]. The biological properties of the fungal RESV produced by *Arcopilus aureus* might also be attributed to the inherent property of the fungus to produce various bioactive metabolites which can have therapeutic implications.

Thus, *Arcopilus aureus* #12VVLPM holds a promise for further strain improvement for fermentative production of resveratrol and subsequent optimization of the fermentation parameters to meet the industrial-scale production of resveratrol.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Bradamante, S., Barenghi, L., & Villa, A. (2004). Cardiovascular protective effects of resveratrol. *Cardiovascular Drug Reviews, 22*, 169–188.
2. Jang, M. S., Cai, E. N., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., & Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science, 275*(5297), 218–220.
3. Saxena, S., & Srivastava, A. (2014). Resveratrol: biological activities and therapeutic potential. *Journal of Pharmaceutical Technology, Research and Management, 2*(2), 145–157.
4. Wang, D. G., Liu, W. Y., & Chen, G. T. (2013). A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*. *Journal of Pharmaceutical Analysis, 3*(4), 241–247.
5. Fremont, L. (2000). Biological effects of resveratrol. *Life Sciences, 66*(8), 663–673.
6. Wang, Q., Xu, J. F., Rottinghaus, G. E., Simonyi, A., Lubahn, D., Sun, G. Y., & Sun, A. Y. (2002). Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain Research, 958*(2), 439–447.
7. Baur, J. A., & Sinclair, D. A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nature Reviews Drug Discovery, 5*(6), 493–506.
8. Zhang, H. W., Song, Y. C., & Tan, R. X. (2006). Biology and chemistry of endophytes. *Natural Product Reports, 23*(5), 753–771.
9. Berman, A. Y., Motechin, R. A., Wiesenfeld, M. Y., & Holz, M. K. (2017). The therapeutic potential of resveratrol: a review of clinical trials. *NPJ Precision Oncology, 1*(1), 35.
10. Tassoni, A., Fornale, S., Franceschetti, M., Musiani, F., Michael, A. J., Perry, B., & Bagni, N. (2005). Jasmonates and Na-orthovanadate promote resveratrol production in *Vitis vinifera* cv. Barbera cell cultures. *The New Phytologist, 166*(3), 895–905.

11. Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews, 67*(4), 491–502.
12. Suryanarayanan, T. S., Thirunavukkarasu, N., Govindarajulu, M. B., Sasse, F., Jansen, R., & Murali, T. S. (2009). Fungal endophytes and bioprospecting. *Fungal Biology Reviews, 23*(1-2), 9–19.
13. Schulz, B., Wanke, U., Draeger, S., & Aust, H. J. (1993). Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research, 97*(12), 1447–1450.
14. Photita, W., Lumyong, S., Lumyong, P., & Hyde, K. D. (2001). Endophytic fungi of wild banana (*Musa acuminata*) at DosiSuthepPui National Park, Thailand. *Mycological Research, 105*(12), 1508–1513.
15. Suryanarayanan, T. S., Venkatesan, G., & Murali, T. S. (2003). Endophytic fungal communities in leaves of tropical forest trees: diversity and distribution patterns. *Current Science, 85*(4), 489–492.
16. Gupta, Y. K., Briyal, S., & Chaudhary, G. (2002). Protective effect of trans-resveratrol against kainic acid-induced seizures and oxidative stress in rats. *Pharmacology Biochemistry and Behavior, 71*(1-2), 245–249.
17. Shi, J., Zeng, Q., Liu, Y., & Pan, Z. (2012). *Alternaria sp*. MG1, a resveratrol-producing fungus: isolation, identification, and optimal cultivation conditions for resveratrol production. *Applied Microbiology and Biotechnology, 95*(2), 369–379.
18. Al-Jumaily, E. F. A., Hamid, G. S., & Ali, K. F. (2014). Synthesis and total phenol content of new resveratrol derivative. *American Journal of Advanced Drug Discovery, (2–3),* 320–329.
19. Park, J., & Boo, Y. C. (2013). Isolation of resveratrol from *Vitis viniferae* caulis and its potent inhibition of human tyrosinase. *Evidence-Based Complementary and Alternative Medicine*. Article ID 645257.
20. Babu, S. K., Kumar, K. V., & Subbaraju, G. V. (2005). Estimation of trans-resveratrol in herbal extracts and dosage forms by high-performance thin layer chromatography. *Chemical and Pharmaceutical Bulletin, 53*(6), 691–693.
21. Wang, X. W., Houbraken, J., Groenwald, J. Z., Meijer, M., Andersen, B., Nielsen, K. F., Crous, P. W., & Samson, R. A. (2016). Diversity and taxonomy of *Chaetomium* and *Chaetomium*-like fungi from indoor environments. *Studies in Mycology, 84*, 145–224.
22. White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications* (Vol. 18, pp. 315–322).
23. Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution, 30*(10), 2725–2729.
24. Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. *Frontiers in Microbiology, 7*, 1538.
25. Gonzalez-Coloma, A., Cosoveanu, A., Cabrera, R., Gimenez, C., & Kaushik, N. (2016). Endophytic fungi and their bioprospection. In S. K. Deshmukh, J. K. Misra, J. P. Tewari, & T. Papp (Eds.), *Fungi: applications and management strategies* (pp. 14–31). CRC Press.
26. Le Cocq, K., Gurr, S. J., Hirsch, P. R., & Mauchline, T. H. (2017). Exploitation of endophytes for sustainable agricultural intensification. *Molecular Plant Pathology, 18*(3), 469–473.
27. Aly, A. H., Debbab, A., & Proksch, P. (2013). Fungal endophytes—secret producers of bioactive plant metabolites. *An International Journal of Pharmaceutical Sciences, 68*(7), 499–505.
28. Kharwar, R. N., Kumar, A., Verma, V. C., & Redman, R. S. (2015). Book chapter Ajit Varma (Endophytes). Endophytic fungi: better players of biodiversity, stress tolerance, host protection and antimicrobial production. *A textbook of molecular biotechnology*, pp 1033–1057.
29. Hubbard, B. P., & Sinclair, D. A. (2014). Small molecule SIRT1 activators for the treatment of aging and age-related diseases. *Trends in Pharmacological Sciences, 35*(3), 146–154.
30. Liu, Y., Nan, L., Liu, J., Yan, H., Zhang, D., & Han, X. (2016). Isolation and identification of resveratrol-producing endophytes from wine grape Cabernet Sauvignon. *Springer Plus, 5*(1), 1029.
31. Langcake, P., & Pryce, R. J. (1976). The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. *Physiological Plant Pathology, 9*(1), 77–86.
32. González, V., & Tello, M. L. (2011). The endophytic mycota associated with *Vitis vinifera* in central Spain. *Fungal Diversity, 47*(1), 29–42.
33. Brum, M. C. P. D., Araujo, W. L. D., Maki, C. S., & Azevedo, J. L. D. (2012). Endophytic fungi from *Vitis labrusca* L. ('Niagara Rosada') and its potential for the biological control of *Fusarium oxysporum*. *Genetics and Molecular Research, 11*(4), 4187–4197.
34. Fisher, P. J., Anson, A. E., & Petrini, O. (1986). Fungal endophytes in *Ulex europaeus* and *Ulex gallii*. *Transactions of the British Mycological Society, 86*(1), 153–156.
35. Roll-Hansen, F., & Roll-Hansen, H. (1979). Ascocoryne species in living stems of *Picea* species: a literature review. *Forest Pathology, 9*(5), 275–280.
36. Rodriguez, R. J., White Jr., J. F., Arnold, A. E., & Redman, A. R. A. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist, 182*(2), 314–330.

37. Kernaghan, G., Mayerhofer, M., & Griffin, A. (2017). Fungal endophytes of wild and hybrid *Vit*is leaves and their potential for vineyard biocontrol. *Canadian Journal of Microbiology, 63*(7), 583–595.
38. Mostert, L., Crous, P. W., & Petrini, O. (2000). Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. *Sydowia, 52*(1), 46–58.
39. Zeng, Q., Shi, J. L., & Liu, Y. L. (2012). Isolation and identification of a resveratrol-producing endophytic fungus from grape. *Food Science, 33*(13), 167–170.
40. Gond, S. K., Verma, V. C., Kumar, A., Kumar, V., & Kharwar, R. N. (2007). Study of endophytic fungal community from different parts of *Aegle marmelos* Correa (*Rutaceae*) from Varanasi (India). *World Journal of Microbiology and Biotechnology, 23*(10), 1371–1375.
41. Kharwar, R. N., Maurya, A. L., Verma, V. C., Kumar, A., GOND, S. K., & Mishra, A. (2012). Diversity and antimicrobial activity of Endophytic fungal community isolated from medicinal plant *Cinnamomum camphora*. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 82*(4), 557–565.
42. Márquez, S. S., Bills, G. F., Acuña, L. D., & Zabalgogeazcoa, I. (2010). Endophytic mycobiota of leaves and roots of the grass Holcuslanatus. *Fungal Diversity, 41*(1), 115–123.
43. Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., Marzani, C., D'Ambrosio, M., di Toppi, L. S., & Pertot, I. (2006). Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. *Phytopathology, 96*(7), 689–698.
44. Casieri, L., Hofstetter, V. A. L. É. R. I. E., Viret, O. L. I. V. I. E. R., & Gindro, K. A. T. I. A. (2009). Fungal communities living in the wood of different cultivars of young *Vitis vinifera* plants. *Phytopathologia Mediterranea, 48*(1), 73–83.
45. Bills, G. F., & Polishook, J. D. (1994). Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia, 86*(2), 187–198.
46. Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406–425.
47. Somrithipol, S. (2004). Coprophilous fungi. In E. B. G. Jones, M. Tanticharoen, & K. D. Hyde (Eds.), *Thai fungal diversity* (pp. 119–128). Thailand: BIOTEC.
48. Soytong, K., & Quimio, T. H. (1989). A taxonomic study on the Philippine species of *Chaetomium*. *The Philippine agriculturist, 72*(1), 59–72.
49. Brewer, D., Jerram, W. A., & Taylor, A. (1968). The production of cochliodinol and a related metabolite by *Chaetomium* species. *Canadian Journal of Microbiology, 14*(8), 861–866.
50. Kanokmedhakul, S., Kanokmedhakul, K., Phonkerd, N., Soytong, K., Kongsaeree, P., & Suksamrarn, A. (2001). Anti-mycobacterial anthraquinone-chromanone compound and diketopiperazine alkaloid from the fungus *Chaetomium globosum* KMITLN0802. *Planta Medica, 68*(9), 834–836.

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RESEARCH ARTICLE

In Vitro Anti-oxidant, Anti-fungal and Anti-staphylococcal Activity of Resveratrol-Producing Endophytic Fungi

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Abstract Trans-resveratrol is an enormously exploited stilbene exhibiting a broad spectrum of biological activities. However, commercial production to meet the demand and supply is very difficult as it is largely extracted from plants such as *Polygonum cuspidatum* making it very expensive. In the present study, resveratrol-producing endophytic fungi have been investigated for their in vitro antioxidant, antifungal and anti-staphylococcal activities. All the four fungal endophytes exhibited significant resveratrol production in the range of 31.3–89.1 mg/L in liquid cultures which was evaluated by high-performance liquid chromatography analysis. The endophytic isolate #12VVLPM in the present study exhibited strong antioxidant property in case of 2,2-diphenyl-1-picrylhydrazyl, Trolox equivalent antioxidant capacity, metal ion chelating, Nitric oxide radical and Hydrogen peroxide with IC_{50} values of 0.11–4.96, 0.28–2.16, 0.12–1.33, 0.08–1.35 and 0.12–1.18 mg/mL, respectively. The maximum inhibition ($57.76 \pm 0.82\%$) was exhibited by #12VVLPM against *Botrytis cinerea*. The present result shows strong correlation between antioxidant activities, anti-staphylococcal as well as antifungal activity.

Significance Statement Resveratrol is extensively being used as a therapeutic moiety, as well as a pharmacophore for development of new drugs due to its multifarious beneficial effects. In the present study, resveratrol-producing endophytic fungi have been investigated for their in vitro antioxidant, antifungal and anti-staphylococcal activities.

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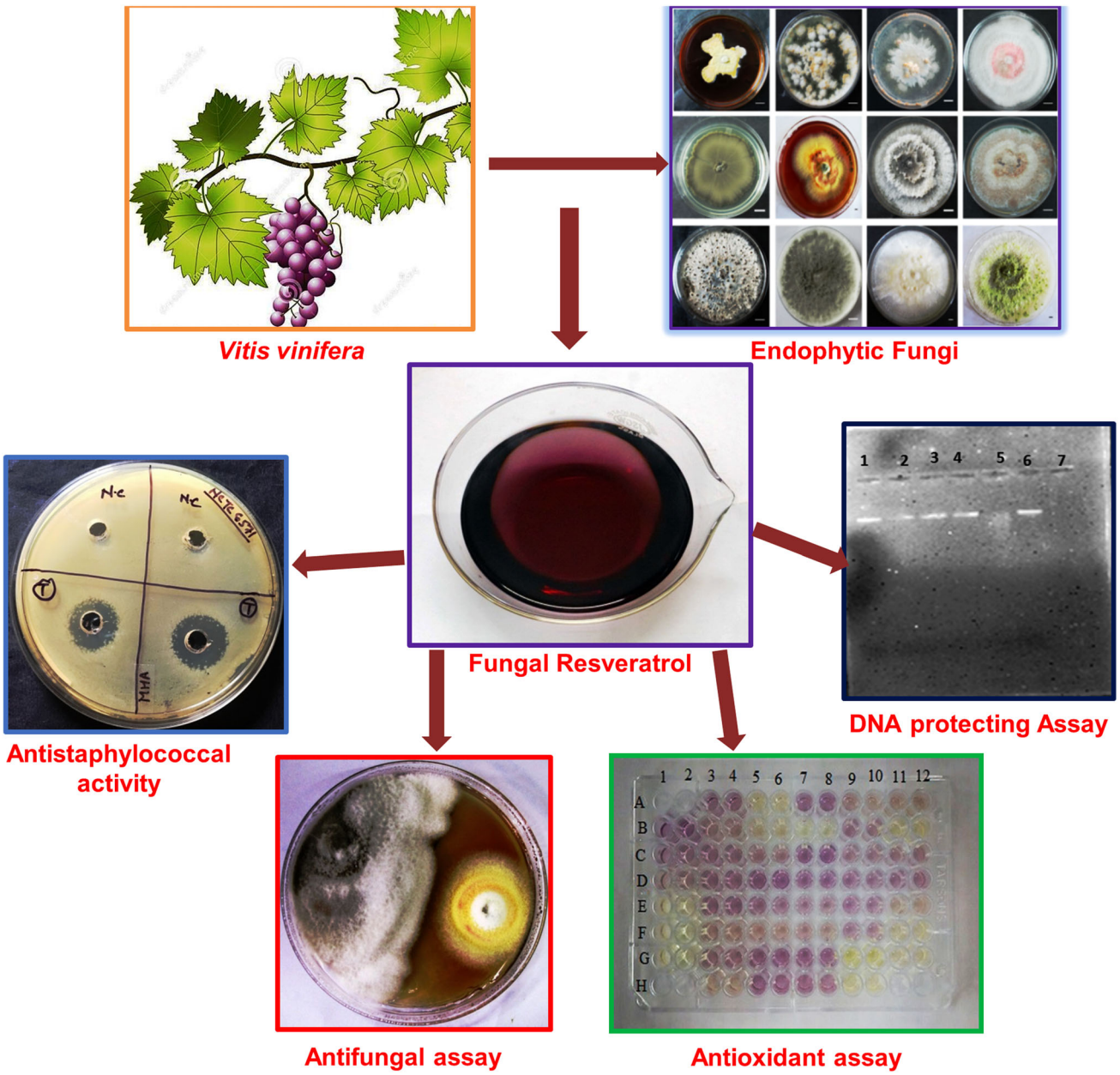
Graphical Abstract



Keywords Endophytes · In vitro antioxidant activity · In vitro antifungal activity · In vitro anti-staphylococcal activity · Resveratrol

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
TEAC	Trolox equivalent antioxidant capacity
FRAP	Ferric reducing antioxidant power
TPC	Total phenolic content
TFC	Total flavonoids content
RESV	Resveratrol
MHA	Mueller Hinton agar
SDA	Sabouraud dextrose agar
MTCC	Microbial type culture collection
ATCC	American type culture collection
Sau	*Staphylococcus aureus*

Introduction

Endophytic fungi are a group of microorganisms which reside within the intra- and intercellular plant tissues for all or a part of their life cycle and maintain an asymptomatic and inconspicuous union with their host plant. These endophytes produce an array of the signal molecules to overcome the inherent defence mechanisms of the host plant which purportedly play a role in aiding the host plant in combating different types of biotic and abiotic stresses. The signal molecules produced by endophytic fungi can produce an array of secondary metabolites which may find applications in agriculture, medicine, and industry. Thus, they offer to be a resource for novel chemical entities [1, 2].

Resveratrol (RESV) or Trans 3, 5, 4′-trihydroxystilbene is a renowned bioactive molecule which garnered much attention in the past decade due to its multifarious therapeutic action on human health such as cardioprotective and neuroprotective agent, exhibiting anti-inflammatory, anti-cancer, anti-ageing effects apart from playing a preventive role in obesity and diabetes [3, 4]. Thus, the therapeutic effects of red wine have been primarily attributed to the transfer of RESV from grapes to wine during winemaking. RESV is produced by the different varieties of the plants including grape (*Vitis vinifera*) as a defense mechanism in response to fungal infection, stress, injury and U.V radiation [5]. Today, RESV is being used as a constituent in health supplements and wellness products apart from being studied as a pharmacophore for drug development.

Numerous antioxidant compounds have been resourced from nature which are being exploited by mankind in diverse products. An antioxidant molecule neutralizes the reactive oxygen species (ROS) and protects it from cellular damages [6]. Limited generation of ROS is an essential part of body's defence mechanism against disorders, cellular signalling system as well as inducing mitogenic response; however, at higher concentrations, these are responsible for oxidative damage to proteins, nucleic acids and lipids and ultimately leading to cell death [6, 7]. Antioxidant molecules block the free radical chain reactions of oxidation thereby delaying the oxidative damage due to excessive ROS production [8, 9]. Globally antimicrobial resistance is a burgeoning problem with limited options. One of the prominent opportunistic pathogen is *Staphylococcus aureus*, which generally resides in the interior nasal cavity of human beings [10]. *Staphylococcus aureus* has exhibited extreme genetic plasticity and developed a variety of drug resistant variants which exhibit a refractory behaviour towards the current armamentarium of antimicrobial drugs [11]. Resveratrol was initially identified as a phytoalexin, a class of natural product expressed during plant–microbe interaction and exhibit inhibitory activity against a spectrum of plant as well as human pathogens [12–15]. In due course, it was also found to be present as a phytochemical. Hence, the present research work is oriented towards the exploration of antioxidant and antimicrobial properties of resveratrol-producing endophytic fungi.

Material and Methods

Instrumentation and Reagents

BIOTEK® Powerwave 340 microplate reader was used for performing different in vitro antioxidant assays. Resveratrol (RESV), 2,2′-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), Trolox and FC reagent were procured from Sigma-Aldrich (USA); gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, streptomycin, sodium sulphate (Na_2SO_4), sodium nitrate ($NaNO_3$), aluminium chloride ($AlCl_3$), ferric chloride ($FeCl_3$) from Hi-Media Labs Pvt. Ltd. (India); methanol (MeOH), acetonitrile (ACN) and conc. sulphuric acid (H_2SO_4) from Merck, Millipore, (USA); a nitrocellulose membrane (NCM) from GE Healthcare and Life Sciences (USA); orthophosphoric acid (H_3PO_4) from Hi-Media (India); and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) from Sigma-Aldrich (USA).

Microorganisms and the Culture Media

The microbial test panel comprised of four plant pathogens, *Botrytis cineria* (MTCC 359), *Colletotrichum gloeosporioides* (MTCC 9623), *Cercospora beticola* (ATCC 24888) and *Rhizoctonia solani* (MTCC 4634) and five clinical surrogate isolates of *Staphylococcus aureus,* viz. NCTC 6571, Sau G9, MTCC 96, MTCC 737, and Sau 902. All the bacterial cultures were revived and maintained on Mueller Hinton agar (MHA), plant pathogens on Potato Dextrose Agar (PDA) and *Candida* was sub-cultured in Sabouraud Dextrose Agar (SDA).

Re-culturing and Maintenance of the Endophytic Fungi

Arcopilus aureus (#12VVLPM), *Fusarium equiseti* (#19VVLPM), *Xylaria psidii* (#22(P) VVLPM), *Fusarium solani* (#4(P) VVLPM) (having GenBank accession numbers MF597785, MH181157, MH142837, MH169229 respectively) were the resveratrol-producing endophytic fungi isolated from *Vitis vinifera* which were selected for the present study. The cultures were revived on PDA plates which were incubated at 26 ± 2 °C for 10 days.

Subsequently, 5-mm mycelial plugs were prepared from 7- to 10-day old culture and inoculated aseptically to PDA slants and vials and incubated at 26 ± 2 °C till fungal mycelia was observed.

Culture Filtrate Production and Isolation of Secondary Metabolite

The culture filtrates were obtained by inoculating 5-mm mycelial plugs of 7-day old culture of the selected endophytic isolates in 250 mL of pre-sterilized potato dextrose broth (PDB) in 500-mL Erlenmeyer flasks which were incubated at 26 ± 2 °C for 10 days at 120 rpm. Culture filtrates so obtained after production were then subjected to liquid–liquid extraction for the probable isolation of bioactive residues/secondary metabolites. The culture filtrate was extracted with ethyl acetate in the ratio of 1:3. The organic layer was pooled out, and the extraction procedure was repeated thrice. The organic layer was dried with the addition of anhydrous sodium sulphate and evaporated in the rotatory evaporator at 45 °C to get the dry crude bioactive fraction. The fraction so obtained was weighed, reconstituted in methanol and again screened for the presence of resveratrol.

Estimation of RESV Production Through High-Performance Liquid Chromatography (HPLC)

The identification and concentration of resveratrol in crude bioactive fractions were determined via HPLC (PerkinElmer–200, USA) using C18 reverse phase discovery column (Sigma-Aldrich, USA) equipped with series-200 pump, dual wavelength detector and total chrome workstation software. About 20 µL of the sample of defined concentration was injected into the HPLC column and eluted using orthophosphoric acid (0.1%, pH-2.3 Sigma-Aldrich) and acetonitrile (Sigma-Aldrich); 45–55% ratio was used as mobile phase with a flow rate of 1 mL/min. The yield of fungal RESV was determined against a standard curve of standard RESV (0.1–1 mg/mL) under similar conditions. Sample (20 µL) was injected into C18 (5 µm, 25 cm × 4.6 mm) reverse phase discovery column (Sigma-Aldrich; 58298), with column oven temperature of 37 °C. The data of the peak area vs. concentration of the standard resveratrol obtained were used to estimate the quantity of fungal resveratrol in crude ethyl acetate (EA) fraction [16].

Screening In Vitro Antioxidant Activity

The in vitro antioxidant activity of the resveratrol-producing endophytic fungi by different methods comprised of using a standard antioxidant or resveratrol as standard. Resveratrol was used as a standard in DPPH scavenging assay, metal ion chelating scavenging assay exclusively while hydrogen peroxide radical scavenging assay uses double standards—resveratrol as well as ascorbic acid. Ascorbic acid was used as a standard in the nitric oxide radical scavenging assay, while Trolox was used in the TEAC assay.

DPPH Scavenging Assay

The ability of fungal extract to scavenge the free radical was determined by recording the change in the optical density of the DPPH radicals (1, 1-diphenyl-2-picrylhydrazyl) according to Ho et al. [17]. Briefly, to 1 mL of DPPH (100 µM in methanol), 50 µL of the fungal extract (10–50 µg/mL) was added and incubated at room temperature for half an hour in the dark. Further, the absorbance was measured at 517 nm using a medium throughput reader (Powerwave 340, BioTek, USA). Methanol was used as the negative control. Resveratrol (10–50 µg/mL) was used as a standard. Free radical scavenging activity was expressed as a percentage. The percentage of scavenging activity was expressed and calculated as following.

$$\text{Free radical scavenging activity } (\%) = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{test}}}{\text{OD}_{\text{control}}} \times 100$$

The test was performed in triplicates, and data were represented as mean ± SD and expressed as µg of resveratrol equivalent/mg of sample.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

This method is based on the ability of an antioxidant molecule to scavenge the ABTS (2, 2′-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) radical, relative to that of the standard antioxidant Trolox. TEAC assay was performed according to Re et al. [18]. The $ABTS^+$ radical was generated by mixing of 7 mM ABTS (in 0.1 M phosphate buffer saline, pH = 7.4) and 2.45 mM potassium persulphate in equal volume, and the mixture was allowed to stand in the dark at room temperature for 16 h to obtain the dark green colour. $ABTS^+$ radicals working solution was prepared by diluting the above solution with phosphate buffer saline to an absorbance of 0.9–1.0 at 734 nm. For carrying out the assay, 10 µL of extract (200–1000 µg/mL) was added with 1.0 mL of diluted $ABTS^+$ solution. The mixture was incubated for 6 min, and a decrease in absorbance was recorded at 734 nm using a medium throughput reader (Powerwave 340, BioTek, USA). Phosphate buffer saline was used as a blank, and working

$ABTS^+$ solution was taken as control. Trolox was used as the standard (200–1000 µg/mL), and radical scavenging capacity was expressed as µg of Trolox equivalent/mg of sample. The percentage of scavenging activity was expressed and calculated by the formula mentioned in the previous Para. The test was performed in triplicates, and data were represented as mean ± SD.

Metal Ion Chelating Scavenging Assay

The ferrous ion chelating potential of the fungal extracts was evaluated according to Danagoudar et al. [19]. In this assay, ferrozine can quantitatively chelate Fe^{2+} ions and form a red-brown coloured compound. Evaluation of colour reduction measured the chelating activity to compete with ferrozine for the ferrous ions. Briefly, the reaction mixture comprised of 1 mL of different concentrations of the fungal extracts (0.2–1.0 mg/mL) and 500 µL of 2 mM of $FeCl_3$. The reaction was initiated after the addition of 200 µL of 5 mM ferrozine. After vigorous shaking, the reaction mixture was left standing at 30 °C for 10 min. Moreover, the decrease in the intensity of the colour was measured at 562 nm against a reagent blank. Resveratrol was used as a standard. The percentage of scavenging activity was expressed as mentioned earlier.

Nitric Oxide Radical Scavenging Assay

Griess Illosvoy reaction was used for estimation of Nitric oxide (NO) radical scavenging activity [20]. The reaction mixture (3 mL) comprised of 2 mL sodium nitroprusside (10 mM), phosphate buffer saline (0.5 mL) and standard solution or fungal extract (0.5 mL) (ethyl acetate fraction: 100–500 µg/mL) which was incubated at room temperature for 2 h. After the incubation, 500 µL of the reaction mixture was mixed with 1 mL sulfanilic acid reagent and allowed to stand for 5 min for diazotization. Further, 1 mL of the naphthylethylene diaminedihydrochloride was added, mixed and allowed to stand for 30 min at room temperature. Absorbance was taken in the form of concentration of nitrite at 546 nm. Ascorbic acid was used as standard, and buffer was taken as a blank. The percentage inhibition was calculated as mentioned earlier.

Hydrogen Peroxide Radical Scavenging Assay

The ability of fungal extract to scavenge hydrogen peroxide was calculated by the method of Ruch et al. [21], with minor modifications. A solution of 43 mM of hydrogen peroxide was prepared in 1 M phosphate buffer (pH 7.4). Various concentrations of fungal extract (0.2–1.0 mg/mL) were added to 600 µL (43 mM) of hydrogen peroxide solution. The intensity of hydrogen peroxide at 230 nm was determined after 10 min of incubation against a blank solution containing phosphate buffer without hydrogen peroxide. Resveratrol and ascorbic acid were used as standards. The percentage of scavenging activity was calculated as mentioned earlier.

λ-DNA Nicking Assay

Oxidative λ-DNA damage protection ability of fungal ethyl acetate extract was assessed by the method of Danagoudar et al. [19]. Briefly, the reaction was performed in an Eppendorf tube containing 1 µL of λ-DNA (0.5 µg) with and without fungal extract (ethyl acetate fraction: 2 µL of 300 µg/mL) in 15 µL TE buffer and incubated with Fenton reagent (3 µL of 2 mM $FeSO_4$, 3 µL of 30% H_2O_2 in Tris buffer 10 mM) in a final reaction volume of 30 µL for 1 h at 37 °C. After that 1 µL of 0.5 mM EDTA (pH 8.0) was added to stop the reaction. TE buffer was used in control. The relative difference between oxidized and native DNA was analysed on 1% agarose gel prepared in Tris-acetate-EDTA buffer (pH-8.5). The gel was run at 70 V for 1 h at room temperature, and after that band intensity was documented using XR + molecular imager Gel documentation system (Bio-RAD, USA).

In Vitro Antifungal Activity

Fungicidal activity of the culture extract of resveratrol-producing fungi was assessed against a panel of plant pathogenic fungi comprising of *Botrytis cinerea* (MTCC 359), *Colletotrichum gloeosporioides* (MTCC 9623), *Cercospora beticola* (ATCC 24888) and *Rhizoctonia solani* (MTCC 4634) by dual culture method. From 7-day old culture of resveratrol-producing endophytic fungi and plant pathogen fungi, 6-mm mycelial agar plugs were prepared aseptically. These were then placed on an SDA plate (90 mm dia) at a distance of 5 cm from each other. Subsequently, the plates were incubated at 28 °C for 15 days. The growth of the endophytic fungi and pathogen was observed regularly, and the mean colony diameter measured radial growth till the 15th day of inoculation. The percentage of inhibition was calculated by the following formula.

$$\text{Percentage inhibition } (\%) = \frac{R1 - R2}{R1} \times 100$$

where $R1$ = radial growth of pathogen; and $R2$ = radial growth of pathogen-inoculated with endophytic fungi. All the tests were performed in triplicates, and their mean ± SD was calculated [22].

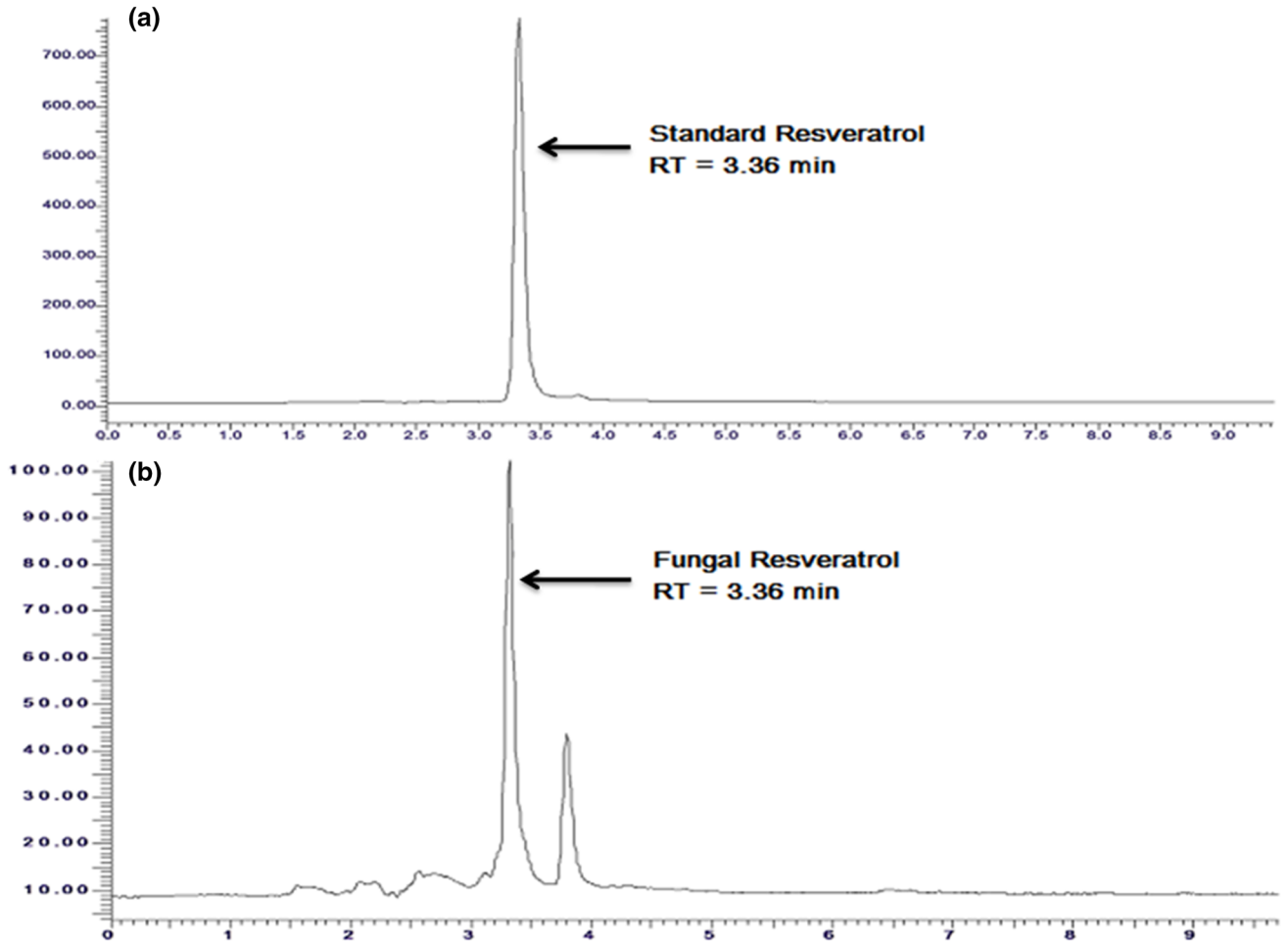


Fig. 1 HPLC spectra of **a** standard resveratrol, **b** EA extract fungal resveratrol

Table 1 Qualitative and quantitative analysis of resveratrol-producing endophytic fungi

Culture code	Culture identification	Quantification of resveratrol by HPLC (mg/L)*
# 12VVLPM	*Arcopilus aureus*	$89.1^{a} \pm 0.10$
#19VVLPM	*Fusarium equiseti*	$52.3^{b} \pm 0.10$
#22(P)VVLPM	*Xylaria psidii*	$35.4^{c} \pm 0.10$
#4(P)VVLNM	*Fusarium solani*	$31.3^{d} \pm 0.11$

Means with different superscript letters (i.e., a, b, c, d) are different by Tukey's post hoc test ($p < 0.05$)

*Data presented are mean ± standard deviation of three replicates

In Vitro Anti-staphylococcal Activity

The extracts obtained were evaluated for their antimicrobial potential against *Staphylococcus aureus* (NCTC 6571, MTCC 96, MTCC 737, Sau G9, and Sau 902) using agar well diffusion assay. Briefly, the plates were swabbed with 18–24 h old 0.5 McFarland adjusted cultures. Wells (of 5 mm) were punched in Mueller Hinton Agar (MHA) plates using a sterile cork borer. Subsequently, 30 µL of the fungal extract (1 mg/mL) was dispensed in each well and allowed to diffuse at 37 °C for 20 min. A similar concentration of streptomycin (Hi-Media) served as a positive control. The plates were incubated overnight at 37 °C. Antimicrobial activity was determined by measuring the zone of inhibition formation around the well [12, 23]. All the tests were performed in triplicates, and their mean ± SD was calculated.

Statistical Analysis

All the assays were performed in triplicates, and the data were represented as mean ± SD. The results were analysed by ANOVA followed by Tukey's post hoc test ($p < 0.05$). Statistical analysis was performed using Graph Pad Prism 7

software, and $p < 0.05$ was considered significant. The concentration that caused 50% scavenging (IC_{50}) of the antioxidant activities was calculated by linear regression.

Results and Discussion

Quantification of Resveratrol Through High-Performance Liquid Chromatography (HPLC)

Presence and quantification of fungal resveratrol were done by HPLC wherein it exhibited a symmetrical peak at RT 3.36 min on C18 reverse phase column similar to that of standard resveratrol. The UV absorption analysis showed a peak representing absorption at 302 nm. The peak area versus resveratrol concentration, obtained in case of the standard resveratrol, was used to estimate the quantity of fungal resveratrol. All the top 4 endophytic fungi produced resveratrol in range of 31.3–89.1 mg/L liquid culture which was confirmed by HPLC analysis (Fig. 1, Table 1). There was a statistically significant difference in mean resveratrol concentrations of the different resveratrol-producing endophytic isolates which was confirmed by one-way ANOVA. HPLC analysis played an important role in identification of potent RESV-producing endophytic fungi. The main keystones for evaluating RESV in the organic extracts have been HPLC which has also been used in earlier studies [3, 8, 16]. Apart from being a potential source of putative phytochemicals, endophytic fungi also produce an array of bioactive moieties which are expressed in response to biotic and abiotic stresses to which plant is exposed from time to time [1, 2]. The accumulation of RESV in plants and specifically in grapes (*Vitis vinifera*) is multifactorial and is generally influenced by plant–microbe interaction or stress conditions like UV-light, mechanical injury apart from grape variety and climatic conditions [3, 8, 16]. Microorganisms have also been genetically manipulated to optimize the production of resveratrol through the fermentation route. Transgenic yeast and *E. coli* have been developed and are being tested for the production of trans-resveratrol through fermentation route [5]. Resveratrol is a billion-dollar molecule from stilbene family which is being studied as a therapeutic molecule as well as a pharmacophore towards the development of new drugs due to its multifarious health benefits. As resveratrol is being widely used as a nutraceutical, cosmeceutical and also as a pharmaceutical moiety, the demand for its bulk production is continually rising [5, 8]. So in the present study, the emphasis was to investigate the in vitro antioxidant, antifungal and antistaphylococcal activities of resveratrol-producing endophytic fungi isolated earlier by Indian grapevine yards.

In Vitro Antioxidant Activities

Free radical damage has been implicated in pathogenic infections, inflammatory response, cardiovascular disorders, ageing and autoimmune disorders like rheumatoid arthritis. Different in vitro assays were used to assess the antioxidant activity. In the present study, there was a statistically significant difference in the in vitro DPPH scavenging activity among the different fungal extracts by one-way ANOVA. Further, post hoc comparisons using Tukey's HSD ($p < 0.05$) indicated that maximum DPPH scavenging was carried out by a fungal extract of #12VVLPM with IC_{50} of 0.11 ± 0.01 mg/mL when compared to resveratrol which was used as a standard with IC_{50} of 0.1 ± 0.011 mg/mL. The least DPPH scavenging was found in the fungal extract of #4(P) VVLNM with IC_{50} of 4.96 ± 0.21 mg/mL (Table 2). The fungal resveratrol in the present study exhibited a strong antioxidant activity in different antioxidant assays such as DPPH (IC_{50} of 0.11–4.96 mg/mL). DPPH method is traditionally used for analysing the preliminary free radical scavenging potential

Table 2 IC_{50} of different fungal extracts evaluated using DPPH, TEAC, metal ion chelating, nitric oxide radical scavenging and hydrogen peroxide scavenging assays

S. no	Sample	IC_{50} (mg/ml)*				
		DPPH scavenging	Trolox equivalent antioxidant scavenging	Metal ion chelating scavenging	Nitric oxide radical scavenging	Hydrogen peroxide scavenging
1.	Standard	$0.1^{d} \pm 0.011$	$0.16^{e} \pm 0.03$	$0.33^{b} \pm 0.02$	$0.37^{d} \pm 0.02$	$0.38^{c} \pm 0.01$
2.	#12VVLPM	$0.11^{d} \pm 0.01$	$0.28^{d} \pm 0.02$	$0.12^{c} \pm 0.03$	$0.08^{e} \pm 0.03$	$0.12^{d} \pm 0.07$
3.	#19VVLPM	$0.48^{c} \pm 0.02$	$0.82^{c} \pm 0.01$	$0.42^{b} \pm 0.02$	$0.61^{c} \pm 0.01$	$0.26^{cd} \pm 0.04$
4.	#22(P)VVLPM	$1.25^{b} \pm 0.03$	$1.51^{b} \pm 0.04$	$1.35^{a} \pm 0.05$	$0.91^{b} \pm 0.01$	$0.75^{b} \pm 0.02$
5.	#4(P)VVLNM	$4.96^{a} \pm 0.21$	$2.16^{a} \pm 0.02$	$1.33^{a} \pm 0.03$	$1.35^{a} \pm 0.01$	$1.18^{a} \pm 0.04$

Means with different superscript letters (i.e., a, b, c…) are different by Tukey's post hoc test ($p < 0.05$)

*Data presented are mean ± standard deviation of three replicates

of microbes and plant extract, and it also corroborates to the inhibition of lipid peroxidation. Antioxidant molecule acts as an electron donor to DPPH, by which it neutralizes its free radical character [8, 9]. Plant and endophytic fungi act as an electron donor due to the presence of phenolic compounds in them. This is also a justification due to which endophytic fungi exhibit the potential to scavenge DPPH free radical scavenging property. The DPPH scavenging has been widely used as a fast, reliable as well as a reproducible parameter to measure the in vitro antioxidant capacity of any biological compound [8, 17]. Reducing potential of any biological compound could serve as an indicator of potential antioxidant property. The two RESV derivatives produced by endophytic *Alternaria* sp. also displayed moderate antioxidant activity by DPPH scavenging [24]. Khanduja and Bhardwaj [25] found that RESV showed stronger antioxidant potential than catechin, myricetin, and fisetin. Shrikanta et al. [26] also observed antioxidant property which was attributed to the presence of RESV in grapes and jamun.

In case of in vitro $ABTS^{+}$ radical scavenging potential, there was a statistically significant difference among the different fungal extracts analysed by one-way ANOVA. The highest scavenging was found in fungal extract (ethyl acetate fraction) of #12VVLPM with IC_{50} value of 0.28 ± 0.02 mg/mL as compared to resveratrol used as a standard which exhibited IC_{50} value of 0.16 ± 0.03 mg/mL, while the least scavenging was recorded in fungal extract of #4(P) VVLNM with IC_{50} value of 2.16 ± 0.02 mg/mL (Table 2). ABTS antioxidant assay is a well-known fast screening method for determination of antioxidant potential of any compound, and it may be a useful tool for high throughput screening of foods for natural antioxidants [18]. The decolonization of ABTS ions is measured by the reduction of free radical cation which is interpreted as percentage inhibition at absorbance of 734 nm (visible range) [18, 27]. In the presence of particular chemical bonds in the endophytic fungal extract, it inhibits the activity of potassium persulfate by which it inhibits the production of ABTS ions. Zhou et al. [27] performed the DPPH and ABTS scavenging assay of all fungal extract using four different media and found that more than 80% of isolates are showing good scavenging activity.

Metal ions play an important biochemical and physiological role in living systems. They help in transportation of oxygen, apart from being cofactors of many enzymes to optimize their catalytic activity. Metals also participate in catalysis reaction and result in oxidative change in lipids, proteins as well as many other cellular biocomponents. The metal ion chelating potential of endophytic fungal extract was measured by the development of ferrous ion ferrozine complex. Ferrozine binds with metal ions and produces a red colour complex which is detected at absorbance at 562 nm. In the present study of metal ion chelating assay, there was a significant statistical difference among different fungal extracts which was analysed using one-way ANOVA. The highest scavenging was present in the fungal extract of #12VVLPM with IC_{50} value of 0.12 ± 0.03 mg/mL as compared to resveratrol which exhibited IC_{50} value of 0.33 ± 0.02 mg/mL which was used as a standard. The least scavenging activity was obtained in the fungal extract of #22(P) VVLPM with IC_{50} value of 1.35 ± 0.05 mg/mL. Further, #22(P)VVLPM and #4(P)VVLNM did not exhibit a significant difference in their mean IC_{50} which was confirmed by Tukey's post hoc analysis ($p < 0.05$) (Table 2).

In case of nitric oxide radical scavenging assay, there was a statistically significant difference among the different fungal extracts by one-way ANOVA. Further, post hoc comparisons using Tukey's HSD ($p < 0.05$) indicated that the mean nitric oxide radical scavenging activity was highest in #12VVLPM with IC_{50} value of 0.08 ± 0.03 mg/mL as compared to resveratrol which was used as standard with IC_{50} value of 0.37 ± 0.02 mg/mL, and the least scavenging was exhibited by the fungal extract of #4(P) VVLNM with IC_{50} value of 1.35 ± 0.01 mg/mL (Table 2). In nitric oxide radical scavenging activity, nitric oxide produced from sodium nitroprusside reacts with free oxygen from nitrite ion, which is inhibited by the antioxidant molecules. Sulphanilamide is converted to a diazonium salt by competing with nitrite in acidic medium. This

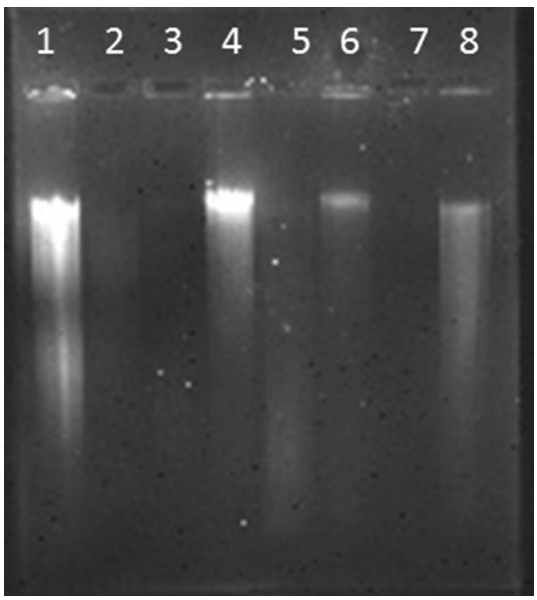


Fig. 2 DNA protection assay; Lane 1: λ-DNA, Lane 2, 3: λ-DNA + Fenton Reagent (FR), Lane 4: λ-DNA + #12VVLPM extract + FR, Lane 5: λ-DNA + #22(P)VVLPM extract + FR, Lane 6, 8: λ-DNA + #19VVLPM extract + FR, Lane 7: λ-DNA + #4(P)VVLNM extract + FR

diazonium salt reacts with NED (N-1-naphthyl-ethylenediamine), resulting in the formation of an azo dye that can be measured quantitatively at 542 nm in visible range using a UV–Vis spectrophotometer [19, 20].

The inhibitory activity of the extract from resveratrol-producing endophytic fungi on the oxidative cell damage produced by hydrogen peroxide and hydroxyl ion with the help of Fenton reagent was assayed through peroxidase assay. In biological systems, many biological end products of lipid peroxidation such as MDA constitute a significant source of cell membrane, obliteration and cell injuries. In the present assay, the ability of fungal extract to scavenge hydrogen peroxide was evaluated and it was observed that a significant statistical difference existed among the different fungal extracts by one-way ANOVA. Further, post hoc comparisons using Tukey's HSD ($p < 0.05$) indicated that the mean scavenging activity was highest in fungal extract of #12VVLPM with IC_{50} value of 0.12 ± 0.07 mg/ml followed by #19VVLPM with IC_{50} value of 0.26 ± 0.04 mg/ml as compared to resveratrol which was used as the standard with IC_{50} value of 0.38 ± 0.01 mg/ml and the least scavenging was exhibited by #4(P) VVLNM with IC_{50} value of 1.18 ± 0.04 mg/ml (Table 2). Shrikanta et al. [26] also observed antioxidant property which was attributed to the presence of RESV in grapes and jamun. Apart from DPPH scavenging potential, RESV also exhibited hydrogen peroxide and superoxide anion radical scavenging activities [28].

DNA Protection Assay

This assay was carried out to understand the possible role of the fungal extracts (ethyl acetate extract) on prevention of damage to DNA by free radicals. In this study, it was found that fungal extracts of resveratrol-producing endophytic fungi exhibited good antioxidant activity and thus prevented the DNA damage (Fig. 2). On the basis of result, the authors can conclude that resveratrol-producing isolates from *Vitis vinifera* have potential activity as a protective compound against oxidative stresses. The present results also corroborate with other studies which demonstrate that polyphenols and stilbenes are able to protect macromolecules from oxidative stresses or enhance the resistance to damages caused by oxidants [19].

In Vitro Antifungal Activity

The emerging and re-emerging appearance of fungal disease encountered in the course of allogeneic bone marrow transplantation, organ transplantation, and cancer therapy demands the screening of novel natural dietary component as an antifungal compounds with better efficacy and elevate compatibility. There are defined numbers of naturally isolated antifungal compounds in practice against various fungal infections. Considering the massive hidden potential of natural products from endophytic fungi as an alternative source of novel antifungal compounds for different applications remains a largely under explored area. Therefore, in this experiment, all the resveratrol-producing endophytic fungi were screened for their antagonistic potential against a panel of plant pathogenic fungi. All the resveratrol-producing isolates significantly inhibited mycelial growth and conidial formation against *Botrytis cinerea* (MTCC 359), *Colletotrichum gloeosporioides* (MTCC 9623), *Cercospora beticola* (ATCC 24888) and *Rhizoctonia solani* (MTCC 4634), which were analysed by one-way ANOVA. The highest inhibition was exhibited by #12VVLPM against *B. cinerea* (57.8 ± 0.8%) followed by *Cercospora beticola* (ATCC24888), while the least inhibition was observed in #22(P) VVLPM and #4(P) VVLNM against *Botrytis cinerea.* A non-significant inhibition was observed in the case of #22(P) VVLNM against the test panel isolates (Fig. 3, Table 3). The maximum inhibition was exhibited by #12VVLPM against *B. cinerea* (57.76 ± 0.82%). Houille et al. [29] demonstrated that dimethoxy RESV derivatives exhibited antifungal activity against *C. albicans*. The inhibitory effect of different compounds of RESV on conidia germination was found to be about 70%. Paulo et al. [13] demonstrated that RESV possessed antimicrobial properties.

In Vitro Anti-staphylococcal Activity

Multidrug resistant (MDR) microbes are becoming more and more prominent in both developed as well as under developed countries due to their refractory behaviour and are implicated in chronic infections. One of the most deadly bacteria which has seen a geometric rise and global spread is MRSA (Methicillin-resistant *Staphylococcus aureus*). This demands exploration of new drug scaffolds in order to combat with MRSA and its refractory variants in the hospital and community settings. Endophytic fungi have already been proven to be fountainheads of novel bioactive compounds. Therefore, in this experiment, all the resveratrol-producing endophytic fungi were also screened for their in vitro anti-staphylococcal activity against a battery of five human pathogenic microorganisms comprising of clinical and standard isolates of *Sau* exhibiting methicillin and multidrug resistance. There was a statistically significant difference in the in vitro anti-staphylococcal activity among the different resveratrol-producing endophytic fungi by one-way ANOVA. The endophytic isolates #12VVLPM and #19VVLPM exhibited highest inhibition against the bacterial test isolates in the panel which was further affirmed by post hoc comparison using Tukey's HSD ($p < 0.05$) (Fig. 3, Table 4).

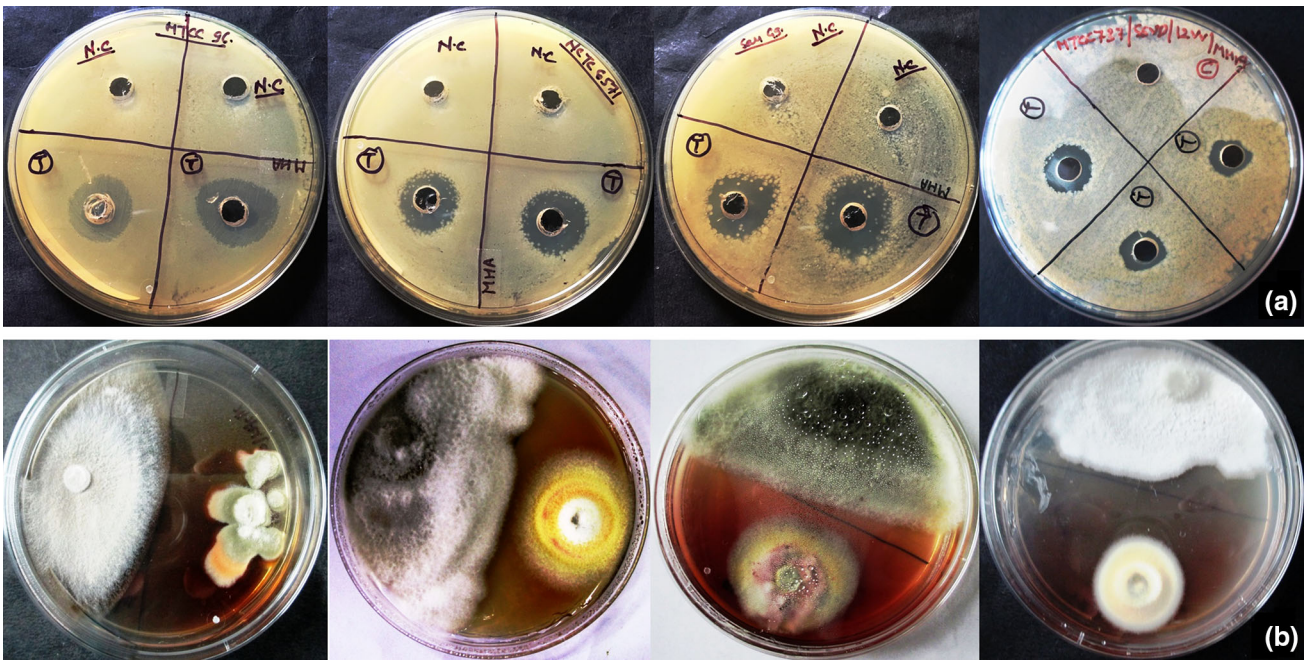


Fig. 3 Plate photo of **a** in vitro anti-staphylococcal against 1) MTCC 96 2) NCTC 6571 3) Sau G9 4) MTCC 737 **b** antifungal activity of the different resveratrol-producing endophytic fungi against 1) MTCC 359 2) MTCC 9623 3) ATCC 24888 4) MTCC 4634

Table 3 Antifungal activity of top resveratrol-producing endophytic fungi

S. no.	Sample	% of inhibition*			
		Botrytis cinerea (MTCC 359)	*Collectrotrichum gloeosporioides* (MTCC 9623)	*Cercospora beticola* (ATCC 24888)	*Rhizoctonia solani* (MTCC 4634)
1	#12VVLPM	$57.76^{a} \pm 0.82$	$55.41^{a} \pm 2.21$	$56.59^{a} \pm 3.08$	$46.83^{a} \pm 3.09$
2	#19VVLPM	$47.28^{b} \pm 1.78$	$45.30^{a} \pm 2.32$	$44.91^{b} \pm 1.70$	$37.92^{a} \pm 3.34$
3	#22(P)VVLPM	$27.08^{c} \pm 3.45$	$30.68^{b} \pm 3.60$	$30.19^{c} \pm 2.84$	$25.66^{b} \pm 2.38$
4	#4(P)VVLNM	$22.47^{c} \pm 1.47$	$23.20^{b} \pm 2.57$	$24.15^{c} \pm 3.44$	$21.93^{b} \pm 1.22$

Means with different superscript letters (i.e., a, b, c…) are different by Tukey's post hoc test ($p < 0.05$)

*Data presented are mean ± standard deviation of three replicates

Table 4 Antibacterial activity of top resveratrol-producing endophytic fungi

S. no.	Sample	% of inhibition*				
		NCTC 6571	Sau G9	MTCC 96	MTCC 737	Sau 902
1.	Positive control	$15.60^{a} \pm 0.10$	$15.70^{a} \pm 0.10$	$17.20^{a} \pm 0.06$	$17.4^{a} \pm 0.10$	$17.00^{a} \pm 0.10$
2.	#12VVLPM	$10.67^{b} \pm 0.15$	$10.33^{b} \pm 0.06$	$11.00^{b} \pm 0.10$	$11.67^{b} \pm 0.15$	$12.00^{b} \pm 0.10$
3.	#19VVLPM	$10.33^{b} \pm 0.15$	$9.67^{bc} \pm 0.12$	$10.67^{b} \pm 0.12$	$11.33^{b} \pm 0.12$	$11.33^{b} \pm 0.06$
4.	#22(P)VVLPM	$9.33^{bc} \pm 0.15$	$9.33^{bc} \pm 0.15$	$12.00^{b} \pm 0.10$	$11.67^{b} \pm 0.15$	$11.67^{b} \pm 0.12$
5.	#4(P)VVLNM	$7.67^{c} \pm 0.15$	$8.33^{c} \pm 0.06$	$7.67^{c} \pm 0.15$	$7.67^{c} \pm 0.10$	$9.67^{c} \pm 0.06$

Means with different superscript letters (i.e., a, b, c…) are different by Tukey's post hoc test ($p < 0.05$)

*Data presented are mean ± standard deviation of three replicates

Table 5 Correlation analysis between antioxidant and antimicrobial activities (n = 5)

Parameter	DPPH	TEAC	Metal ion chelating	Nitric oxide	Hydrogen peroxide	*B. cinerea*	MTCC9623	ATCC24888	MTCC4634	NCTC 6571	Sau G9	MTCC 96	MTCC 737	Sau 902
DPPH		*0.0032***	*0.0262*	*0.0024*	*0.1937*	*0.0233*	*0.0061*	*0.0113*	0.0172	*0.0392*	*0.0195*	*0.4017*	*0.2281*	*0.1727*
TEAC	0.997*		*0.0202*	*0.005*	*0.2417*	*0.0131*	*0.0022*	*0.0031*	0.0067	*0.0625*	*0.0353*	*0.4632*	*0.2793*	*0.2142*
Metal ion chelating	0.974	0.979		*0.0413*	*0.2601*	*0.0037*	*0.0091*	*0.0123*	0.0104	*0.0760*	*0.0854*	*0.5537*	*0.3431*	*0.3027*
Nitric oxide	0.998	0.995	0.959		*0.2071*	*0.0337*	*0.0124*	*0.0157*	0.0229	*0.0496*	*0.0154*	*0.3910*	*0.2260*	*0.1600*
Hydrogen peroxide	0.806	0.758	0.739	0.793		*0.2961*	*0.2435*	*0.2817*	0.3013	*0.0634*	*0.1264*	*0.1021*	*0.0273*	*0.0723*
B. cinerea	0.977	0.987	0.997	0.966	0.704		*0.0056*	*0.0044*	0.0024	*0.0933*	*0.0844*	*0.5773*	*0.3671*	*0.3105*
MTCC9623	0.994	0.998	0.991	0.988	0.757	0.944		0.0017	0.0036	*0.0623*	*0.0472*	*0.4897*	*0.2963*	*0.2392*
ATCC24888	0.989	0.997	0.988	0.984	0.718	0.996	0.998		0.0007	*0.0835*	*0.0588*	*0.5286*	*0.3316*	*0.2647*
MTCC4634	0.983	0.993	0.989	0.977	0.699	0.998	0.996	0.999		*0.0949*	*0.0717*	*0.5600*	*0.3572*	*0.2899*
NCTC 6571	0.961	0.937	0.924	0.95	0.937	0.907	0.938	0.916	0.905		*0.0254*	*0.2496*	*0.1062*	*0.0996*
Sau G9	0.98	0.965	0.915	0.985	0.874	0.916	0.953	0.941	0.928	0.975		*0.2618*	*0.1282*	*0.0790*
MTCC 96	0.598	0.537	0.446	0.609	0.898	0.423	0.51	0.471	0.44	0.75	0.738		*0.0331*	*0.0612*
MTCC 737	0.772	0.721	0.657	0.774	0.973	0.633	0.704	0.668	0.643	0.894	0.872	0.967		*0.0199*
Sau 902	0.827	0.786	0.697	0.84	0.928	0.689	0.761	0.735	0.71	0.9	0.921	0.939	0.98	

*Pearson's correlation coefficient; **significance level of Pearson's correlation coefficient (in italics)

Paulo et al. [13] demonstrated that RESV possessed antimicrobial properties against a battery of Gram-positive and Gram-negative bacteria. The RESV was found to exhibit antimicrobial activity against surrogate, methicillin sensitive and methicillin-resistant *S. aureus*. Similarly, Ma et al. [12] also demonstrated that RESV displayed potent antimicrobial activity against foodborne pathogen. The present study clearly indicates that resveratrol-producing endophytic fungi possess strong antimicrobial activity against all the tested clinical isolates as well as plant pathogenic fungi (Tables 3, 4). As the demand for natural and safe chemical entities in agriculture and medicine is growing for applications in agriculture sector as well as therapeutic entities, endophytic fungi offer to be a prolific resource of these bioactive compounds which could replace synthetic chemicals [12, 13, 23, 29]. In this context, fungal resveratrol also holds a promise for use as a therapeutic moiety as well as a crop protection agent, i.e., biofungicide.

Correlation Between In Vitro Antioxidant and Antimicrobial Activity

To explore the quantitative relationship between in vitro antioxidant activity and antimicrobial activity, a correlation analysis was carried out. The relationship between the above parameters was statistically evaluated and presented in Table 5. DPPH, TEAC, metal ion chelating and nitric oxide scavenging activity and antibacterial assay indicated a significant positive correlation. Similarly, the antifungal activity also exhibited a positive correlation with the different antioxidant assays. A similar correlation was observed by the resveratrol-producing endophytic fungi between the antifungal activity and the antibacterial activity (Table 5). The positive correlation occurred between in vitro antimicrobial and the antioxidant activity (i.e., IC_{50}), despite different methods. In the present study too, it was found that lower the IC_{50} value higher is the scavenging potential. #12VVLPM exhibited the highest scavenging antioxidant activity. The present study supports the free radical scavenging property of resveratrol-producing endophytic fungi depending upon the polyphenolic content. All the resveratrol-producing endophytic fungi demonstrate positive and significant correlation among DPPH, TEAC, metal ion chelating, nitric oxide and hydrogen peroxide as well as antimicrobial with coefficient in a range of 0.44–0.999 (Table 5). This was in accordance with the earlier study by Balik et al. [30] wherein the highest correlation occurred between the antioxidant activity, and total polyphenols content (0.8336–0.9952 mg/kg; $p < 0.0001$) regardless of the method used. A relatively wide range of the correlation coefficient between the estimated antioxidant activity and the contents of selected polyphenols was also presented by Fernández-Pachón et al. [31]. The antimicrobial activity was positively correlated to the RESV content, and it was in accordance with earlier studies wherein RESV has been shown to exhibit activity against *B. cinerea* and *C. albicans* [29].

Conclusion

Resveratrol has brought attention due to its overgrowing demand not only as a health supplement as well as a possible role in the natural antimicrobial compound. To the best of our knowledge, this is the first study that concurrently examined the phenolic content, anti-staphylococcal activity and antioxidant potential of the resveratrol-producing endophytes isolated from different varieties of *Vitis vinifera* from India. These results indicate that resveratrol-producing isolates from *Vitis vinifera* harbour rich, strong antioxidant with strong antifungal activity as well as anti-staphylococcal activity, therefore exhibiting its possible future role in health supplements and food preservation.

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Author's Contribution Prof. SS conceived and designed the present study, while VD performed all experiments, analysed the data. Prof. SS and VD jointly wrote the publication.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest to publish this manuscript.

References

1. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67(4):491–502
2. Suryanarayanan TS, Thirunavukkarasu N, Govindarajulu MB, Sasse F, Jansen R, Murali TS (2009) Fungal endophytes and bioprospecting. Fungal Biol Rev 23(1–2):9–19
3. Parker JA, Arango M, Abderrahmane S, Lambert E, Tourette C, Catoire H, Néri C (2005) Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. Nat Genet 37(4):349
4. Petrovski G, Gurusamy N, Das DK (2011) Resveratrol in cardiovascular health and disease. Ann N Y Acad Sci 1215(1):22–33
5. Wang DG, Liu WY, Chen GT (2013) A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*. J Pharmaceut Anal 3(4):241–247
6. Ramana KV, Reddy A, Majeti NV, Singhal SS (2018) Therapeutic potential of natural antioxidants. Oxidative Med Cell Longev. https://doi.org/10.1155/2018/9471051

7. Ratz-Łyko A, Arct J (2018) Resveratrol as an active ingredient for cosmetic and dermatological applications: a review. J Cosmet Laser Therapy. https://doi.org/10.1080/14764172.2018.1469767
8. Simioni C, Zauli G, Martelli AM, Vitale M, Sacchetti G, Gonelli A, Neri LM (2018) Oxidative stress: the role of physical exercise and antioxidant nutraceuticals in adulthood and aging. Oncotarget 9(24):171–181
9. Berman AY, Motechin RA, Wiesenfeld MY, Holz MK (2017) The therapeutic potential of resveratrol: a review of clinical trials. NPJ Precis Oncol 1(1):35
10. Mehton RK, Meshram V, Saxena S, Chhibber M (2016) Synthesis and anti-staphylococcal activity of 2, 4-disubstituted diphenylamines. J Braz Chem Soc 27(7):1236–1244
11. Saxena S, Gomber C (2010) Surmounting antimicrobial resistance in the millennium superbug: *Staphylococcus aureus*. Open Med (previously Central European Journal of Medicine) 5(1):12–29
12. Ma DS, Tan LTH, Chan KG, Yap WH, Pusparajah P, Chuah LH, Goh BH (2018) Resveratrol—potential antibacterial agent against foodborne pathogens. Front Pharmacol 9:102
13. Paulo L, Ferreira S, Gallardo E, Queiroz JA, Domingues F (2010) Antimicrobial activity and effects of resveratrol on human pathogenic bacteria. World J Microbiol Biotechnol 26(8):1533–1538
14. Mora-Pale M, Bhan N, Masuko S, James P, Wood J, McCallum S, Koffas MA (2015) Antimicrobial mechanism of resveratrol-trans-dihydro dimer produced from the peroxidase-catalyzed oxidation of resveratrol. Biotechnol Bioeng 112(12):2417–2428
15. Deshmukh S, Gupta M, Prakash V, Saxena S (2018) Endophytic fungi: a source of potential antifungal compounds. J Fungi 4(3):77
16. Dwibedi V, Saxena S (2018) *Arcopilus aureus*, a resveratrol-producing endophyte from *Vitis vinifera*. App Biochem Biotechnol 186(2):476–495
17. Ho R, Violette A, Cressend D, Raharivelomanana P, Carrupt PA, Hostettmann K (2012) Antioxidant potential and radical-scavenging effects of flavonoids from the leaves of *Psidium cattleianum* grown in French Polynesia. Nat Prod Res 26(3):274–277
18. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity is applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26(9–10):1231–1237
19. Danagoudar Josh CG, Sunil Kumar R, Poyya J, Nivya T, Hulikere MM, AnuAppaiah KA (2017) Molecular profiling and antioxidant as well as the anti-bacterial potential of polyphenol producing endophytic fungus-*Aspergillus austroafricanus* CGJ-B3. Mycology 8(1):28–38
20. Patel A, Patel A, Patel A, Patel NM (2010) Determination of polyphenols and free radical scavenging activity of *Tephrosia-purpurea* Linn. leaves (Leguminosae). Pharmacogn Res 2(3):152
21. Ruch RJ, Cheng SJ, Klaunig JE (1989) Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 10(6):1003–1008
22. Ghildiyal A, Pandey A (2008) Isolation of cold tolerant anti-fungal strains of *Trichoderma* sp. from glacial sites of Indian Himalayan region. Res J Microbiol 3(8):559–564
23. Meshram V, Saxena S, Kapoor N (2012) In vitro anti-staphylococcal potential of endophytic fungi from *Aegle marmelos*. J Pure App Microbiol 6(4):1859–1868
24. Wang J, Cox DG, Ding W, Huang G, Lin Y, Li C (2014) Three new resveratrol derivatives from the mangrove endophytic fungus *Alternaria* sp. Mar Drugs 12(5):2840–2850
25. Khanduja KL, Bhardwaj A (2003) Stable free radical scavenging and anti-peroxidative properties of resveratrol compared in vitro with some other bioflavonoids. Indian J Biochem Biophys 40(6):416–422
26. Shrikanta A, Kumar A, Govindaswamy V (2015) Resveratrol content and antioxidant properties of underutilized fruits. J Food Sci Technol 52(1):383–390
27. Zhou J, Diao X, Wang T, Chen G, Lin Q, Yang X, Xu J (2018) Phylogenetic diversity and antioxidant activities of culturable fungal endophytes associated with the mangrove species *Rhizophorastylosa* and *R. mucronata* in the South China Sea. PloS ONE 13(6):e0197359
28. Gülçin İ (2010) Antioxidant properties of resveratrol: a structure-activity insight. Innov Food Sci Emerg Technol 11(1):210–218
29. Houille B, Papon N, Boudesocque L, Bourdeaud E, Besseau S, Courdavault V, Clastre M (2014) Antifungal activity of resveratrol derivatives against *Candida* species. J Nat Prod 77(7):1658–1662
30. Balik J, Kyselakova M, Vrchotová N, Tříska J, Kumšta M, Veverka J, Lefnerová D (2008) Relations between polyphenols content and antioxidant activity in vine grapes and leaves. Czech J Food Sci 26:S25–S32
31. Fernández-Pachón MS, Villano D, Troncoso AM, García-Parrilla MC (2006) Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity. Anal Chim Acta 563(1–2):101–108

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ORIGINAL ARTICLE

Isolation and enhancement of resveratrol production in *Xylaria psidii* by exploring the phenomenon of epigenetics: using DNA methyltransferases and histone deacetylase as epigenetic modifiers

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Abstract
Resveratrol is an important stilbene which is having a high demand due to its therapeutic, cosmeceutical and nutraceutical activities. 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 the 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 (52.32 µg/mL) and by 10 µm AZA (48.94 µg/mL) followed by 10 µm SAHA (41.10 µg/mL) and 5 µm AZA (37.72 µg/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 the 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 the epigenetic variants as compared to wild strain. In the case of TEAC also the same trend as in the case of DPPH was obtained.

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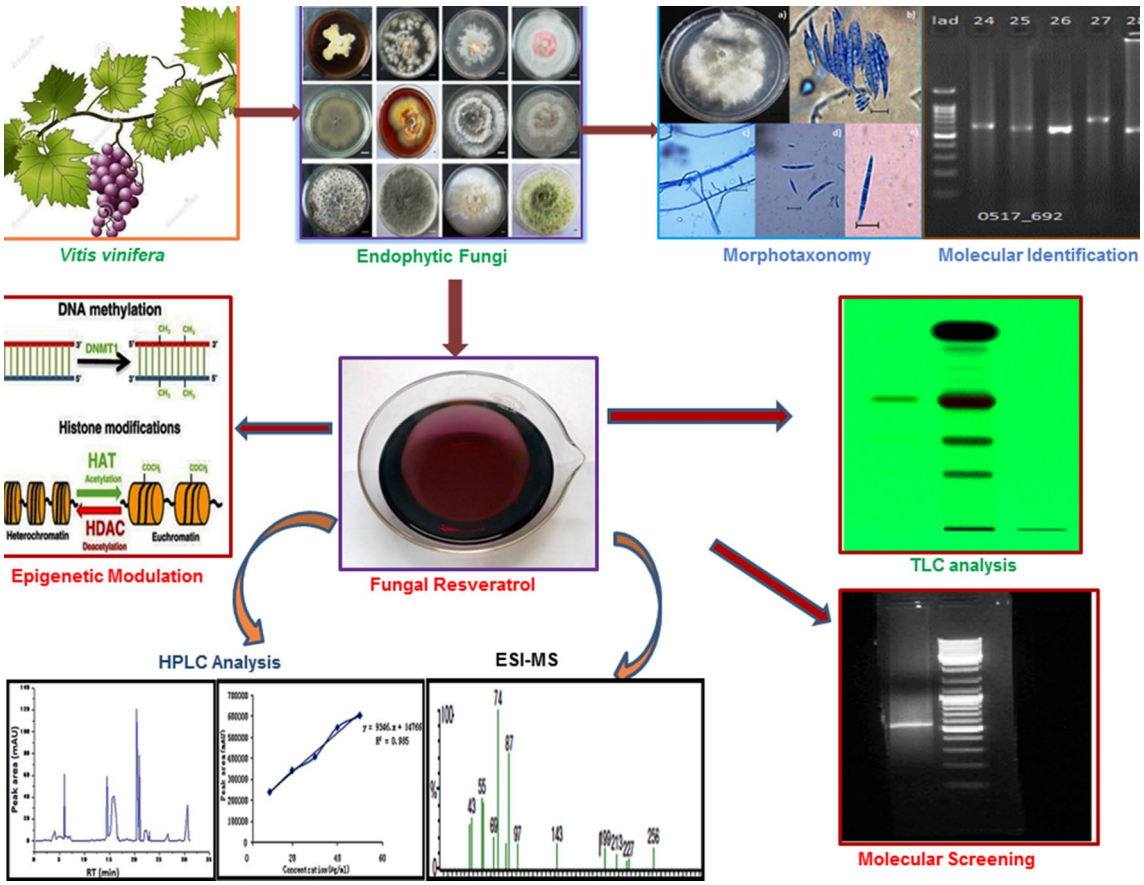
Graphical abstract



Keywords Endophytic fungi · Resveratrol · Epigenetic modification · HPLC · *Xylaria psidii*

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
TEAC	Trolox equivalent antioxidant capacity
FRAP	Ferric reducing antioxidant power
TPC	Total phenolic content
TFC	Total flavonoids content
RESV	Resveratrol
MHA	Muller Hinton agar
SDA	Sabouraud dextrose agar
MTCC	Microbial type culture collection
ATCC	American type culture collection
Sau	*Staphylococcus aureus*

Introduction

Resveratrol (RESV) is a natural polyphenol of stilbene family which is represented by two benzene rings associated via isopropyl moiety separated by a double bond [1]. Resveratrol was first discovered in the 1940s from the roots of *Veratrum grandifolium* (White hellebore) and subsequently in 1960s from *Polygonum cuspidatum* which were traditionally used as a component of Chinese and Japanese medicines [2, 3]. However, the medicinal properties of resveratrol came into the limelight when it was observed that the French people exhibited very few symptoms of coronary heart diseases despite French cuisine comprising a large number 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 [4]. In addition to various properties like anti-cancer, anti-inflammatory, anti-diabetic, anti-aging [5, 6]; RESV has made a spot for itself in agriculture, medicine, food and cosmetic industry as a boon molecule. RESV also possess an unusual antifungal activity against *Botrytis cinerea* which is the most important fungal pathogen of grape vines [7]. These outstanding properties of resveratrol make it a molecule of high appeal among researchers ever since its discovery.

Various sources of resveratrol comprise of grapes, peanuts, chocolate, tea and other berry species [8]. Currently,

RESV is commercially extracted from the roots of *Polygonum cuspidatum,* which is expensive as well as time-consuming process. Moreover, this may also have a negative ecological impact since the extensive annihilation of the plant is carried out. Many techniques have been employed for the enhancement of secondary metabolite production like plant cell culture technology wherein cells are cultivated in bioreactors to produce a secondary metabolite using hairy root culture, *rol,* and *STS* gene transformation [9, 10]. However, the significant disadvantage of this technique is genomic instability which leads to the silencing of various other gene clusters [11]. As there is global demand for RESV molecule, it has become imperative to explore alternative methods for bulk production of RESV.

Endophytic fungi are a particular class of fungi which resides inside the plant tissue and maintain an asymptomatic and inconspicuous union with their hosts, minimum for a season of their life cycle. Endophytic fungi exhibit an exciting property of producing compounds analogous to those present in the host plant as a result endophytes are today recognized as an essential source for the production of various medicinal molecules of plant origin for direct use as a drug [12]. Various techniques are these days employed on endophytes to target the enhancement of the secondary metabolite production like pathogen attack, UV irradiation, and genetic modification using *STS* genes or *rol* genes [13, 14] or by using biosynthetic precursor or chemical elicitors.

Epigenetics allows us to target a large number of fungi without any prior knowledge of their genome. Therefore, epigenetic modulators can be used as a strategy to epigenetically regulate the gene transcription of endophytic fungi. An epigenetic modification like histone deactivation and DNA methylation controls the putative biosynthesis of gene clusters [15]. Therefore, epigenetics can be utilized as a strategy for modulating the gene expression and enhancing the production of a target secondary metabolite. Chemical elicitors like suberoylanilide hydroxamic acid (SAHA) and 5-azacytidine (AZA) 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 catalyses the transfer of methyl group to the DNA apart from getting incorporated into the genetic material which leads to the disassembly of polyribosomes, defective methylation and acceptor function of tRNA thereby inhibiting production of specific proteins [15, 16]. SAHA acts as histone deacetylase inhibitor which by acting as a chelator for zinc ions present in the active site of the histone deacetylase leads to the accumulation of the acetylated histones and acetylated protein further inhibiting the gene expression [17, 18].

Thus, in the present work, we report an endophytic fungus #22(P) VVLPM as a promising candidate for the production of resveratrol and also intend to enhance resveratrol production by developing epigenetic variants of endophytic fungus #22(P) VVLPM having high titer value of RESV in free fermenting conditions.

Materials and methods

Instrumentation and reagents

HPLC analysis was carried out using Perkin Elmer HPLC (Series 200) with C-18 reverse phase Discovery column (4.6 mm ID × 150 mm L; Sigma Aldrich) for the determination of resveratrol content. BIOTEK® Powerwave 340 microplate reader was used for different anti-oxidant assays. Resveratrol (RESV), 2,2′-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid (ABTS), Trolox and FC reagent was purchased from TCI (Japan); 2,2-diphenyl-1-picrylhydrazyl (DPPH), Streptomycin, Sodium sulphate (Na_2SO_4), Aluminium chloride ($AlCl_3$), Ferric chloride ($FeCl_3$) from Hi-Media Labs Pvt. Ltd. (India). Methanol (MeOH), Acetonitrile (ACN) and Conc. Sulphuric acid (H_2SO_4) from Merck, Millipore, (USA), a Nitrocellulose membrane (NCM) from GE Healthcare and Life Sciences (USA), Orthophosphoric acid (H_3PO_4) from Hi-Media (India).

Isolation of fungal endophytes and their preservation

Healthy, mature, symptomless leaves were collected from the wine of *Vitis vinifera* from the National Center for Grapes, Pune Maharashtra. The collected leaves were stored in sterile zip pouches and stored at 4 °C. The collected sample was washed thoroughly under running tap water for 20–30 min and then washed with double distilled water to remove external debris. Subsequently, the leaf and stem samples were surface sterilized by dipping them in 2% solution of sodium hypochlorite for 5 min followed by 70% ethanol for 45 s, followed by dipping in 30% ethanol for the 30 s and drying them aseptically. Finally, 5 × 5 mm segments from the sterilized leaves were placed on Potato Dextrose Agar (PDA) [19]. The potency of surface sterilization of leaves was analyzed by a leaf imprint method [19, 20]. The plates were then incubated at 28 ± 2 °C for 10 days with 12 h light and 12 h dark cycles. Emerging hyphae after 14 days incubation were picked and transferred on PDA for further analysis. Pure cultures of the endophytic fungi so obtained were stored on PDA slants enriched with 10% glycerol.

Culture filtrate production

The endophytic isolate #22(P) VVLPM was subjected to culture filtrate production by inoculating 5 mm fungal mycelial disc of 8–10 day old fungal culture to 50 mL pre-sterilized

Potato dextrose broth (PDB) medium in 250 mL Erlenmeyer flasks. The flasks were then incubated at 28 ± 2 °C, 120 rpm for 10 days. After the incubation, the spent broth was separated from mycelia by filtration through Whatman filter paper 4 followed by centrifugation at 15,000 rpm for 5 min and then passing it through 0.2 µM nitrocellulose membrane to get cell-free filtrate [21–23].

Liquid–liquid extraction and TLC analysis

The fungal filtrate and ethyl acetate were taken up in a ratio of 1:3 and extracted three times. The organic layers so obtained were pooled and dehydrated by addition of anhydrous sodium sulphate. Subsequently, the solvent fraction was evaporated in a rotary evaporator at 30 °C to dryness to get the coarse fraction. The coarse fraction of the cultures was further fractionated by subjecting to preparative thin layer chromatography (PTLC). The TLC plate of 0.5 mm thickness was prepared by coating silica gel (Sigma Aldrich; 381276) onto 20 × 15 × 5 mm clean glass plates (Merck Millipore; RP-8F254S) which was activated by incubating at 100 °C for 2 h before use. Subsequently, the sample was spotted onto activated TLC plate just 1 cm above the edge of the plate with the help of capillary tube (Sigma Aldrich; Z114960). Simultaneously, the TLC chamber was saturated with different solvent systems (binary/tertiary) for 30 min. The TLC plate with the sample was then placed inside the saturated TLC chamber and allowed to run for 30 min when the solvent front reached the marked level. Subsequently, the TLC plate was taken out and allowed to air dry. The chromatogram was developed by visualizing the TLC plate under UV light (Thermo Fisher Scientific; UVGL-58). Resveratrol (0.1 mg/mL) was used as the standard for the comparison of retention factor (R_f) value. The R_f value of each band was obtained as the ratio of distance moved by solute to that of solvent [23, 24].

High-performance liquid chromatography (HPLC)

The stock solutions of standard resveratrol, fungal crude extracts and AZA, SAHA treated samples were prepared (0.1–1 mg/mL) and 1 mg/mL of methanol respectively and stored in dark and cool place to avoid the oxidation and isomerisation of trans-resveratrol to cis form. Subsequently the samples were sonicated for 15 min, followed by filtration through a syringe filter with cellulose membrane of 0.22 µm (GE Healthcare and Life Sciences, Merck, Millipore USA). The identification and concentration of RESV in crude bioactive fractions was determined via HPLC (PerkinElmer-200, USA) using C18 reverse phase discovery column (Sigma Aldrich, USA) equipped with series − 200 pump, dual wavelength detector, and total chrome workstation software. About, 20 µL of the sample of defined concentration was injected into the HPLC column and eluted using orthophosphoric acid (0.1%, pH 2.3, Sigma Aldrich, USA) and acetonitrile (Sigma Aldrich, USA), 45–55% ratio was used as mobile phase with a flow rate of 1 mL/min. The yield of fungal RESV was determined against a standard curve of standard RESV (0.1–1 mg/mL) under similar conditions. The peak area versus concentration of the standard as well as the number of peaks and peak height were used to estimate the concentration of resveratrol [22, 23, 25].

LC–MS analysis

The residue of fungal resveratrol from *Xylaria psidii* and standard resveratrol were analyzed by LC–MS (Waters, Micromass Q-TOF micro using Waters Alliance 2795 separation module) to confirm the presence of resveratrol. The confirmation was done on the basis of molecular ion of standard and fungal resveratrol by comparing their molecular ions as well as the fragmentation pattern [26, 27].

Morphotaxonomy of resveratrol producing endophytic fungus

For morphotaxonomic studies, the endophytic fungi were grown over different media i.e., Water agar (WA), Czapek Dox Agar (CDA), Grape leaf agar (GLA), Potato Dextrose Agar (PDA), Cornmeal agar (CMA), *Fusarium* minimal media (FMA), Synthetic nutrient deficient agar (SNA) for 30 days at 28 ± 2 °C and 98% relative humidity (with 12 h of photoperiod). Colony growth rate, appearance, along with its microscopic features like hyphae characteristics, conidia formation, and other cellular bodies were critically observed under Nikon stereo zoom microscope (Nikon, Japan). All the micrometric observations were carried out using Nikon NIS element software with at least 30 observations per structure [28–30].

Molecular identification of the resveratrol producing endophytic fungus

For genomic DNA extraction, mycelia of 3–4 days old culture (approx. 0.1–0.2 g) was scraped off with the sterile inoculation loop and crushed to excellent powder in a sterile mortar and pestle under liquid nitrogen. Further DNA extraction was done by using the Wizard Genomic DNA purification kit (Promega, Madison, WI) as per manufacturer's instructions. The Internal Transcribed Spacer (ITS) region 1, 5.8 S and 2 was amplified using ITS 1 and ITS 4 primers [31]. The reaction mixture (25 µL) comprised of 1.5 U of Taq DNA Polymerase in 10 × Taq buffer containing 25 mM $MgCl_2$ (Bangalore GeNei, Bengaluru, India), 2.5 mM of dNTP, 1 µL of extracted genomic DNA, 10 mM of each primer. The conditions for the polymerase chain

reaction were 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s followed by final extension at 72 °C for 10 min. The ITS amplicons so obtained were examined using a 1.5% agarose gel under UV light in Bio-Rad Gel documentation System using Quantity 1-D analysis software (Bio-Rad, Hercules, CA). The PCR products were purified with Wizard SV gel and PCR clean-up system (Promega, Madison, WI) as per manufacturer's instructions. The obtained PCR products were sequenced at Eurofins (Bangalore, India).

The final sequence was then obtained by assembling the sequences using Sequencher ver.5 (www.genecodes.com) and submitted to the GenBank under the accession number MH142837. The sequence similarity search of the ITS sequence was performed using the BLAST algorithm tool of NCBI. The selected sequences of reference taxa obtained using BLAST were then aligned with the ITS sequence of the endophytic fungi using the Clustal W option in MEGA 6. The aligned sequences were trimmed to make alignment uniform, and the aligned files were saved in FASTA and MEGA format. The evolutionary relationship was inferred by employing the Maximum Likelihood method using Tamura and Nei Model [32]. A thousand bootstraps were used to assess the evolutionary history. Gaps were considered as missing data.

Induction of epigenetic modulation using chemical elicitors

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

The wild-type endophytic fungus *Xylaria psidii* was subjected to epigenetic modulation using 5-azacytidine (AZA) and suberoylanilide hydroxamic acid (SAHA) as chemical elicitors. The stock solutions of 1 mg/mL of AZA and 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 plates were stored in an incubator till further use at the temperature of 28 ± 2 °C.

The endophytic fungus #22(P) VVLPM was point inoculated at the center of the AZA and SAHA enriched PDA plates respectively at the concentrations mentioned above. The plates were then incubated at 28 ± 2 °C for 10 days.

Production of culture filtrates and culture filtrate extraction and biomass calculation

For resveratrol production, 50 mL of pre-sterilized potato dextrose broth (PDB, HiMedia, India) in 250 mL Erlenmeyer flask (Borosil, India, 4980021) was aseptically inoculated with a 5 mm mycelial disc of a week old fungal culture. The flask was then incubated at 28 ± 2 °C, 120 rpm for 10 days. After the culmination of the incubation period, the mycelium was separated from the spent broth by filtration through Whatman paper 4 (Sigma Aldrich, USA, Z240567) followed by centrifugation at 15,000 rpm for 5 min and then passing it through the 0.2 µm nitrocellulose membrane (Merck Millipore, USA, PR04066) for making it cell-free. Further, the biomass of fungal spores was jotted down, for which the filter paper containing fungal spores was dried at 60 °C for overnight in a hot air oven, and the weight of dried spores was calculated by subtracting from the pre-observed weight of filter paper.

Biological assays

DPPH scavenging assay

The free radical scavenging activity of the culture filtrate of #22(P) VVLPM AZA and SAHA epigenetic variants was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals, according to the procedure described in the literature [33] 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 the 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 a negative control. Resveratrol (10–50 µg/mL) was used as a standard. The test was performed in triplicate, and the data were reported as mean ± SD. Percentage of free radical scavenging (%FRS) at different concentration was calculated using the formula:

$$\%\text{Free radical scavenging} = \frac{(ODcontrol - ODsample)}{ODcontrol} \times 100$$

Trolox equivalent antioxidant assay (TEAC)

TEAC was analyzed by using the method described by Re et al. [34]. The assay utilizes the ability of an antioxidant to scavenge $ABTS^{+}$. 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 h at room temperature in the dark. This ABTS mixture was diluted in PBS to an absorbance of 0.9–1.0 at 734 nm using BIOTEK® Powerwave 340 microtiter plate reader. To carry out the reaction 1 mL of working ABTS solution was added to 10 µL of the extract and the reaction mixture was allowed to stand for 6 min. The decrease in absorbance was recorded at 734 nm. Phosphate buffer saline was used as a blank,

and ABTS was used as a control. The test was performed in triplicate, and the data were reported as the mean ± SD (Standard deviation). Percentage of free radical scavenging (%FRS) at different concentration was calculated using the formula.

$$\%\text{Free radical scavenging} = \frac{(ODcontrol - ODsample)}{ODcontrol} \times 100$$

Stability study of epigenetic variant

The epigenetic variants were sub-cultured up to five generations on AZA and SAHA enriched PDA plates in which the maximum RESV concentration appeared during HPLC analysis of the first epigenetic variants. Subsequently the stability of the variants was also evaluated using HPLC and different in vitro antioxidant assays.

Statistical analysis

All the assays were performed in triplicates, and the data were represented as mean ± standard deviation. The results were analyzed by ANOVA followed by Tukey post hoc test ($p < 0.05$). Statistical analysis was performed using Graph Pad Prism 7 software and $p < 0.05$ was considered significant.

Results

Isolation and preservation of endophytic #22(P) VVLPM

In the present study, an endophytic fungus was isolated from the surface sterilized leaves of *Vitis vinifera* (Shiraz) from the Pune, Maharashtra. Culture isolate was preserved on PDA slants and vials supplemented with 10% (w/v) glycerol and incubated at 26 ± 2 °C for the growth and stored at 4 °C for maintenance of culture.

Sub culturing of #22(P) VVLPM on AZA and SAHA PDA plates

The fungal isolates of #22(P) VVLPM inoculated on the PDA plates supplemented with different concentration of AZA and SAHA, produced variation in the morphological characteristics of #22(P) VVLPM (Fig. 1). Changes in colony diameter of the subcultured #22(P) VVLPM over PDA plates with different concentrations of AZA and SAHA were noted after the incubation of 10 days (Fig. 1). It was observed that the diameter of the colony decreased after the concentration of 20 µM of AZA and SAHA due to the toxicity at higher concentrations (Fig. 1).

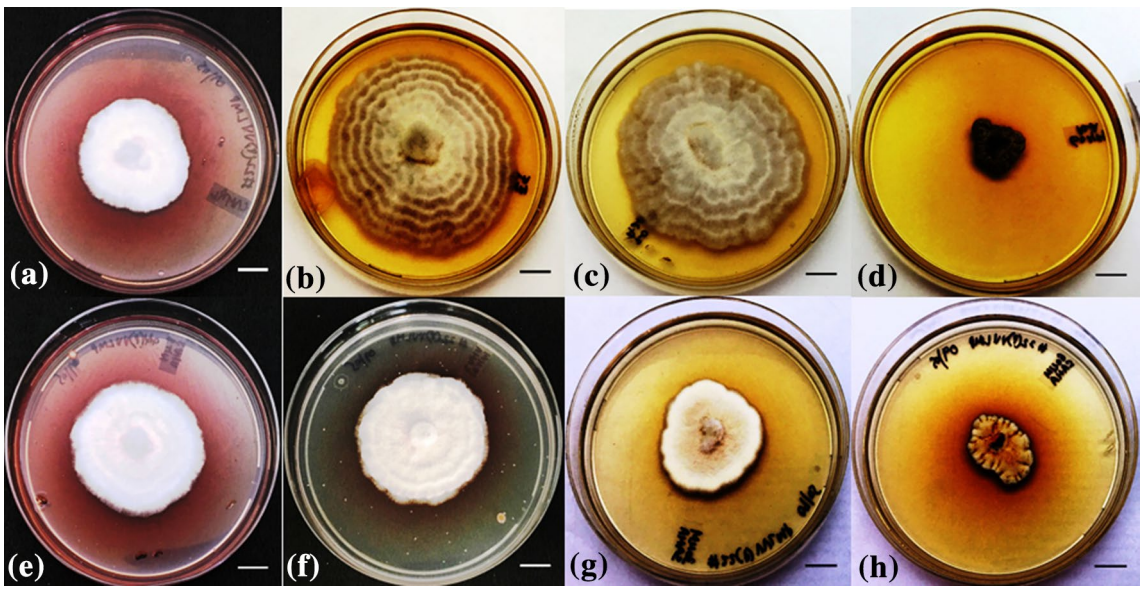


Fig. 1 Epigenetic variants of #22(P)VVLPM **a** Control (#22(P) VVLPM); **b** AZA 10 µm on PDA medium; **c** AZA 20 µm on PDA medium; **d** AZA 60 µm on PDA medium; **e** SAHA 1 µm on PDA medium; **f** SAHA 10 µm on PDA medium; **g** SAHA 30 µm on PDA medium; **h** SAHA 60 µm on PDA medium (Bar: 10 mm)

Production and extraction of culture filtrates

It was found that the volume of spent broth has reduced with the notable change in colour and characteristic smell of culture filtrates due to the production of secondary metabolites. The culture filtrates were filtered after 10 days of incubation using Whatman filter paper 4. The biomass of culture filtrates was also recorded. It was found that the weight of the biomass increased up-to-the concentration of 10 µM and then decreased from 20 µM as the concentration increases. It was also observed that there is a positive correlation between the colony diameter and the dry weight of biomass (Supplementary Figs. 1, 2).

Extraction and TLC of resveratrol from the endophytic fungus

The cell-free filtrate of #22(P) VVLPM was subjected to liquid–liquid extraction by ethyl acetate, and subsequently, the residue was reconstituted in methanol. The crude ethyl acetate fraction of #22(P) VVLPM was subjected to different combinations of solvents to achieve proper separation.

The best separation of crude EA extract of #22(P) VVLPM was observed in dichloromethane: Chloroform in the proportion of 5:5 which gave 5 different bands. The R_f value of Band 1 to Band 5 was 0.125, 0.25, 0.28, 0.325, and 0.650, respectively. The standard Resveratrol exhibited R_f value of 0.650 which was similar to that of the R_f value of Band 5 (Fig. 2) of crude ethyl acetate extract of #22(P) VVLPM.

HPLC for affirmation of resveratrol producing isolates

Liquid chromatography is a prevalent method for qualitative and quantitative analysis. The standard graph of resveratrol in a concentration range of 0.1–1 mg/mL was constructed with a linearity of (R^2 = 0.9986). The good fitting curve was indicated in this range due to the higher value of regression coefficient. Further, the precision of fitting was confirmed based on the standard error (S.E.) at 95% confidence interval for the values of intercept (5E + 07) and slope (− 2E + 06). Confirmation of fungal resveratrol was confirmed by HPLC analysis, which exhibited a symmetrical peak at retention time of 11.5 min on C18 reverse phase column similar to that of standard RESV (Fig. 3). The peak area versus RESV concentration, obtained in case of the standard RESV was used to estimate the quantity of fungal RESV present in the spent broth. Out of 26 epigenetic variants of #22(P) VVLPM which were obtained after treatment with different concentrations of AZA and SAHA it was found that 10 µM of AZA and 5 µM SAHA exhibited highest Resveratrol concentration of 48.94 µg/mL and 52.32 µg/mL respectively as compared to control (35.43 µg/mL) and on increasing the concentration of AZA and SAHA a decrease in resveratrol concentration was observed (Table 1).

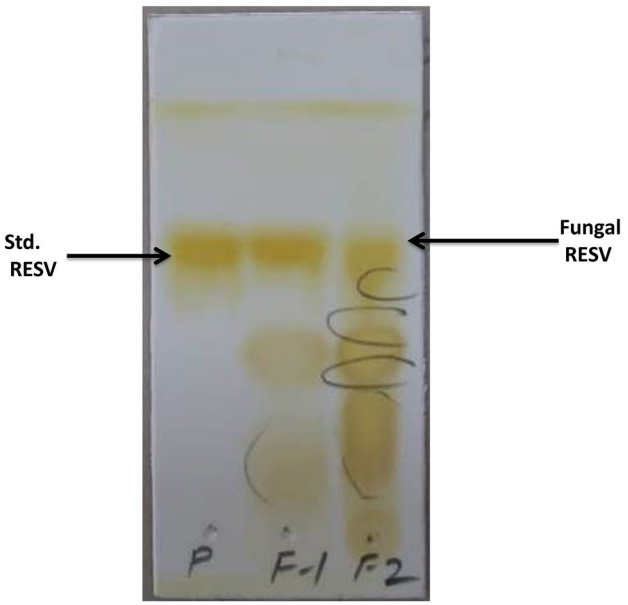


Fig. 2 TLC profile of crude extract of #22(P) VVLPM using dichloromethane: chloroform solvent system, in the ratio of 5:5 as the mobile phase. The R_f values of Band 1-4 was ranging between 0.650 and 0.125

LC–MS analysis

For further confirmation of the presence of fungal resveratrol in the bioactive ethyl acetate residue obtained from the spent broth of #22(P) VVLPM, LC–MS was carried out which revealed the presence of resveratrol. Further MS analysis revealed that the fungal resveratrol and standard resveratrol exhibited the same base peak at 228.1 m/z (Fig. 4).

Effect of AZA concentration on resveratrol production

Through HPLC analysis it was found that there was a significant increase in resveratrol production when chemical elicitor AZA were added in the culture medium. An increase of *38.13%* (48.94 µg/mL) was recorded over the basal concentration of 35.43 µg/mL without any treatment in the case of AZA 10 µM (Table 2). The decrease in concentration of resveratrol was also noted as compared to control after the treatment of AZA at the concentration of 10 µM (Table 2).

Effect of SAHA concentration on resveratrol production

Through HPLC analysis it was found that there was a significant increase in resveratrol production when

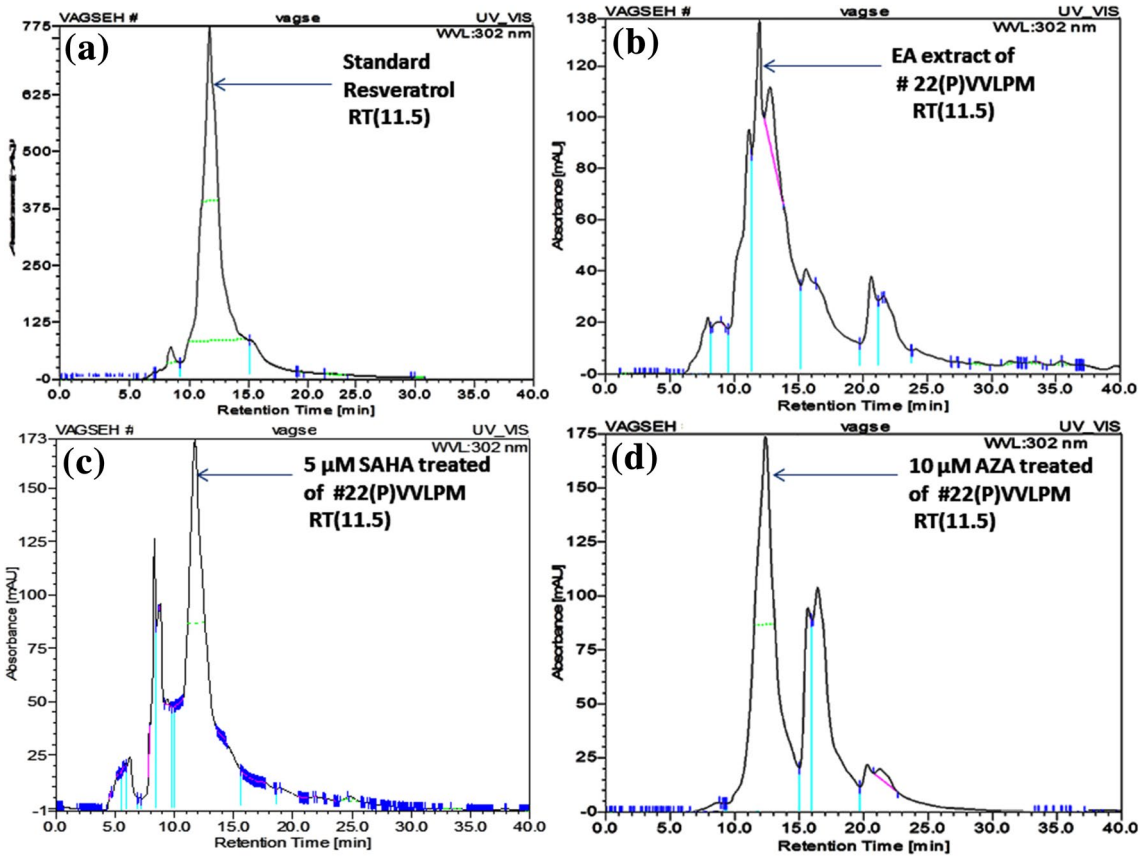


Fig. 3 HPLC-UV_{302nm} chromatogram of **a** Standard resveratrol solution (0.1–1 mg/mL) RT(11.5), **b** ethyl acetate extract of crudefungal resveratrol from #22(P)VVLPMRT(11.5), **c** 5 µm SAHA treated of #22(P) VVLPM RT(11.5), **d** 10 µm AZA-treated of #22(P) VVLPM RT(11.5)

Table 1 Morphological characteristics of the epigenetic variants of *Xylaria psidii* as compared to the wild #22(P) VVLPM strain

Characteristics	Wild (#22(P) VVLPM)	AZA (10 µm)	SAHA (5 µm)
Diameter (mm)	54.5 ± 0.14	56.5 ± 0.37	55 ± 0.61
Colony color	White velvety filamentous mycelia growth, produces red wine colour	A white velvety filamentous colony with a red wine colour. Pink pigment around the center of the growth	A white velvety filamentous colony with dark red wine colour. Pink pigment around the center of the growth
Color (base)	Red-wine color	Red-wine color with pink pigmentation around the center of the growth	Red-wine color with pink pigmentation around the center of the growth
Margin	Smooth margin	Cottony smooth margin	Cottony smooth margin
Odour	Fruity smell	Phenolic smell (Sweet and Terry)	Phenolic smell (Sweet and Terry)
Dry weight (gm/L)	12.43 ± 0.65	15.57 ± 0.95	16.51 ± 1.12
Concentration of RESV (µg/mL)	35.43 ± 0.16	48.94 ± 0.33	52.32 ± 0.24

The results presented are a compilation of data from at least three cultures of each organism. Numerical values are mean ± SD

chemical elicitor SAHA were added in the culture medium. An increase of *47.67%* (52.32 µg/mL) was recorded over the basal concentration of 35.43 µg/mL without any treatment in the case of SAHA 5 µM (Table 2). A decrease in concentration of resveratrol was also noted as compared to control after the treatment of SAHA at the concentration of 5 µM (Table 2).

Identification of resveratrol producing a fungal isolate

Classical morphotaxonomy

The identification of resveratrol producing endophytic fungus *Xylaria psidii* (#22(P) VVLPM) was done on the basis of

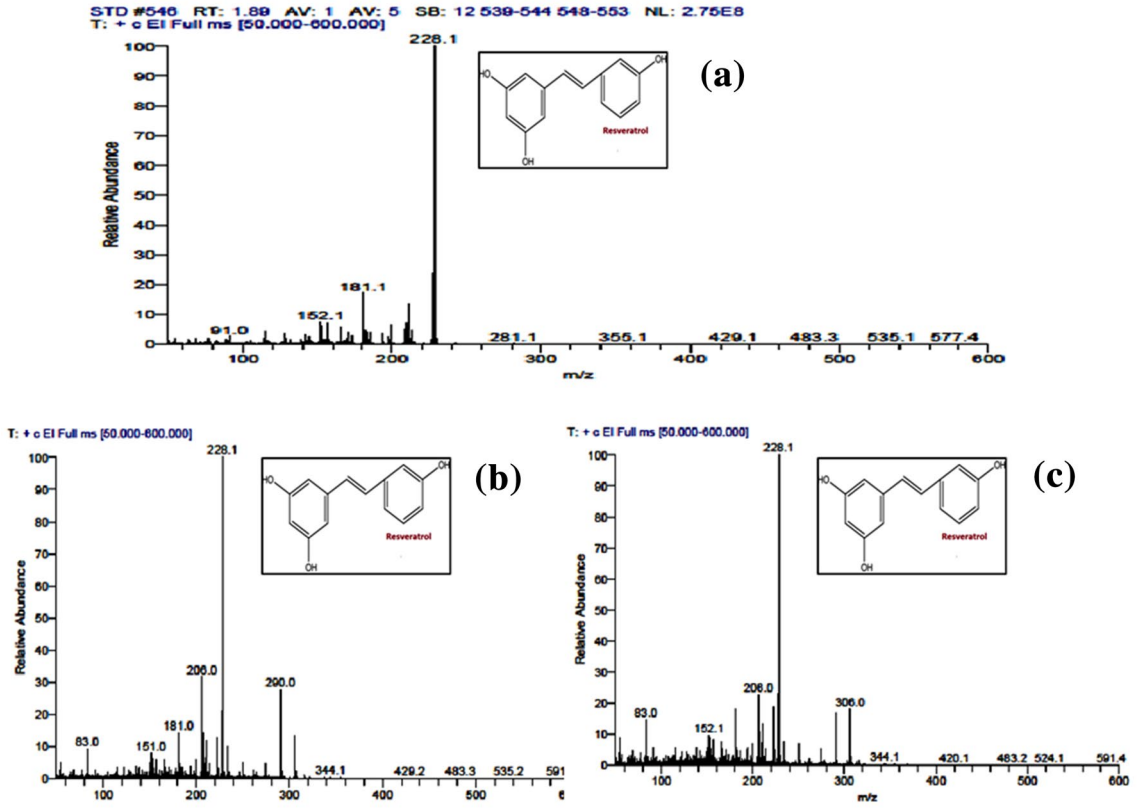


Fig. 4 Mass spectrometric analysis of **a** standard resveratrol, **b** fungal resveratrol 5 µm SAHA treated of #22(P) VVLPM, **c** fungal resveratrol 10 µm AZA-treated of #22(P) VVLPM

Table 2 The concentration of RESV and antioxidant activity of top AZA and SAHA epigenetic modifiers of #22(P) VVLPM

Treatment	Conc. of RESV (µg/mL)*	%increase or decrease (%)	DPPH (%FRS)*	TEAC (%FRS)*	% increase or decrease	
					DPPH (%)	TEAC (%)
Control	35.43 ± 0.16	–	$19.26^{f} \pm 0.48$	$52.86^{f} \pm 0.22$		
Standard	–	–	$60.05^{a} \pm 1.65$	$72.01^{b} \pm 0.94$		
AZA 3	35.01 ± 0.33	− 1.19	$23.83^{e} \pm 0.49$	$55.18^{e} \pm 0.30$	23.73	4.39
AZA 5	37.72 ± 0.74	6.46	$25.57^{de} \pm 0.32$	$60.31^{d} \pm 0.27$	32.76	14.09
AZA 10	**48.94 ± 0.33**	**38.13**	**$30.92^{c} \pm 0.74$**	**$70.83^{b} \pm 0.65$**	**60.54**	**34.00**
SAHA 5	**52.32 ± 0.24**	**47.67**	**$33.82^{b} \pm 0.28$**	**$74.28^{a} \pm 0.26$**	**75.60**	**40.52**
SAHA 10	41.10 ± 0.80	16.00	$29.78^{c} \pm 0.17$	$63.97^{c} \pm 0.20$	54.62	21.02
SAHA 20	32.65 ± 0.72	− 7.85	$27.19^{d} \pm 0.30$	$59.30^{d} \pm 0.46$	41.17	12.18

Bold signifies the treatment wherein there was an enhancement in the resveratrol production by different epigenetic modifiers

*Data presented are the mean ± standard deviation of three replicates. Means with different superscript letters are different by Tukey's post hoc test ($p < 0.05$)

its specific morphological and reproductive characteristics. Colonies of #22(P) VVLPM on PDA produced wine red colour and orange colour (Fig. 5a). 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 30 days of incubation with 12 h photoperiod (Fig. 5a–d). Young colonies are usually white by aerial mycelium becoming red or orange due to its red pigment exudation (Fig. 5a, b).

Though the selected fungal isolate was cultivated on diverse media, it remained in vegetative state i.e. did not produce any reproductive structure like stromata, conidia, conidiogenous cells or ascospores after 2–9 weeks of incubation (Fig. 5a–d). Further, the isolate did not sporulate

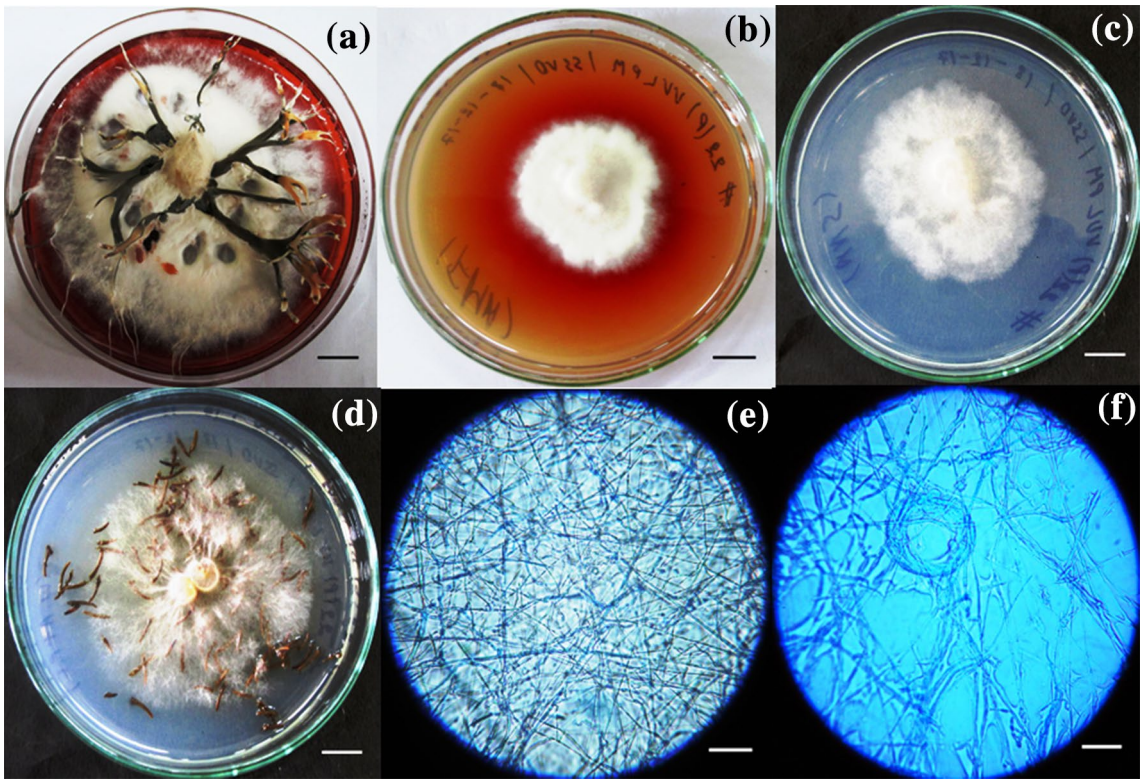


Fig. 5 Colony morphology and microscopic features of #22(P) VVLPM (*Xylaria* sp). **a** PDA (pH 7) Red wine in colour, **b** Over FMA Media Reddish Brown in color, **c**, **d** Over SNA and PLA Media white in colour, **e**, **f** microscopic feature on SNA and PLA. Bars: (**a**–**d**) 10 mm; (**e**, **f**) 10 µm. (Color figure online)

even after providing stress conditions like incubation under complete darkness and ultraviolet radiation, but it could be identified by their specific stromata (Fig. 5a). Stromata are upright, 4–5 cm long, branched or unbranched, single or in clusters and extended to the fertile cylindrical or conical apex (Fig. 5a). Further identification using molecular technique revealed that the fungus belongs to the *Xylaria* sp.

Molecular identification of resveratrol producing fungus

The resveratrol producing endophytic fungi were initially identified using morphotaxonomic studies; however, their final identification was confirmed by carrying out the molecular phylogeny based on ITS sequences. BLAST analysis of ITS sequence of #22(P) VVLPM exhibited a 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 ITS sequence of #22(P) VVLPM. In this phylogenetic tree #22(P) VVLPM clustered *Xylaria psidii* isolate SUT124 in clade I. Hence from morphological as well as phylogenetic analysis #22(P) VVLPM was assigned the name *Xylaria psidii* (Fig. 6). The ITS sequence of #22(P) VVLPM has been submitted in GenBank with accession no. MH142837.

Biological assays

Antioxidant assays

Free radical damage has been implicated in pathogenic infections, inflammatory response, cardiovascular disorders, ageing and autoimmune disorders like rheumatoid arthritis. Different in vitro assays were used to assess the antioxidant activity. In the present study the culture filtrate of *Xylaria psidii* was evaluated for its antioxidant potential activity using DPPH and ABTS assays. The best activity and percentage of free radical scavenging was observed in concentration 10 µM AZA and 5 µM SAHA, after that there was decline in free radical scavenging activity when we increase the concentration of AZA and SAHA.

DPPH scavenging assay

There was a statistically significant difference in the in vitro DPPH scavenging activity among the different fungal extracts by one way ANOVA. It was observed that the DPPH scavenging activity was significantly enhanced in the ethyl acetate extract of spent broth treated with 10 µM AZA and 5 µM of SAHA exhibiting the highest

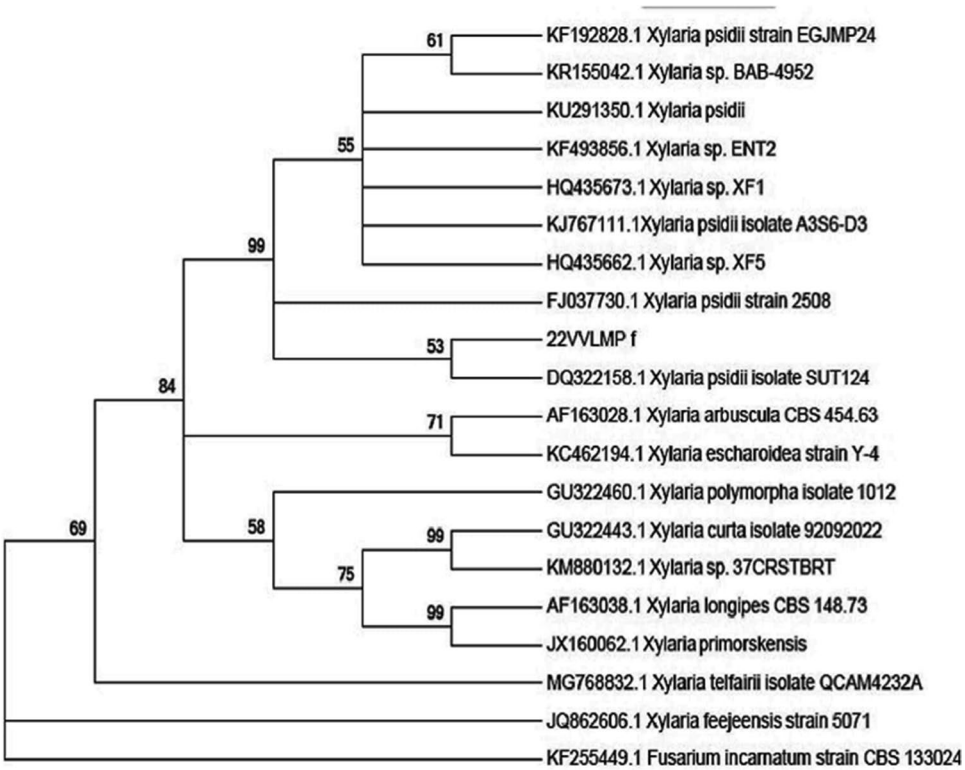


Fig. 6 *Maximum* Likelihood tree based on the ITS1-5.8S-ITS2 region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)

% free radical scavenging activity, i.e., 30.92 ± 0.74 and 33.82 ± 0.28. It was also observed that there was significant positive correlation between DPPH scavenging assay and resveratrol concentration because they both were highest at 10 µM AZA and 5 µM of SAHA. A similar correlation was observed by the resveratrol production, fungal biomass and diameter. 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 19.26 ± 0.48 (Fig. 7c, d) (Table 2).

Trolox equivalent antioxidant capacity (TEAC) assay

In the in vitro TEAC assay a significant difference was observed in the fungal extracts by one way ANOVA.

It was observed that the TEAC was significantly enhanced in the ethyl acetate extract of spent broth treated with 10 µM AZA and 5 µM of SAHA which also exhibited the highest % free radical scavenging activity, i.e., 70.83 ± 0.65 and 74.28 ± 0.26 in the epigenetic variants of #22(P) VVLPM which was more as compared to control (52.86 ± 0.22) (Fig. 7a, b) (Table 2). In case of TEAC here too positive correlation between TEAC scavenging assay, resveratrol concentration as well as biomass and diameter.

Stability studies

To determine the stability of the epigenetic mutants AZA 10 µM and SAHA 5 µM the mutated culture was subcultured up to 5 generations. The concentration of resveratrol was further analysed by using HPLC. It was observed that even after five generations the concentration of resveratrol produced by the mutant AZA 10 µM and SAHA 5 µM did not decrease significantly.

Discussion

Ever since the discovery of "Taxol" producing endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia*, led the foundation for exploiting endophytic fungi as a novel source of biologically active secondary metabolites. Endophytic fungi have been immensely explored and exploited for the production of putative phytochemicals such as Taxol [35], Camptothecin [36], Podophyllotoxin [37], deoxypodophyllotoxin [36] and Vincristine [38]. Apart from being a potential source of putative phytochemicals, endophytic fungi also produce an array of bioactive moieties which are expressed in response to biotic and abiotic stresses to which plant is exposed from time to time [36]. Recent advancements in the fungal molecular genetics have established that fungi have many cryptic genes (phenotypically silent or unexpressed

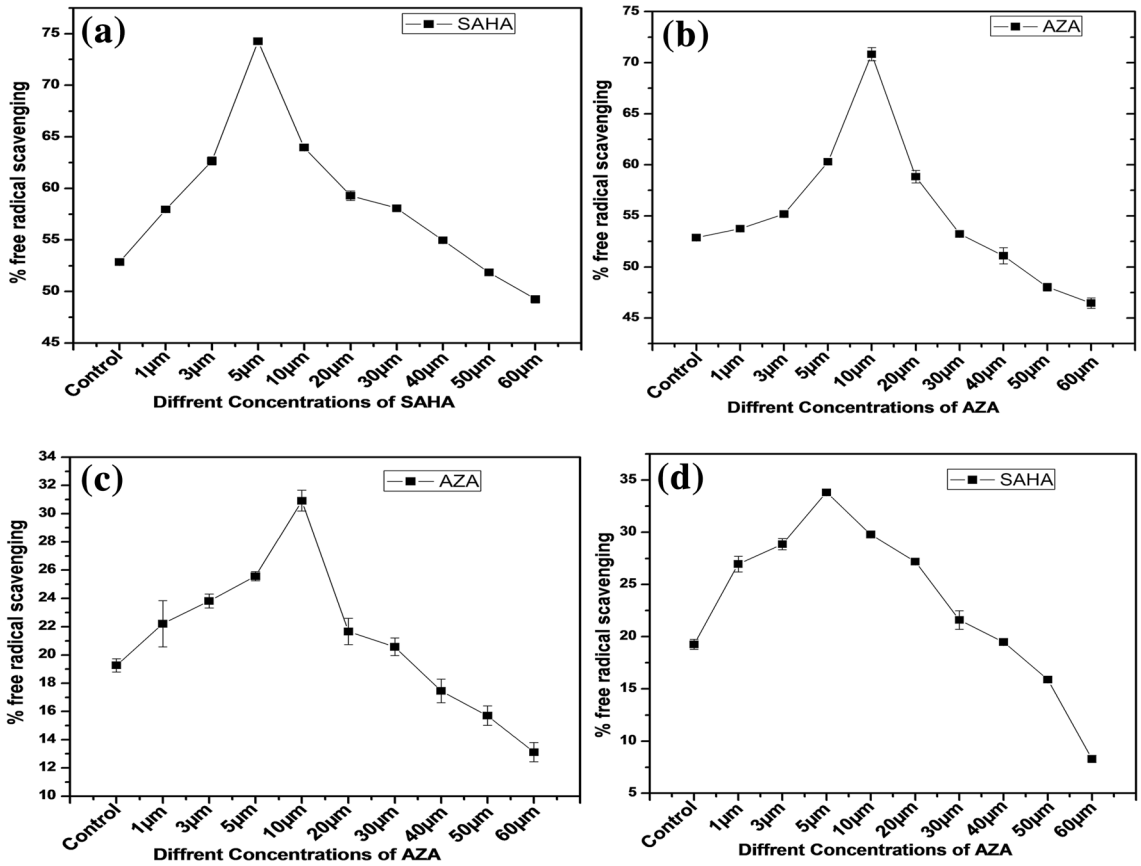


Fig. 7 Antioxidant activity of *Xylaria psidii* treated with different concentrations of AZA and SAHA using DPPH, ABTS assay, **a** ABTES AZA, **b** ABTES SAHA, **c** DPPH AZA, **d** DPPH SAHA

gene). These genes can be expressed by epigenetic modifiers like microbial and chemical elicitors, precursors, and through epigenetic modifications which leads to enhancement of novel secondary metabolite that could be exploited as a nutraceutical, pharmaceutical or in agrochemical industries [39, 40].

Epigenetic modifiers such as HDAC inhibitors (SAHA) and DMNTs (AZA) inhibitors represent an exciting chemical tool to express a cryptic or silent gene which is unexpressed under standard laboratory condition. Earlier the successful attempt of epigenetic modulation have been made using 5- azacytidine in mycodiesel producing endophytic fungus *Hypoxylon* sp. (CI-4), producing a vast 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 [39]. Li et al. [41] performed the co-culturing experiment of endophytic fungi *Camporesia sambuci* (FT1061) and *Epicoccum sorghinum* (FT1062) and reported a new N-methoxypyridone analog, together with four known compounds. All these research have suggested 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 metabolites.

Resveratrol is natural polyphenolic stilbene, first identified as a significant 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 can promote the longevity of *Saccharomyces cerevisiae*. Due to these reasons, resveratrol is now widely used as a nutraceutical and in cosmetics [25, 42]. *Xylaria psidii* (# 22(P) VVLPM) is a member of family *Xylariales,* though *Xylaria* sp. has been previously reported from *Vitis vinifera* [23, 43]. In the current research, a study was performed for enhancement of resveratrol production via using a different concentration of chemical elicitor in free fermentative medium to modulate the secondary metabolite production.

Thin layer chromatography is routinely used for separation and identification of compounds in a mixture which is generally carried out by using a standard along with the mixture to be resolved. Our study shows a similar retention factor of our fungal resveratrol to that of standard resveratrol

(plant derived), i.e. 0.65. HPLC analysis further played a crucial role for the identification as well as quantification of resveratrol. HPLC analysis I revealed that RESV concentration was maximum and enhanced in the case of treatment with 5 µM SAHA and by 10 µM AZA followed by 10 µM SAHA and 5 µM AZA. In earlier reports, *Colletotrichum gloeosporioides* an endophytic fungus, was also reported to have an increase in cryptic compounds as well as their antimicrobial potential by using epigenetic modifiers, grape extract and turmeric extracts [17].

Endophytic fungi are famed for their antimicrobial as well as antioxidant potential [44]. The fungal resveratrol producing 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 the 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 compared to wild strain. In the case of TEAC also same trend as in the case of DPPH was obtained, whereas in the case of TPC and TFC an increment was observed in fungal samples treated with 3 µM AZA and SAHA, 5 µM AZA and SAHA, 10 µM AZA and SAHA when compared to #22(P) VVLPM control. Khanduja and Bhardwaj [45] in their study found that RESV showed stronger antioxidant potential than Catechin, Myricetin, and Fisetin. Shrikanta et al. [44] also observed antioxidant property which was attributed due to the presence of RESV in grapes and jamun.

Epigenetic modifiers such as HDAC inhibitors (SAHA) and dMNTs (AZA) inhibitors represent an exciting chemical tool to express a cryptic or silent gene which is unexpressed under standard laboratory condition. In the present study, the effect of two different epigenetic modifiers, i.e., HDAC inhibitors (SAHA) and dMNTs (AZA) inhibitors on the metabolic profile of *Xylaria psidii* (#22(P) VVLPM) was studied. A significant effect on the secondary metabolite production was observed in HPLC profiles of the extract obtained after epigenetic induction when compared to extract obtained without addition of epigenetic modifiers. An HPLC and LC–MS spectrum shows an enhancement of *47.67%* (52.32 µg/mL) in case of SAHA 5 µM and *38.13%* (48.94 µg/mL) in case of AZA 10 µM, resveratrol concentration which was earlier present in very less amount (35.43 µg/mL) in the crude ethyl acetate extract. A similar observation was made by some other researchers as carried out by Lopes et al. [46] wherein they used a range of epigenetic modifiers on *Nigrospora* sp. SS67 and they found that epigenetic modifiers increase the production of the secondary metabolites. Establishment of a high producing endophytic epigenetic mutant strain for industrial production of resveratrol is a genuine metabolic engineering challenge. All these studies vigorously advocate that fungi also have many genes like other eukaryotic microbes which remain cryptic under normal lab conditions, and need some epigenetic modifiers to activate fungal gene transcription, leading to the fungal strain improvement for production of known as well as novel secondary metabolites.

Conclusion

Considering the increased demand for resveratrol in pharmaceutical, food as well as cosmetic industries the enhancement of resveratrol production, using the epigenetic modifiers such as HDAC inhibitors (SAHA) and dMNTs (AZA) inhibitors was 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. Activation of silenced genes was more efficient with HDAC inhibitors (SAHA) than with dMNTs (AZA) inhibitors. This method is more effective than others as it is cost-effective, reliable supply and high purity as compared to extraction from plant materials. The present study shows a positive correlation between resveratrol content and the antioxidant potential.

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Author contribution Prof. Sanjai Saxena conceived and designed the present study, while Vagish Dwibedi and Shreya Kalia performed all experiments, analyzed the data. Prof. Sanjai Saxena and Vagish Dwibedi jointly wrote the publication.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Kasiotis KM, Pratsinis H, Kletsas D, Haroutounian SA (2013) Resveratrol and related stilbenes: their anti-aging and anti-angiogenic properties. Food Chem Toxicol 61:112–120
2. Lee TH, Seo JO, Baek SH, Kim SY (2014) Inhibitory effects of resveratrol on melanin synthesis in ultraviolet B-induced pigmentation in Guinea pig skin. Biomol Ther 22(1):35
3. Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. Nat Prod Rep 23(5):753–771
4. Bradamante S, Barenghi L, Villa A (2004) Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 22(3):169–188

5. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW et al (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275(5297):218–220
6. Kundu JK, Surh YJ (2008) Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. Cancer Lett 269(2):243–261
7. Langcake P, Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Physiol Plant Pathol 9(1):77–86
8. Burns J, Yokota T, Ashihara H, Lean ME, Crozier A (2002) Plant foods and herbal sources of resveratrol. J Agric Food Chem 50(11):3337–3340
9. Lijavetzky D, Almagro L, Belchi-Navarro S, Martínez-Zapater JM, Bru R, Pedreño MA (2008) Synergistic effect of methyl jasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures. BMC Res Notes 1(1):132
10. Donnez D, Jeandet P, Clément C, Courot E (2009) Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. Trends Biotechnol 27(12):706–713
11. Venugopalan A, Srivastava S (2015) Endophytes as in vitro production platforms of high-value plant secondary metabolites. Biotechnol Adv 33(6):873–887
12. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67(4):491–502
13. Kobayashi S, Ding CK, Nakamura Y, Nakajima I, Matsumoto R (2000) Kiwifruits (*Actinidia deliciosa*) transformed with a Vitis stilbene synthase gene produce piceid (resveratrol-glucoside). Plant Cell Rep 19(9):904–910
14. Kiselev KV (2011) Perspectives for production and application of resveratrol. Appl Microbiol Biotechnol 90(2):417–425
15. Cherblanc F, Chapman-Rothe N, Brown R, Fuchter MJ (2012) Current limitations and future opportunities for epigenetic therapies. Future Med Chem 4(4):425–446
16. Cherblanc FL, Davidson RW, Di Fruscia P, Srimongkolpithak N, Fuchter MJ (2013) Perspectives on natural product epigenetic modulators in chemical biology and medicine. Nat Prod Rep 30(5):605–624
17. Sharma VK, Kumar J, Singh DK, Mishra A, Verma SK, Gond SK et al (2017) Induction of cryptic and bioactive metabolites through natural dietary components in an endophytic fungus *Colletotrichum gloeosporioides* (Penz.) Sacc. Front Microbiol 8:1126
18. Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH (2008) Epigenetic remodeling of the fungal secondary metabolome. Org Biomol Chem 6(11):1895–1897
19. Schulz B, Wanke U, Draeger S, Aust HJ (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol Res 97:1447–1450. https://doi.org/10.1016/S0953-7562(09)80215-3
20. Petrini O, Sieber TN, Toti L, Viret O (1993) Ecology, metabolite production, and substrate utilization in endophytic fungi. Nat Toxins 1(3):185–196
21. Gupta YK, Briyal S, Chaudhary G (2002) Protective effect of trans-resveratrol against kainic acid-induced seizures and oxidative stress in rats. Pharmacol Biochem Behav 71(1–2):245–249
22. Shi J, Zeng Q, Liu Y, Pan Z (2012) *Alternaria* sp. MG1, a resveratrol-producing fungus: isolation, identification, and optimal cultivation conditions for resveratrol production. Appl Microbiol Biotechnol 95(2):369–379
23. Dwibedi V, Saxena S (2018) *Arcopilus aureus*, a resveratrol-producing endophyte from *Vitis vinifera*. Appl Biochem Biotechnol 186:476–495
24. Babu SK, Kumar KV, Subbaraju GV (2005) Estimation of trans-resveratrol in herbal extracts and dosage forms by high-pe$_{Rf}$ormance thin-layer chromatography. Chem Pharm Bull 53(6):691–693
25. Wang DG, Liu WY, Chen GT (2013) A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*. J Pharm Anal 3(4):241–247
26. Dincheva I, Badjakov I, Tsvetkov I, Dzhambazova T, Kondakova V, Batchvarova R (2011) GC–MS determination of secondary metabolites in red wine from Bulgarian wild grape (*Vitis vinifera* ssp. sylvestris. J Mt Agric Balkans 14(6):1331–1343
27. Ramirez-Lopez LM, DeWitt CA (2014) Analysis of phenolic compounds in commercially dried grape pomace by high-performance liquid chromatography-electrospray ionization mass spectrometry. Food Sci Nutr 2(5):470–477
28. Ellis MB, Ellis JP (1985) Microfungi on land plants. An identification handbook. Croom Helm Ltd., Kent
29. Barnett HL, Hunter BB (1998) Illustrated genera of imperfect fungi
30. Nagamani A, Kunwar IK, Manoharachary C (2006) Handbook of soil fungi. IK International, New Delhi
31. White TJ, Bruns T, Lee SJWT, Taylor JL (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc 18(1):315–322
32. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biol Evol 30(12):2725–2729
33. Ho R, Violette A, Cressend D, Raharivelomanana P, Carrupt PA, Hostettmann K (2012) Antioxidant potential and radical scavenging effects of flavonoids from the leaves of *Psidium cattleianum* grown in French Polynesia. Nat Prod Res 26:274–277
34. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity was applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26(9–10):1231–1237
35. 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
36. Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol 19(7):792–798
37. Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. J Nat Prod 69(8):1121–1124
38. Aly AH, Debbab A, Proksch P (2011) Fungal endophytes: unique plant inhabitants with grand promises. Appl Microbiol Biotechnol 90(6):1829–1845
39. Ul-Hassan SR, Strobel GA, Booth E, Knighton B, Floerchinger C, Sears J (2012) Modulation of volatile organic compound formation in the mycodiesel-producing endophyte *Hypoxylon* sp. CI-4. Microbiology 158(2):465–473
40. Kumar J, Sharma VK, Singh DK, Mishra A, Gond SK, Verma SK et al (2016) Epigenetic activation of antibacterial property of an endophytic Streptomyces coelicolor strain AZRA 37 and identification of the induced protein using MALDI TOF MS/MS. PLoS ONE 11(2):e0147876
41. Li C, Sarotti AM, Yang B, Turkson J, Cao S (2017) A new N-methoxypyridone from the co-cultivation of Hawaiian endophytic fungi *Camporesia sambuci* FT1061 and *Epicoccum sorghinum* FT1062. Molecules 22(7):1166
42. Jiménez JB, Orea JM, Ureña AG, Escribano P, De La Osa PL, Guadarrama A (2007) Short anoxic treatments to enhance trans-resveratrol content in grapes and wine. Eur Food Res Technol 224(3):373–378
43. Mostert L, Crous PW, Petrini O (2000) Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. Sydowia 52(1):46–58

44. Shrikanta A, Kumar A, Govindaswamy V (2015) Resveratrol content and antioxidant properties of underutilized fruits. J Food Sci Technol 52(1):383–390
45. Khanduja KL, Bhardwaj A (2003) Stable free radical scavenging and antiperoxidative properties of resveratrol compared in vitro with some other bioflavonoids
46. Lopes AA, da Silva DB, Lopes NP, Pupo MT (2012) Epigenetic modulation changed the secondary metabolite profile in the endophyte *Nigrospora sphaerica* SS67. Planta Med 78(14):PL38

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