Effect Of Azacytidine on Resveratrol Production by *Arcopilus aureus*

A THESIS

submitted in partial fulfilment of the requirement of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted By

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June 2022

CERTIFICATE

This is to certify that Ankita's thesis, "Effect of Azacytidine on Resveratrol Production by Arcopilus aureus," which she is submitting with the roll number 302001002 in part fulfilment of the requirements for the award of a degree in Master of Science in Biotechnology from the Thapar Institute of Engineering and Technology in Patiala, Punjab, is a genuine work that was created with the guidance and inspiration of Dr. Sanjai Saxena.

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DECLARATION

By submitting this statement, I affirm that the work described in the thesis entitled "**Effect Of Azacytidine on Resveratrol Production by *Arcopilus aureus***" in partial fulfilment of the requirements for the award of degree of Master in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala is my own laboratory work carried out during the period of February 2022 to June 2022, under the supervision of Dr. Sanjai Saxena, Professor, Department of Biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is to verify that, to the best of my knowledge, the candidate's above statement is accurate and true.

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ACKNOWLEDGEMENT

This project needed a lot of direction and help from many people in order to succeed and produce the desired results. I would like to take this opportunity to express my sincere gratitude to Dr. Sanjai Saxena, Professor of the Department of Biotechnology at the Thapar Institute of Engineering and Technology in Patiala, Punjab, for his honorable project guidance, insightful suggestions, insightful comments, gracious behavior, and friendly assistance throughout the course of the investigation and preparation of this manuscript.

I respect and thank my seniors, Ms. Gurleen Kaur Sodhi, Ms. Shreya Gupta, Ms. Ankita Arora, Ms. Ramandeep Kaur and Mr. Pushkar Bharti, whose careful direction, unwavering zeal, and frequent encouragement were a big help during my dissertation. I truly appreciate them for offering their frank and enlightening perspectives on a variety of issues pertaining to this report. I want to express my sincere gratitude to Ms. Gurleen Kaur Sodhi in particular for her unfailing support and willingness to lend a hand.

I also want to convey my sincere gratitude to my mother, Mrs. Archana Bansal, and my father, Mr. Raman Kumar, for their unwavering support and inspiration.

In closing, I would like to express my gratitude to all the experts whose insightful comments and suggestions on this report inspired me to better it. I want to express my gratitude to everyone who helped me finish my report, both intrinsically and extrinsically.

(Ankita)

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LIST OF ABBREVIATION

S. NO.	ABBREVIATION	FULL FORM
1.	µg	Micro Gram
2.	µL	Micro liters
3.	AZA	5-Azacytidine
4.	µM	Micro Molar
5.	DPPH	1,1-Diphenyl-2-Picrylhydrazyl
6.	PDA	Potato Dextrose Agar
7.	CMA	Corn Meal Agar
8.	WA	Water Agar
9.	HPLC	High Performance Liquid Chromatography
10.	TLC	Thin Layer Chromatography
11.	mg	Milli Gram
12.	mL	Milli liter
13.	PDB	Potato Dextrose Broth
14.	TFC	Total Flavonoid Content
15.	TPC	Total Phenolic content
16.	UV	Ultra Violet

EXECUTIVE SUMMARY

Resveratrol is a major constituent of red wine and exhibits multifarious properties. This polyphenolic flavonoid was first extracted from plant sources but the extensive process and purification were very expensive. Due to its nutraceutical, cosmeceutical and therapeutic properties there is a huge global demand of resveratrol. A cost-effective alternative to produce resveratrol involves using endophytic fungi. Endophytic fungi show exciting property of producing host secondary metabolites owing to horizontal gene transfer. They live in symbiotic relationship with the host. Both benefits from each other in the terms that host provide the fungus a shelter to live while the endophytic fungi help the host to fight against the biotic and abiotic stresses. Endophytic fungi strengthen the defense mechanism of the host by secreting secondary metabolites. These secondary metabolites are not only consumable by humans in their diet but also can be directly or indirectly used in the medicinal preparations. Many resveratrol producing endophytic fungi were studied. For enhancing the resveratrol amount, certain strategies like UV radiation, pathogen attack, and metal salt addition were employed but the generation of instability of genomic content directed the study towards epigenetic modifications. The current study directs to certain strategies that could help to increase the amount of resveratrol produced by the endophytic fungi. Out of hundreds of studied endophytic fungi, *Arcopilus aureus*, proved to be the most promising endophytic fungi for the commercial production of resveratrol till date. So, this isolated endophytic fungus from *Vitis vinifera* was cultured in the laboratory. Addition of epigenetic modifier – AZA was done to observe the change in the amount of resveratrol produced. The different concentrations of this modifier were then subjected to TLC for quantification, out of which the 10 µM concentration exhibited the maximum production of resveratrol and had the highest antioxidant property, maximum TPC and TFC content. This directed the study that the *Arcopilus aureus* when cultured after the addition of AZA at 10 µM, it will provide the maximum amount of resveratrol. All the studies and experiments advocate the theory that epigenetic chemical modifiers can be used to enhance the production of secondary metabolites in the endophytic fungi.

Keywords: Resveratrol, *Arcopilus aureus*, chemical elicitor AZA, TLC.

CHAPTER 1

INTRODUCTION

1. Introduction:

Resveratrol (RSV), chemically known as trans-3,4,5-trihydroxystilbeneis a phytochemical, first isolated from white hellebore (*Veratrum grandiflorum* O. Loes) during the 19th century (Rajasekaran, *et al.*, 2011; Cocetta, *et al.*, 2021). It is a derivative of resorcinol after which it was named as resveratrol. The seemingly contradictory epidemiological finding that French people have a low incidence of coronary heart disease (CHD) despite eating a diet high in saturated fats was attributed to resveratrol content in red wine. It also started the nutraceutical and medicinal research on this compound (Ndlovu *et al.*, 2019). Resveratrol has immense health benefits such as antioxidant, anti-inflammatory, anti-ageing, cardioprotective, anticancer properties etc., (Akter *et al.*, 2021; Otero *et al.*, 2021; Ralte *et al.*, 2022). These properties further lead to lowering of blood pressure, decrease in LDL cholesterol oxidation, increasing insulin sensitivity etc., (Li, *et al.*, 2014; Banez *et al.*, 2020). Besides this, it is also studied to have benefits in controlling the neural disorders like Parkinson's and Alzheimer (Sun *et al.*, 2010; Mathew *et al.*, 2019). Currently, the global market of resveratrol was reported at 69 million USD in the year 2017 and projects an increase of 9.1% by 2026 (Tiwari, *et al.*, 2021).

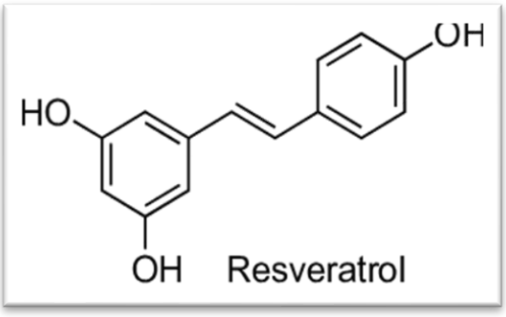

Figure1.1: Structure of Resveratrol (Omran *et al.*, 2020)

Owing to the major health benefits, resveratrol was subsequently isolated from the medicinal plant *Polygonum cupsidatum* for commercial production but the process was quite cost intensive (Lin, *et al.*, 2014). Thus, there is a need to find a novel, cost-effective and sustainable alternative for resveratrol production. Since, it is a plant derivative, the utilization of symbiotic association of endophytic fungi and plants has been explored for production of various plant compounds (Bielecka *et al.*, 2022). Endophytes ("endo" = within, "phyte" = plant) are microorganisms that live inside plants, particularly leaves, stems, and roots, without harming the

host (Porras-Alfaro *et al.*, 2011). Endophytes are ubiquitous, with a high biodiversity, and can be found in nearly every plant species on the planet, with each plant host having one or more than one endophyte (Strobel & Daisy, 2003). Endophytic fungi are understood to be plant mutualists because they acquire sustenance and protection from the host plant, while the host plant gains greater competitive powers and resistance to herbivores, diseases, and abiotic stresses (Nanda *et al.*, 2019; Uzma *et al.*, 2019). It spends the majority of its life cycle invading the host plant's healthy tissues, either inters- or intra-cellularly, without creating apparent indications of infection (Tan & Zou, 2001). Endophytic fungi exhibit an interesting property of producing host secondary metabolites or photochemical owing to the gene acquisition (horizontal gene transfer) due to its co-evolution with the host plant (Bormann *et al.*, 2015; Khan, 2018). They have the tendency to produce certain signaling molecules to help them combat the host's defense mechanism (Hughes & Sperandio, 2008). These signaling molecules of the endophytic fungi also aid to produce secondary metabolites which may be agriculturally, therapeutically or industrially important (Satheesan & Sabu, 2020).

Resveratrol is synthesized via phenylalanine pathway (He, *et al.*, 2020). The condensation between 4-coumaroyl CoA and 3 molecules of malonyl CoA produce trans form of resveratrol which can isomerize to cis form but the trans form is more stable and bioactive (Hertweck, 2009; Thapa *et al.*, 2019). These polyphenolic flavonoids arbitrated by many biosynthetic pathways and gene expression alterations (Gianchecchi & Fierabracci, 2020). Previously, various endophytic fungi like *Penicillium* sp., *Fusarium* sp., *Aspergillus* sp., *Arcopilus* sp. etc. (Dwibedi & Saxena, 2018)., have been reported to produce resveratrol but in copious amounts. In order to increase the amount of resveratrol produced by the endophytic fungi, many techniques like UV radiation, metal salt addition, pathogen infection etc. were employed but these caused the genomic instability leading to silencing of various gene clusters (Dwibedi *et al.*, 2019). Whereas, on exploration of genetic modification via chemical elicitors enables targeting many endophytic fungi at the same time without knowing their genome (Dubey & Jeon, 2017). Epigenetically regulating the gene transcription of the endophytic fungi by certain modifications was studied (Dwibedi *et al.*, 2019). This opened the doors for genetic modifications like DNA methylation and histone deactivation. These processes direct the biosynthesis of gene clusters so modulating these processes will help

to regulate the gene transcription; hence modified gene expression will enhance the production of secondary metabolites (Osbourn, 2010; Brakhage, 2013).

Some of the chemical elicitors are valproic acid, 5-azacytidine (AZA), sodium butyrate, jasmonic acid, suberoylanilide hydroxamic acid (SAHA) etc., (Mishra *et al.*, 2021). DNMT inhibitors work by reactivating genes that have been abnormally silenced due to DNMT methylation and restoring their normal activity (Gnyszka *et al.*, 2013). By integrating themselves into the developing DNA strand, these inhibitors covalently bind DNMTs. There are two types of DNMT inhibitors: nucleoside and non-nucleoside inhibitors (Huang *et al.*, 2021). 5-azacitidine and 5-aza-2′-deoxycytidine (decitabine) are nucleoside inhibitors that are usually produced from cytidine and require DNA integration to be active (Yoo & Jones, 2006). Curcumin and procaine are non-nucleoside inhibitors with a variety of chemical structures that bind directly to DNMTs (Singh *et al.*, (2013). 5-azacytidine induce demethylation of DNA by inhibiting DNA methyltransferase enzyme (Stresemann *et al.*, 2006). DNA methyltransferase enzyme catalysis the transfer of methyl group to DNA to the transcription initiation site (Dubey & Jeon, 2017). This does not allow various transcription factors to come, bind and initiate the transcription process to form new genes and proteins (Alberini, 2009). So, inhibiting these DNMTs will allow the transcription factors to bind to transcription initiation site, form a complex and thereby doing the gene expression (Baylin *et al.*, 2001). This will allow certain proteins (secondary metabolites) to be formed which could not be formed before (González-Menéndez *et al.*, 2016)

Considering the above findings, this study is designed to optimize a concentration of AZA at which maximum resveratrol production could be achieved using agar plate and fermentation broth assay. The confirmation of the optimized concentration was done using thin layer chromatography technique (TLC) in comparison to the standard resveratrol. Further bioassays involving effect of different concentrations of AZA on antioxidant potential, phenol and flavonoid content were also studied.

CHAPTER 2

REVIEW OF LITERATURE

2. Review of literature

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) belongs to polyphenols family (Shankar *et al.*, 2007). It is a stilbene, a form of natural phenol, and a phytoalexin generated by a variety of plants in reaction to injury or pathogens like bacteria or fungi (Tiku, 2018). Grape skin, blueberries, raspberries, mulberries, and peanuts are all good sources of resveratrol in food (Shrikanta *et al.*, 2015). There seems to be no solid proof that resveratrol lengthens life or significantly affects any human disease, but considering research in experimental analysis of human ailments and its extensive use as a nutritional supplement (Pasinetti *et al.*, 2015). The molecule has two benzene rings attached by isopropyl separated by double bond (Dwibedi *et al.*, 2019). The molecule exists in *trans* and *cis* forms but the *trans* isomeric form is more stable than *cis* form (Jhanji & Sajish, 2021).

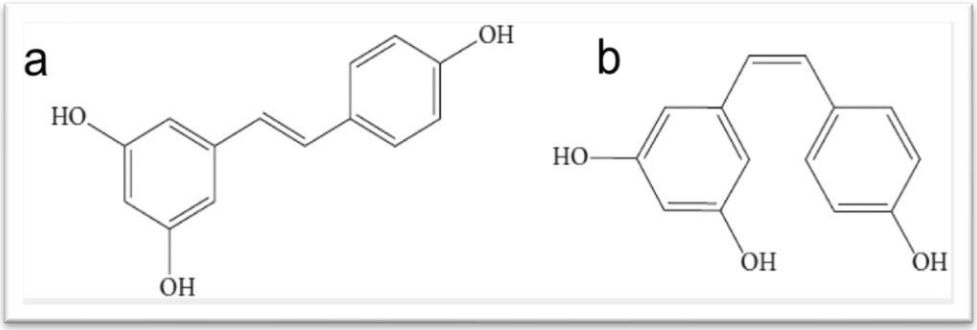

Figure 2.1: a) *trans* form b) *cis* form of resveratrol (Fiod Riccio *et al.*,2020)

In the Shikimate pathway, condensation of malonyl CoA and p-coumaroyl CoA forms the resveratrol (Ibrahim *et al.*, 2021). Plants generally manufacture resveratrol through the phenylpropanoid pathway in nature (PPPN) (Schouteden *et al.*, 2015; Lu *et al.*, 2021). This mechanism was also used to produce resveratrol in endophytic fungus, according to a prior study. PPPN starts with the production of phenylpropanoid acids from the aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr) obtained through the shikimate pathway (Rodriguez *et al.*, 2014; Lu *et al.*, 2021). There are two branches in this phase. Tyr dis-amination to generate p-coumaric acid is catalyzed by Tyrosine ammonia-lyase (TAL). The 4-coumarate coenzyme A ligase (4CL) then combines p-coumaric acid with coenzyme A (CoA) to form 4-coumararoyl-CoA (Zang *et al.*, 2019). Cinnamic aid is generated as a result of Phe catalyzed by phenylalanine ammonia-lyase (PAL) in a distinct branch (Qin *et al.*, 2022). Cinnamate 4-hydroxylase (C4H) and 4CL, in either

sequence, continually catalyze cinnamic acid to produce 4-coumaroyl-CoA (Singh *et al.*, 2009). Finally, stilbene synthase (STS) catalyzes the reaction between one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA to produce resveratrol (Yu *et al.*, 2008). STS is a member of the type III polyketide synthases (PKSs), which also includes chalcone synthase (CHS) (Austin & Noel, 2003). Although CHS is responsible for the biosynthesis of naringenin, there has been evidence of cross-reactivity between CHS and STS, with CHS creating resveratrol and STS forming naringenin (Austin *et al.*, 2014). As a result, the genes PAL/TAL, 4CL, C4H, and STS/CHS are important in the synthesis of resveratrol (Lu *et al.*, 2021).

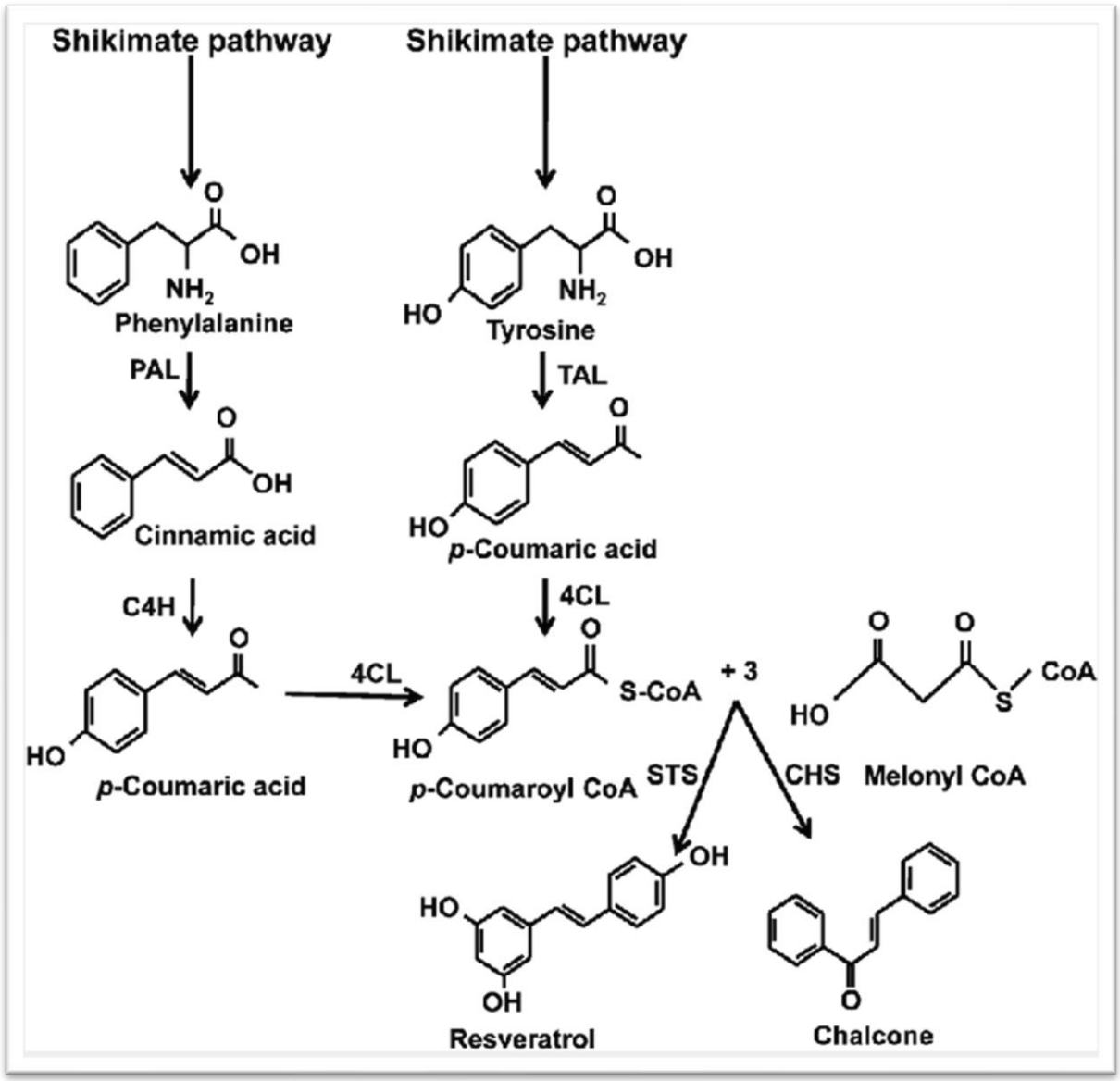

Figure 2.2: Biosynthesis of Resveratrol (Hasan *et al.*, 2013)

It was first isolated from *Polygonum cuspidatum* for the medicinal uses in Japan and China (Bi *et al.*, 2021). Various other families in the plant kingdom have the potential to produce resveratrol like *Liliaceae*, *Pinaceae*, *Vitaceae*, *Moraceae* (Bavaresco *et al.*, 2012). Many parts of the grape plant like stem, seed, skin and leaves can produce resveratrol but grape skin possess the highest amount (Hasan & Bae, 2017). The grape variety and phase of ripening accounts for the resveratrol amount (de Rosas *et al.*, 2022). About 2000 years back, the first use of grape as medicine was done via an ayurvedic preparation "darakchasava", prepared from *Vitis vinifera*, and acts as cardiotonic (Duke, 2022).

The secondary metabolite like resveratrol protects the plant from external attack by fungi like *Botrytis cinerea* as it has anti-fungal properties (Chamkhi *et al.*, 2021). There is noticeable increase amount of secondary metabolite's production under certain environmental, pathogenic attack, biotic or abiotic stresses (Yang *et al.*, 2018; Jan *et al.*, 2021). After the 'French paradox' came into limelight that explained that there is low French cardiovascular mortality rate owing to their red wine consumption, there has been a keen interest in inspecting the other properties of resveratrol (Schwarcz, 2005).

2.1 Medicinal uses of Resveratrol

2.1.1 Anti-oxidant:

Reactive Oxygen Species (ROS) are formed after the consumption of oxygen during respiration or immune mediated functions (Sies *et al.*, 2022). These ROS can be hydroxyl radicles, superoxide anion radicles, hydrogen peroxide etc. These ROS cause peroxidation of lipids, damage biomolecules and DNA (Bertin & Averbeck, 2006; Juan *et al.*, 2021). Failure to scavenge these ROS led to various serious diseases (Drummond *et al.*, 2011). Resveratrol has been found to have the capability to scavenge these ROS (Qi *et al.*, 2021), it was proved by carrying out the DPPH (1, 1, diphenyl-2-picrylhydrazyl).

2.1.2 Anti-microbial:

Resveratrol was identified as phytoalexin, a low molecular weight compound having biological activity against pathogens, especially Botrytis cinerea (Jeandet *et al.*, 2021). It was found that resveratrol inhibited the growth of Helicobacter pylori responsible for ulcer, dyspepsia etc. (Thein

et al., 2021), inhibited fungi *Trichophyton* species. Thus, giving a scope for resveratrol being a natural anti-microbial therapeutic compound (Floris *et al.*, 2021).

Table 2.1: Natural sources of resveratrol

SOURCES	AMOUNT OF RESVERATROL	REFERENCE
FOOD PRODUCTS		
Peanut butter	0.27 to 0.70 µg/gm	Lee *et al.*, 2003
Raisins	0.0005-0.003 µg/gm	Gupta *et al.*, 2014
Peanuts	0.01-0.07 µg/gm	Gupta *et al.*, 2014
Green grapes	0.02-0.32 µg/gm	Gupta *et al.*, 2014
Black grapes	0.95-1.88 µg/gm	Gupta *et al.*, 2014
Blueberries	16 ng/gm	Mukherjee *et al.*, 2010
Cranberry raw juice	0.2 mg/L	Mukherjee *et al.*, 2010
FUNGI		
Arcopilus sp. (Leaf)	89.1 µg/ml	Dwibedi & Saxena, 2018
Botryosphaeria sp. (Leaf)	37.3 µg/ml	Dwibedi & Saxena, 2018
PLANTS		
Polygonum cupsidatum	296-377 µg/gm	Gupta *et al.*, 2014
Arachis hypogeal	0.09 to 0.30 µg/gm	Lee *et al.*, 2004
Vitis rotundifolia	50-100 µg/gm	Singh *et al.*, 2015

2.1.3 Anti-ageing:

The maintenance of age by cosmetics through stemming the development of degenerative tissues giving a healthy appearance (Li *et al.*, 2021). An enzyme family called Sirtuins exhibited anti-ageing property (Covarrubias, *et al.*, 2021). Resveratrol is examined to have the capability to initiate the SIRT 1 enzyme (Covarrubias *et al.*, 2021).

2.1.4 Anti-carcinogenic:

The tumor cell growth is advanced by the COX (cyclooxygenase) by converting the arachidonic acid into prostaglandins (Tu *et al.*, 2022). Resveratrol was studied to be a potent molecule having the capability to suppress the proliferation of tumor cells (Guo *et al.*, 2021). Resveratrol decreases

the levels of estrogen and insulin growth factors 1 (IGF-1) by suppressing ERK-1/2 gene (Hipólito-Reis*et al.*, 2022). The expression of cell survival protein is decreased when the resveratrol suppresses the PKG signaling (Feng *et al.*, 2013). Resveratrol has been discovered to suppress events linked to tumor initiation (Mgbonyebi *et al.*, 1998). In human leukemia HL-60 cells, resveratrol therapy inhibited free radical generation caused by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Bhat & Pezzuto, 2002). Resveratrol's anti-oxidant properties have already been discussed. Resveratrol is an efficient scavenger of hydroxyl group and superoxide anion, as well as radicals produced by cells and stimulated by metals/enzymes (Ko *et al.*, 2017). It also protects against lipid peroxidation in cell membranes and ROS-induced DNA damage. Resveratrol also has anti-mutagenic properties, as evidenced by its ability to reduce the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine in *Salmonella typhimurium* strain TA100 (Zhao *et al.*, 2018). Resveratrol has been suggested as a potential chemo preventive drug, and the anti-carcinogenic characteristics have been proven in a variety of animal models (Athar *et al.*, 2007).

2.1.5 Neuroprotective:

Resveratrol is effective in the case of brain injury or brain seizures, making it a potent neuroprotective agent (Folbergrová *et al.*, 2021). It is effective against oxidative stress and delays the neuro-degeneration after the beta-amyloid plaque formation (Guo *et al.,* 2022). Resveratrol decreases the levels of COX-2 and TNF-alpha mRNA to overcome the chronic inflammation, mitochondrial dysfunction, dopaminergic neuron loss and oxidative stress with the help of ???–hydroxydopamine (Foti Cuzzola *et al.*, 2011; Iranshahy *et al.*, 2022).

2.1.6 Anti-Inflammatory

The body's primary response to damage is inflammation (Naik *et al.*, 2017). Despite the fact that inflammation is an important part of tissue repair, it is generally understood that uncontrolled or chronic inflammation is harmful, leading to progressive tissue damage (Wynn, 2008). Since inflammation was acknowledged to have a crucial role in various diseases, researchers have concentrated on foods high in polyphenols with anti-inflammatory and immunomodulatory characteristics in recent years (de Sá Coutinho *et al.*, 2018). This includes inhibiting the enzymes responsible for the synthesis of pro-inflammatory mediators, such as cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2), or inhibiting the enzymes responsible for the synthesis of pro-

inflammatory mediators, such as cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2), through the inhibitory effect of resveratrol on transcription (AP-1) (Das & Das, 2007; Yar *et al.*, 2010). Resveratrol, as a phenolic molecule, has a low bioavailability and, more critically, a quick elimination from the plasma (Walle *et al.*, 2004). Recent interest in resveratrol's various protective properties suggests that it could be a viable clinical alternative to anti-inflammatory medications (Koushki *et al.*, 2018)

2.1.7 Anti-diabetic

In animals with hyperglycemia, resveratrol lowers blood glucose levels (Shahi *et al.*, 2011). Increased intracellular glucose transport appears to be the primary cause of this impact (Illsley, (2000). Resveratrol has also been shown to have actions that may aid in the protection of diabetic cells (Bitterman *et al.*, 2015). The capacity of resveratrol to lower insulin secretion was proven in tests on pancreatic islets; this effect was validated in rats with hyperinsulinemia, in which resveratrol reduced blood insulin levels (Brasnyó *et al.*, 2014). Furthermore, resveratrol has been proven to decrease cytokine function as well as reduce oxidative damage to pancreatic tissue (Palsamy & Subramanian, 2010). Resveratrol appears to improve insulin activity in rats with insulin resistance, according to studies (Brasnyó *et al.*, 2011). Reduced adiposity, changes in the activity of enzymes, gene expression are all part of the complex mechanism by which resveratrol increases insulin function (Aguirre *et al.*, 2014). By encouraging Beta cells to synthesis more insulin and regulating glycosylated hemoglobin creation rate, resveratrol administration lowered glycosylated hemoglobin levels while also improving plasma insulin levels, displaying an insulin mimic effect (Palsamy & Subramanian, 2010).

2.1.6 Anti-fungal:

Botrytis cinerea the fungus which has its most potent host of wine grapes (Nifakos *et al.*, 2021). It usually causes 'botrytis bunch rot'. The literature studies the fungicidal property of the secondary metabolite especially against *Botrytis cinerea* (Dwibedi & Saxena, 2020). The dual culture method was proceeded to culture the two fungi on the same culture plate at a distance of 5 cm for 15 days (Dwibedi & Saxena, 2020). The percentage inhibition by various fungus cultures was tested against *Botrytis cinerea*. The fungal diseases are emerging very rapidly during the transplantations, cancer therapies etc., thus anti-fungal supplements are required against fungal infections (Asdaq

et al., 2021). Endophytic fungi being a natural source for anti-fungal compounds would largely reduce the manufacturing cost. Thus, many resveratrol producing endophytes were experimented to study the fungal inhibition percentage against *Botrytis cinerea*. The highest inhibition was shown by *Arcopilus aureus* (#12VVLPM) against *Botrytis cinerea* of about 57.76±0.82% (Dwibedi & Saxena, 2020).

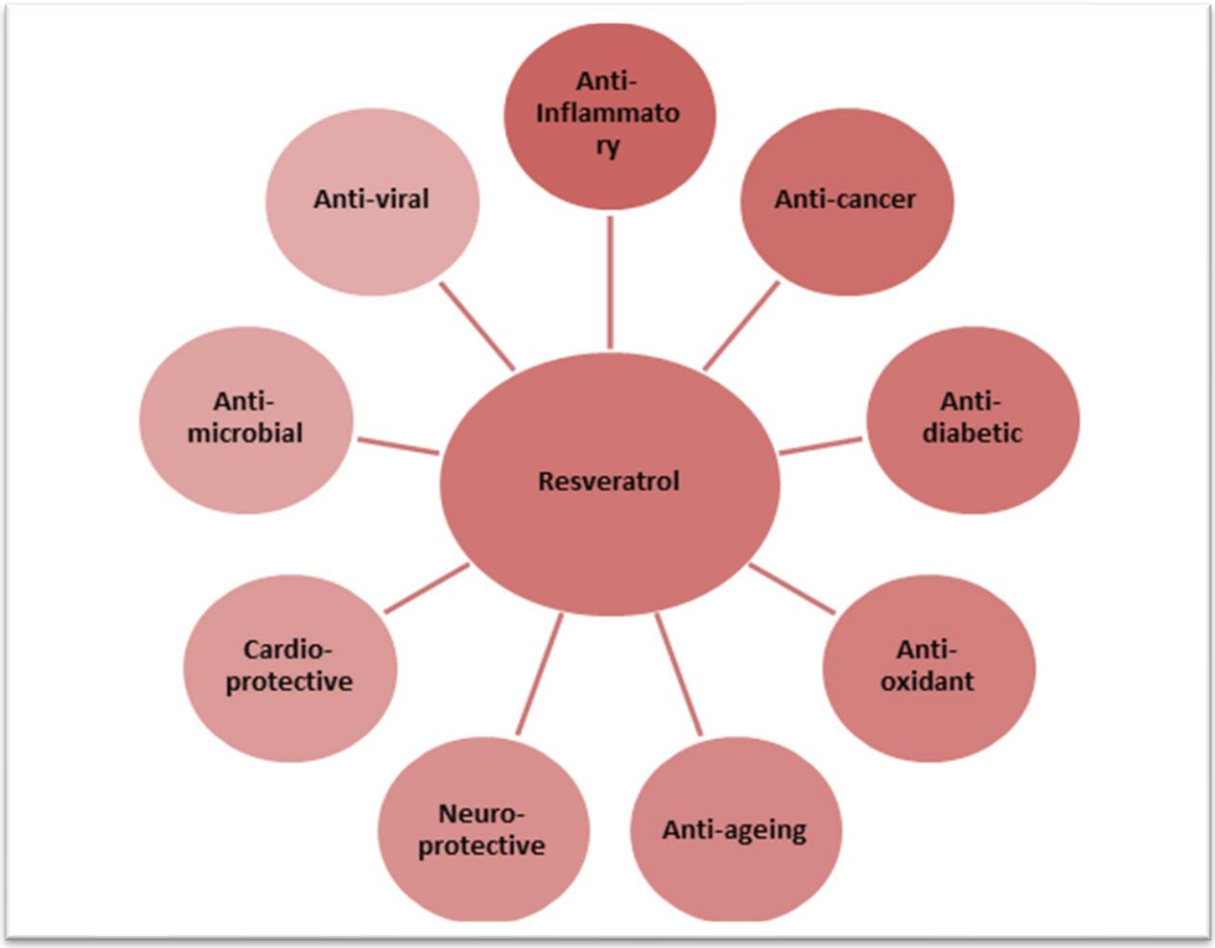

Figure 2.3: Biological activities of Resveratrol (Saxena & Srivastava, 2014)

2.1.7 Anti-staphylococcal:

Due to the refractory behavior of the multi-drug resistant microbes, they are becoming more prevalent in the infections (Juhász *et al.*, 2021). Methicillin resistant *Staphylococcus aureus* is one of the most prominent causes of infection in the hospitals (Thimmappa *et al.*, 2021). Resveratrol being a bioactive compound produced by an endophyte was experimented for its anti-staphylococcal activities (Nischitha & Shivanna, 2022). #12VVLPM that is *Arcopilus aureus* showed

the highest inhibition against *Staphylococcus aureus* (Dwibedi & Saxena, 2020). Thus, it emerged its new property of being an anti-staphylococcal (Gibbons, 2004).

2.2. Factors affecting amount of resveratrol production:

Resveratrol till now has been studied as a pharmacophore and its various advantages (Micale *et al.*, 2021). It was then studied for epigenetic modulation through various supplements, elicitors, precursor and coculturing (Dwibedi & Saxena, 2022). These were added to increase the amount of resveratrol produced by the endophytes fungus *Arcopilus aureus*. The study showed that the grape seed could enhance the production of resveratrol by 27.7% but the grape skin only brought about 13.65% increase (Dwibedi & Saxena, 2022). Precursors such as p-coumaric acid and cinnamic acid were introduced for resveratrol biosynthesis (Shin *et al.*, 2012). The extracellular production of resveratrol was seen to have increased by 1.35% with p-coumaric acid while with the cinnamic acid, the increase was about 0.27%. Elicitors like salicylic acid and cyclodextrins does not bring any marginal increase in the production amount of resveratrol (Hasan & Bae, 2017). The co-culturing impacted the production by 9.4% when co-cultured with Fusarium species. Using the natural supplements, there was also an increase in the anti-oxidant activity which was tested by DPPH and ABTS scavenging assay (Dwibedi & Saxena, 2022). Natural supplements when used followed by coculturing, the increase in the anti-staphylococcal and anti-fungal activities were also observed (Dwibedi & Saxena, 2022). Natural supplement is huge by product in the wine industry; thus, it can be used as a modulator rather than a waste (Rudra *et al.*, 2015). This will lead to a cost-effective production of resveratrol by fermentation process.

Table 2.2: Effect of different factors on resveratrol production (Dwibedi & Saxena, 2022)

Treatment	**% Increase or decrease in resveratrol concentration**
#12VVLPM + grapeseed	+ 27.71
#12VVLPM + grapeskin	+ 13.65
#12VVLPM + p-coumaric acid	+ 1.35
#12VVLPM + cinnamic acid	+ 0.27
#12VVLPM + salicylic acid	- 28.87
#12VVLPM + #19VVLPM	+ 9.36

2.3 Endophytic fungi: Endophyte term was coined by Aton de Bary in 1866 (Wilson, 1995), wherein he referred endophytes as the microorganisms that could reside in the internal tissues of the host plant despite causing any stress or disease to the same (Mishra *et al.*, 2022). Host and endophytes live in symbiotic relationship where the endophytes intake nutrients from the host and in return help the host to combat environmental stress by enhancing host's defense mechanism (Nanda *et al.*, 2019; Lee *et al.*, 2021). Today, fungi have widened its utility arena by producing certain bioactive molecules (Maithani *et al.*, 2022). The need of these compounds drove the research in exploring more of their potential and ways to increase the production of the molecules in higher amount (Craik *et al.*, 2013). In the filamentous fungi, gene clusters are responsible for the biosynthesis of secondary metabolites (Walton, 2000; Wang et al., 2022). Latest evidence on the fungal genome suggests that transcriptional regulation of the potential production of gene clusters can be influenced by epigenetic modifications such deacetylation of histones and methylation of DNA (Williams *et al.*, 2008). Without having access to the genomic sequences beforehand, a wide variety of fungus can benefit from epigenetic regulation of gene transcription (Cherblanc *et al.*, 2012; Dubey & Jeon, 2017).

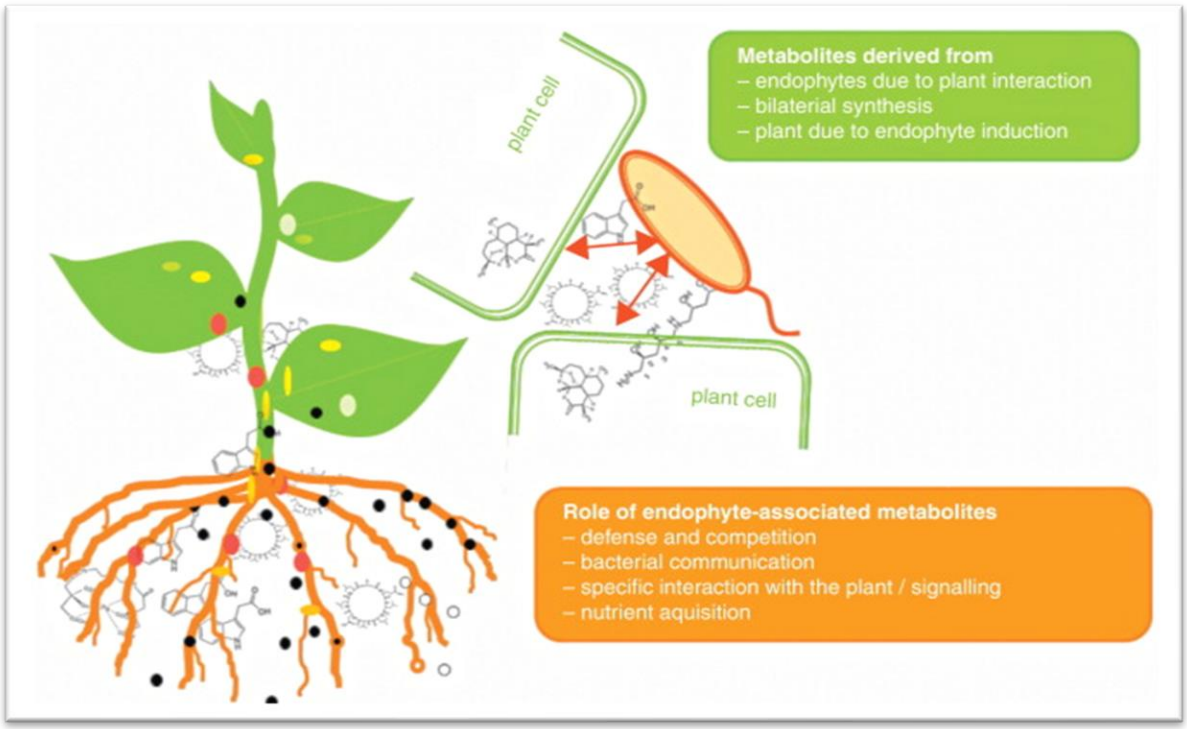

Figure 2.4: Schematic diagram of host-endophyte interaction (Brader *et al.*, 2014)

For this, various endophytic fungi were isolated from different varieties of *Vitis vinifera* collected from different parts of India. 53 endophytic fungi were examined, out of which only 29 isolates resulted positive for phenolic's presence (Dwibedi & Saxena, 2018). There have been many compounds which were isolated from the *Arcopilus* fungi like chaetomanone, ergosterol, chaetoglobosin C, echinuline etc (References missing). The literature stated the reason behind biological properties of resveratrol might be the inherent properties of fungi to produce therapeutic bioactive molecules.

Table 2.3: Endophytic fungi sources of resveratrol (Dwibedi & Saxena, 2018)

Endophytic fungi	**Plant part**	**Amount of Resveratrol (µg/mL)**
Arcopilus sp.	Leaf	89.1
Botryosphaeria sp.	Leaf	37.3
Aspergillus sp.	Stem	23.9
Aspergillus sp.	Leaf	22.4
Penicillium sp.	Leaf	15.3
Alternaria sp.	Leaf	24.1

2.4. Epigenetic modifiers:

The studies were being carried out to increase the resveratrol production by various methods, one such was via the activation of cryptic biosynthetic pathway (Dwibedi & Saxena, 2022). Recent research has found striking parallels between the defense mechanisms induced by general elicitors and animal innate immunity, leading to the enticing hypothesis that identification of general elicitors leads to plant innate immunity (Nürnberger *et al.*, 2004). Elicitors operate as signal substances at low concentrations, delivering information to the plant to initiate defense, as opposed to toxins, which may act only at greater concentrations and/or harm the plant without active plant metabolism (Montesano *et al.*, 2003; Zhao *et al.*, 2005; Kong *et al.*, 2019).

Histone acetyltransferases (HATs) and histone deacetylases are two enzyme groups that regulate histone acetylation (HDACs) (Kruhlak *et al.*, 2001, recent references missing). Higher-order chromatin structure is associated with transcriptional gene suppression, and the latter encourages it (Li & Reinberg, 2011). HATs and HDACs aren't just interested in histone proteins

(Garber, 2007). Non-histone proteins like as p53, signal transducer and activator of transcription (STAT), transcription factor E2F, and others may be directly acetylated by HDACs (Spange *et al.*, 2009). As a result, HDACs don't just play an epigenetic role. DNA methylation and histone modifications are, nonetheless, strongly linked (Cedar & Bergman, 2009). Specific methyl CpG binding proteins (MBPs), which operate as adapters between methylation DNA and chromatin modifying factors, may quickly recognize methylated CpG sites in gene promoter regions (Ripperger & Merrow, 2011). MBPs can enlist the help of co-repressors like HDAC, methyltransferase, and chromatin remodeling factors to form a protein complex that controls gene expression (Kardooni, 2015). When the promoter region of a gene is methylated, transcription factors have a hard time recognizing it, and the gene is suppressed (Chatterjee & Vinson, 2012).

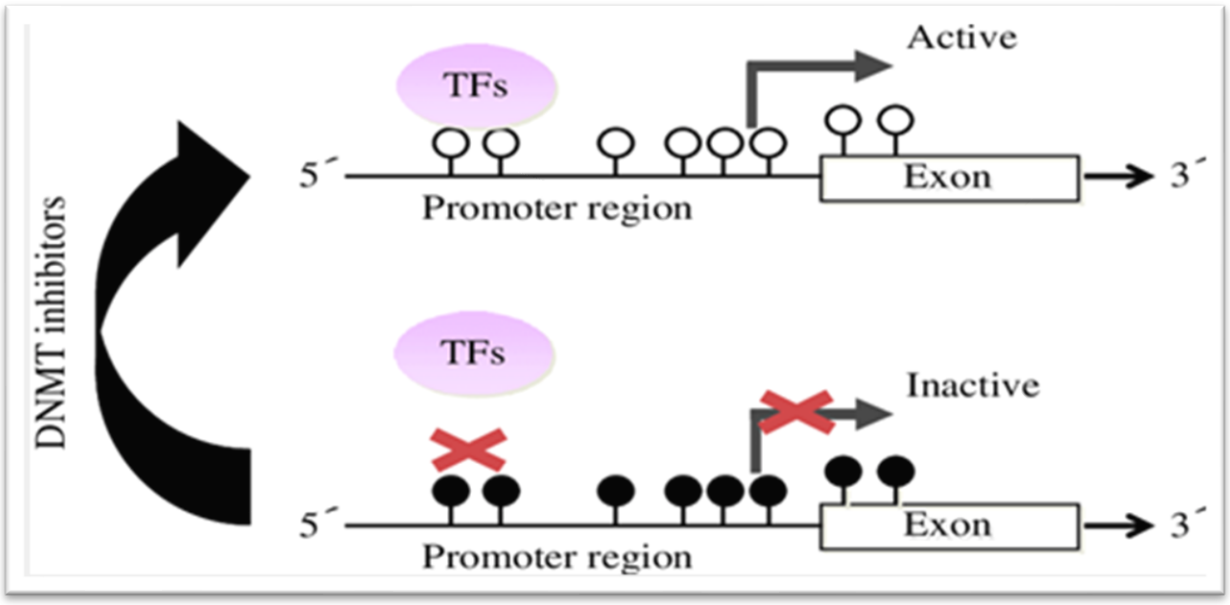

Figure 2.5: Types of epigenetic modulations: DNA Methylation (Kondo *et al.*, 2008)

The endophytic fungi *Xylaria psidii* was isolated from *Vitis vinifera* plant and with HPLC it was studied that enhanced production can be done by treating it with 5µM SAHA (52.32 µg/mL) and 10 µM AZA (48.94 µg/mL) followed by 10 µM SAHA and 5µM AZA (Dwibedi & Saxena, 2019). The two different types of elicitors: HDAC inhibitors (SAHA) and dMNTs inhibitors (AZA) brought a significant increase in the production amount of resveratrol (Dwibedi & Saxena, 2019). There was also an increase in the anti-oxidant property as compared to the wild strain. Both DPPH and

TEAC test showed the significant increase of anti-oxidant property (Dwibedi & Saxena, 2019). The best results were displayed by 5µM SAHA and 10 µM AZA (Dwibedi & Saxena, 2019). This directed the study on to the path that the elicitors might bring a noticeable amount of change in the production.

CHAPTER 3

AIM OF THE STUDY

3. Aim of the study

The aim of the present study is:

1. To optimize the concentration of elicitor AZA at which maximum resveratrol production is achieved qualitatively and quantitatively
2. To study the effect of different concentrations of AZA on antioxidant potential of *Arcoplius aureus* by estimation of total phenolic and total flavonoid content

CHAPTER 4

MATERIALS AND METHODS

4. Materials and Methods

Procurement of #12VVLPM (*Arcopilus aureus*) was done from the pre-existing repository maintained by Dr. Sanjai Saxena, Professor, Thapar Institute of Engineering and Technology, Patiala.

4.1 Preparation of PDA (Potato Dextrose Agar) plates

To make PDA media of 1000 mL, 200 grams potato infusion was filter through 8 folds of muslin cloth. The pH was maintained at 5.36 followed by addition of1.5% dextrose and agar (Mohinudeen *et al.*, 2022). The media was autoclaved at 121°C at 15 psi. The autoclaved media was dispensed into sterile Petri Dishes (dimensions 90×15 mm) in the Laminar air flow hoof (LAF) aseptically.

4.2 Revival of culture

#12VVLPM cultures previously isolated from *Vitis vinifera* was revived on the PDA media. After every 8 days, subculturing was done to maintain the culture. The isolated cultures were sub cultured by point inoculating the PDA plates in the center and incubating for 8-9 days at 26°C 2°C. After every 24 hours of incubation, morphological characteristics such as colony color, texture, size, and pigmentation were recorded.

4.3 Classical Morpho-taxonomy

The isolated culture of *Arcopilus aureus* (#12VVLPM) was grown over different media viz Water Agar, Potato Dextrose Agar, Corn Meal Agar to study the morphological features. Colony shape, diameter, pigment production etc. were studied. For this, a clean and alcohol wiped glass slide was taken. A water drop was placed over it. Mycelial mass was taken and placed on the water with the help of fine needle. It was followed by teasing of the culture with fine tip needle. A single drop of Lacto-phenol cotton blue stain was added followed by placing a cover slip of dimensions 18×10mm over it gently to avoid formation of air bubbles (Parthasarathy *et al.*, 2014). The slide was then observed under 10X, 40X and 100X using microscope (de Arruda *et al.*, 2013)

4.3.1 Corn Meal Agar

For100 ml of media, 1.7 g of Corn meal Agar was added to 100 ml distilled water. The media was autoclaved at 121°C at 15 psi. The autoclaved media was poured into sterile Petri Dishes (dimensions 90×15 mm) in the Laminar air flow hoof (LAF) aseptically.

4.3.2 Water Agar

To make Water Agar of 100 ml, 1.5g of Water Agar was dissolved in 100 ml of distilled water. The media was autoclaved at 121°C at 15 psi. The autoclaved media was poured into sterile Petri Dishes (dimensions 90×15 mm) in the Laminar air flow hoof (LAF) aseptically.

Further, #12VVLPM Determined by different factors such colony structure, color, pigment production, and culture diameter were investigated. A clear glass slide was taken and covered with a water drop enabling microscopic analysis. Mycelial mass was taken and deposited over the drop of water with the aid of a fine needle. A fine-tipped needle was used to correctly tease it, then Lacto-phenol cotton blue was used to stain it. It was covered with an 18×10 mm cover-slip to prevent the production of air bubbles. The slide was then mounted with DPX and examined using a binocular microscope at 10X, 40X, and 100X (Shukla *et al.*, 2022).

4.4 Preservation of culture

From the 8 days old culture, 5 mm diameter mycelial disc were transferred to the PDA slants containing 10% glycerol (v/v). They were kept in incubation until growth was observed and then were stored at 4ºC in the cold storage for further use.

4.5 Induction of epigenetic modulation using chemical elicitors

4.5.1 Screening

1 mg/mL stock solution of chemical elicitors AZA (5-azacytidine) was prepared and kept at -4°C in a falcon wrapped with aluminum foil. PDA plates of AZA's different concentrations 1µM, 3µM, 5µM, 10µM, 20µM, 30µM, 40µM, 50µM, 60µM, 70µM, 80µM, 90µM and 100µM were prepared (Dwibedi *et al.*, 2019). Point inoculation of an 8 days old culture was done and kept at 27°C in the incubator for growth. The diameters of the plates were noted after 10 days of incubation.

4.5.2 Production of culture

5mm mycelial disc of *Arcopilus aureus* from 8 days old culture were inoculated in the PDB supplementing with AZA's different concentrations 1µM, 3µM, 5µM, 10µM, 20µM, 30µM, 40µM, 50µM, 60µM, 70µM, 80µM, 90µM and 100µM (Dwibedi *et al.*, 2019). The flasks were kept on the rotatory shaker at 120 rpm at 27°C for 10 days to observe the growth.

4.5.3 Culture filtration and biomass calculation

The spent broth was filtered against blotting paper to get a cell free filtrate. To note the biomass, the filter paper having fungal spores dried overnight at 52±2°C in hot air oven was weighted. The pre-weight of empty filter paper and post-weight of filter paper with fungal spores after drying was noted to calculate dry biomass weight. The formula used was (Dwibedi *et al.*, 2019):

Dry biomass weight = Final weight of filter paper – Empty weight of filter paper

4.5.4 Liquid-Liquid extraction

The culture filtrate obtained for both wild strain and epigenetically modified strains were filtered through blotting paper and the cell free filtrate was collected. It was extracted with ethyl acetate (EA) in the 1:3 (filtrate: ethyl acetate) ratio (Dwibedi & Saxena, 2018). After repeating the process thrice, the organic layers were pooled. These layers were dried over anhydrous sodium sulfate. The obtained organic layer was evaporated in vacuum at 50°C to obtain bioactive fraction residue. The residue was then reconstituted in methanol at 1mg/mL concentration for TLC analysis and anti-oxidation test.

4.6 Quantification by TLC

Preparative thin layer chromatography was used to further fractionate the coarse fraction of the cultures (PTLC). TLC plates with a thickness of 0.5 mm were made by depositing silica gel (Sigma Aldrich; 381276) onto 20×15× 5 mm clean glass plates and activating them at 100 °C for 2 hours before use. The material was then accurately spotted onto an active TLC plate at 1 cm above the plate's edge using a capillary tube (Sigma Aldrich; Z114960). The TLC chamber was simultaneously submerged for 30 minutes in a number of different solvents. When the solvent front reached the appropriate level, the TLC plate with the sample was put inside the saturated TLC chamber as well as left to run for 30 minutes. Following that, the TLC plate was removed and allowed to air dry. By viewing the TLC plate under UV light, the chromatogram was created (Thermo Fisher Scientific; UVGL-58). For the analysis of retention factor (Rf) values, resveratrol (0.1 mg/mL) was considered as the reference (Mulaudzi *et al.*, 2021). The Rf value of each band was calculated as the ratio of the distance travelled by the solute to the distance travelled by the solvent (Ejaz *et al.*, 2020).

4.7 Bioactivity test of Epigenetic variants

4.7.1 Anti-oxidant test

With only small tweaks from the method described in the literature (Shi et al., 2012), the free radical scavenging activity of the culture filtrate of AZA epigenetic variants of #12VVLPM was assessed using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. In a nutshell, 230 l of DPPH (100 M in methanol) and 20 µl of the culture filtrate were combined well. The solution was let to sit at room temperature in the dark for half an hour. After incubation, the absorbance was assessed at 517 nm using a microtiter plate reader by BIOTEK® Power wave 340. Methanol was employed as the negative control and functional DPPH as the positive control. As a benchmark, resveratrol (10–50 g/ml) was used. The test was run in triplicate, and the results were presented as mean ± standard deviation. The percentage free radical scavenging activity of the fungal extract was calculated as:

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

4.7.2 Total Flavonoid test

Total flavonoid content was determined using procedure mentioned in the literature (Pękal & Pyrzynska, 2014). To the 300 µl of 1mg/ml of fungal extract 1200 µl of deionized water was added. 90 µl of NaNO3 (5%) was added. The reaction mixture was incubated at 27±2°C for 5 minutes. Following that, 90 µl of AlCl3 (10% w/v) was introduced, and 600 µl of 1N NaOH were added to the reaction mixture after 60 seconds of incubation. It was prepared up to 3 ml in volume. At 510 nm, the absorption was detected. As a benchmark, quercetin (50–250 µg/ml) was used. The concentration of the sample was further determined after a regression line was plotted between the concentrations of the sample. The test results were presented as mean± SD and were taken in triplicate.

4.7.3 Total phenolic test

Total phenolic content was determined using Folin-Ciocalteu's (FC) reagent and procedure mentioned in the literature (Kaur & Kapoor, 2002). Gallic acid (50 µg/ml - 250 µg/ml) was used as standard. To 200µl of 1mg/ml fungal extract,3.0 ml of deionized water and 200 µl of FC reagent was added. This was followed by incubation at room temperature (27±2°C) for 10 minutes. Then 400 µl of Na_2CO_3 (6%w/v) was added and the reaction mix was allowed to stand at room temperature (27±2°C) for 1 hour. At 760 nm, the absorbance was studied. The concentration of

the sample was then determined after a regression line was plotted between the concentrations of the sample. The test results were presented as mean± SD in triplicate.

CHAPTER 5

RESULTS

5. Results

5.1 Subculturing and Maintenance of fungal isolates

Endophytic culture of #12VVLPM was procured from the repository maintained by Dr. Sanjai Saxena, Professor, Department of Biotechnology at Thapar Institute of Engineering and Technology was inoculated on PDA plates and incubated at 27ºC ± 2ºC for 10 days. The isolate was sub-cultured every 10-12 days on fresh media to maintain it in active state.

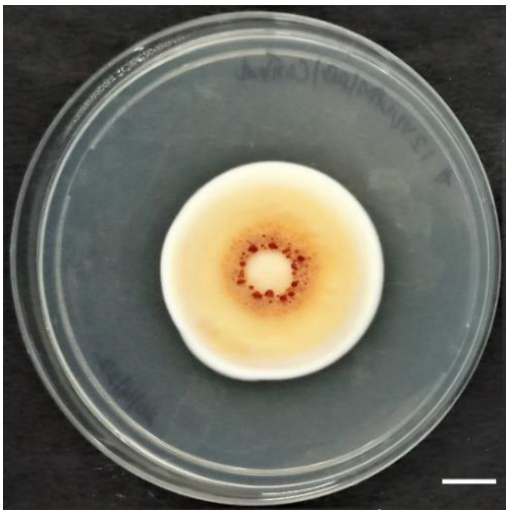

Figure 5.1: Pure culture of endophytic fungi #12VVLPM. (Bar 10 mm)

5.2 Preservation of culture isolates

After revival the isolate #12VVLPM was preserved on PDA slants and vials which were supplemented with 10% (w/v) glycerol. They were incubated at 26ºC ± 2ºC for the growth and stored at 4°C for maintenance of culture and use in the future.

Figure 5.2: Preserved #12VVLPM slants for maintenance of culture

5.3 Classical Morpho taxonomy of #12VVLPM

The culture of *Arcopilus aureus* was grown on different media viz. PDA, CMA and water agar (WA) (figure 5.3), out of which the growth was observed on PDA and CMA. The culture growth on water agar was not significant even after 20 days of inoculation. The colonies were found to be white velveteen with radial crevices and red wine pigmentation on PDA media while there was no pigmentation found on the CMA media. The colonies rapidly grew on PDA media appearing white due to aerial mycelium. The average diameter of 45 ± 2 mm was observed, floccose on different media after 10 days of incubation. Studying the microscopic characters, the fungus did produce ovate, reniform brown ascospores after 12 weeks with diameter of 9.0-10.0×12.0-13.4 µm (Figure 5.3 e). The hyphae were thick, septate and brown in color.

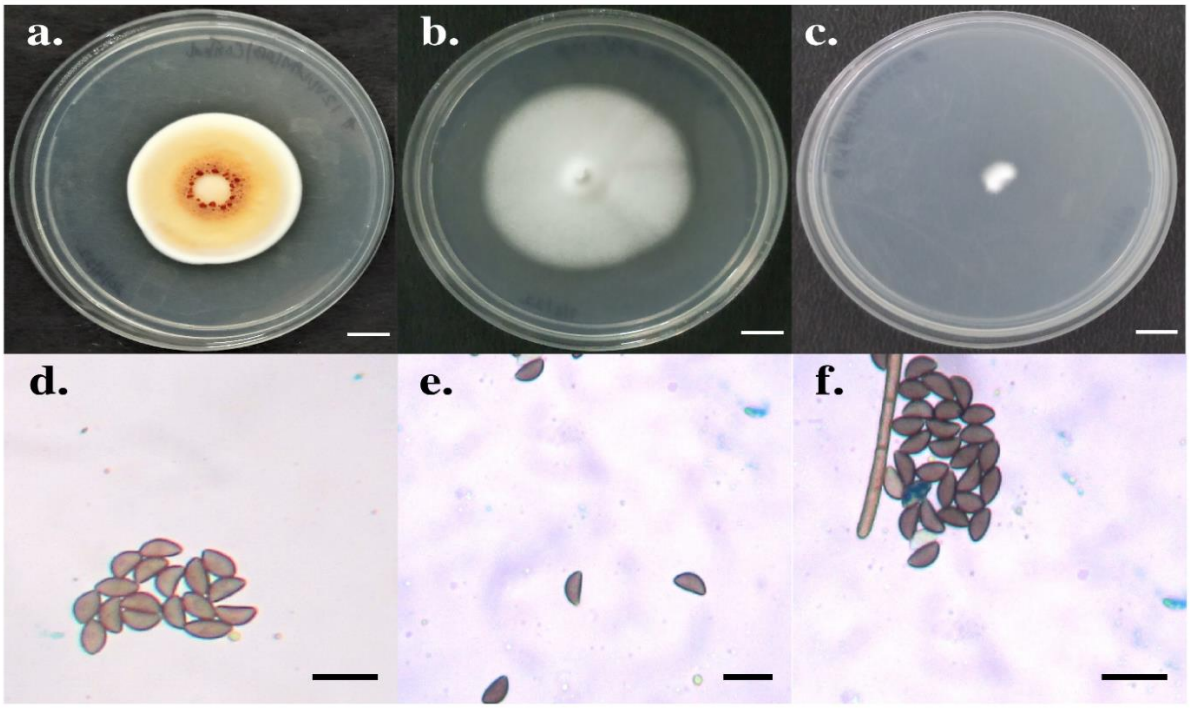

Figure 5.3: #12VVLPM grown on different media **a.** PDA medium; **b.** Corn Meal Agar medium; **c.** Water Agar (Bar 10 mm); Microscopic features of #12VVLPM: **d., e**. Lunate shaped ascospores on PDA; **f.** Brown septate hyphae and ovate ascospores in shape. (Bar 10 µm)

5.4 Induction of epigenetic modulation using chemical elicitors

5.4.1 Screening

The fungal isolate of *Arcopilus aureus* inoculated on the PDA plates with different concentration of AZA produced different colony diameters and morphological characteristics. The colony

diameter of AZA variants and the wild strain were recorded every day for 10 days. The colony diameter of wild strain of #12VVLPM was 4 cm on the 10th day. The fungal type that had been exposed to 10 µM AZA had the largest colony diameter, measuring 4.6 cm. on the 10th day (Figure 5.4). The colony diameter gradually increased from 1 µM till 10 µM whereas a gradual decrease till 60 µM was observed following which it was constant up to 100 µM (Figure 5.5).

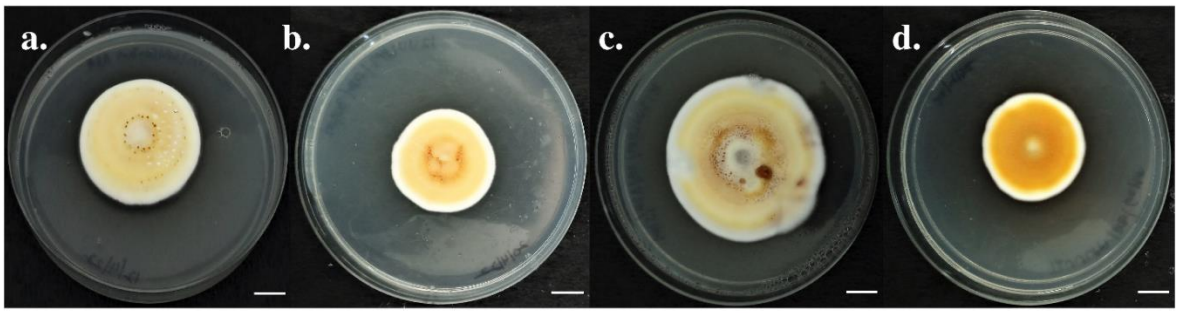

Figure 5.4: Epigenetic variants of #12VVLPM **a.** Control (#12VVLPM); **b.** AZA 3 µM; **c.** AZA 10 µM; **d.** AZA 90 µM. Bar, 10 µM

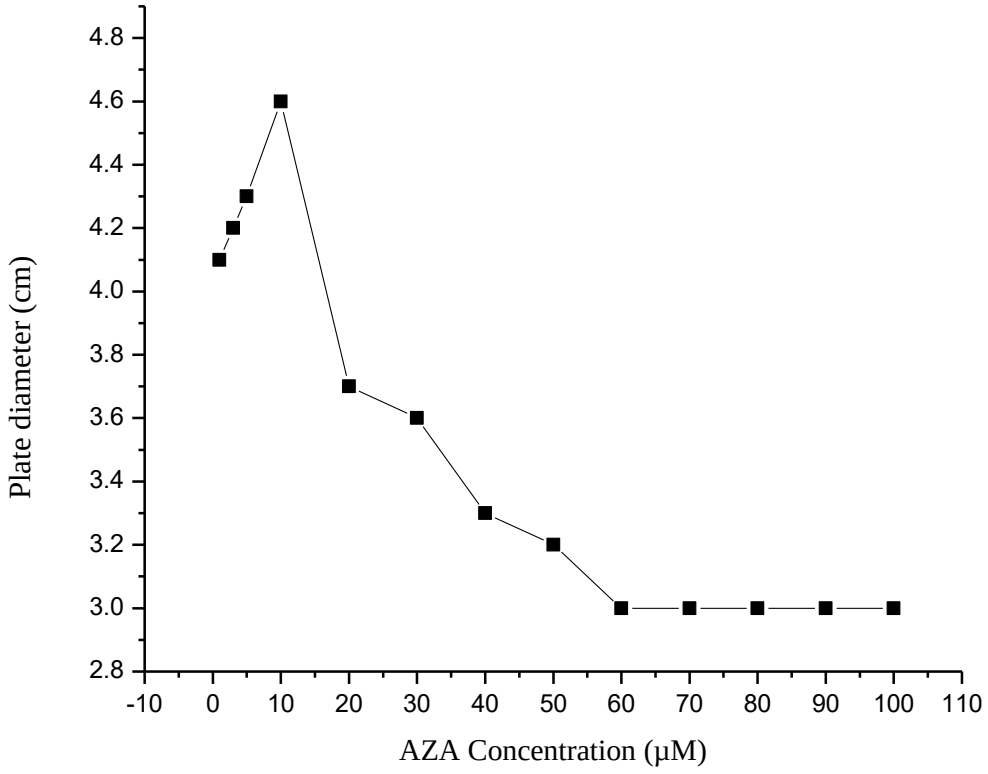

Figure 5.5: Diameter of #12VVLPM on different concentrations of AZA

5.4.2 Culture filtration and biomass calculation

The fungal isolates of *Arcopilus aureus* inoculated in PDB with different concentration of AZA produced different biomass weights and intensity of odor. Due to the synthesis of secondary metabolites, it was noticed that the volume of spent broth was decreased along with a notable change in color and distinctive odor of culture filtrates. After 8–10 days of incubation, the culture filtrates were filtered using Whatman filter paper 4. Additionally, the biomass of culture filtrates was noted. The wild strain's biomass was 2.656 ± 0.031 g. Cell free extract of a fungal variation treated with 10 µM AZA displayed the greatest biomass of 4.179 ± 0.158 g amongst AZA variants. The biomass grew from the concentration of 1 µM to 10 µM, then gradually decreased up to 100 µM. (Figure 5.6).

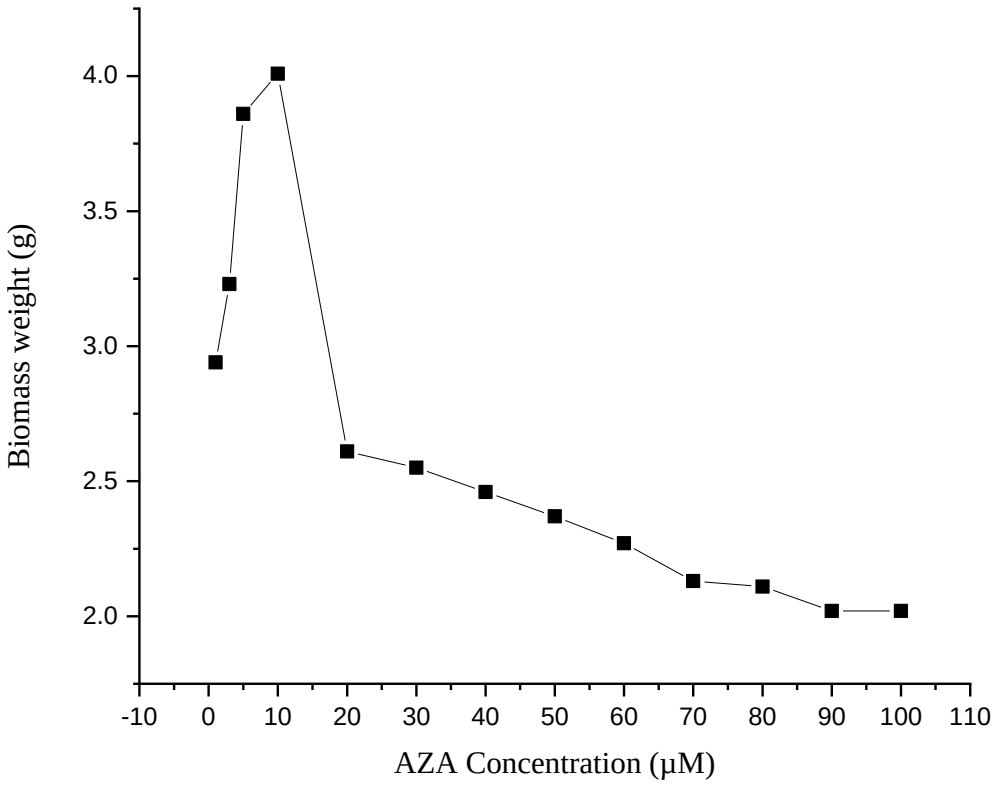

Figure 5.6: Biomass weight of AZA variants of #12VVLPM

The mean diameter and biomass of control and AZA treated #12VVLPM is shown in Table 5.1

Table 5.1: Diameter and Biomass of different concentrations of AZA of #12VVLPM

Sr. No.	Concentration (µM)	Diameter (cm)	Biomass (g)
1.	**10 µM**	**4.60±0.00^{a}**	**4.18±0.16^{a}**
2.	5 µM	4.30±0.00^{b}	3.91±0.07^{b}
3.	3 µM	4.20±0.00^{bc}	3.22±0.01^{c}
4.	1 µM	4.10±0.00^{cd}	2.91±0.03^{d}
5.	Control	4.00±0.00^{d}	2.66±0.03^{e}
6.	20 µM	3.76±0.15^{e}	2.61±0.01^{ef}
7.	30 µM	3.66±0.06^{e}	2.53±0.02^{efg}
8.	40 µM	3.30±0.00^{f}	2.43±0.03^{fgh}
9.	50 µM	3.20±0.00^{f}	2.34±0.03^{ghi}
10.	80 µM	3.03±0.06^{g}	2.12±0.04^{ij}
11.	90 µM	3.03±0.06^{g}	2.11±0.17^{j}
12.	70 µM	3.00±0.00^{g}	2.13±0.02^{ij}
13.	100 µM	3.00±0.00^{g}	2.02±0.02^{j}
14.	60 µM	3.00±0.00^{g}	2.23±0.03^{hij}

*The data represents mean ± SD; values with different superscript letters are different by Tukey's post-hoc test ($p<0.05$).

5.5 Culture production and solvent extraction

Culture isolate #12VVLPM was exposed to fermentation in Potato Dextrose Broth in order to produce a secondary metabolite. After incubation for nine days, the culture filtrates were filtered using Whatman filter paper 4. The extraction was carried out using ethyl acetate in the ratio 1:3. The organic layer were dried over anhydrous sodium sulfate and evaporated in vacuum at 50°C to obtain bioactive fraction residue. The yield obtained was 5.9 mg/ml (control #12VVLPM), 3.25 mg/ml (1 µM), 4.42 mg/ml (3 µM), 9.40 mg/ml (5 µM) and 10.42 mg/ml (10 µM), giving highest yield in 10 µM concentration.

5.6 Confirmation by TLC

To accomplish adequate separation, various solvent mixtures were applied to the crude ethyl acetate fraction of #12VVLPM. The best separation of #12VVLPM crude EA extract was observed in dichloromethane: chloroform in the proportion 1:1 which gave 5 different bands (Figure 5.7). The R_f values of Bands were Band 1 (0.88), Band 2 (0.63), Band 3 (0.37), Band 4 (0.29) and Band 5 (0.13). The standard Resveratrol gave the R_f value 0.65 which is very close to Band 2 R_f value. When under the UV light of 365 nm, the Bands appeared to be violet like that of standard resveratrol.

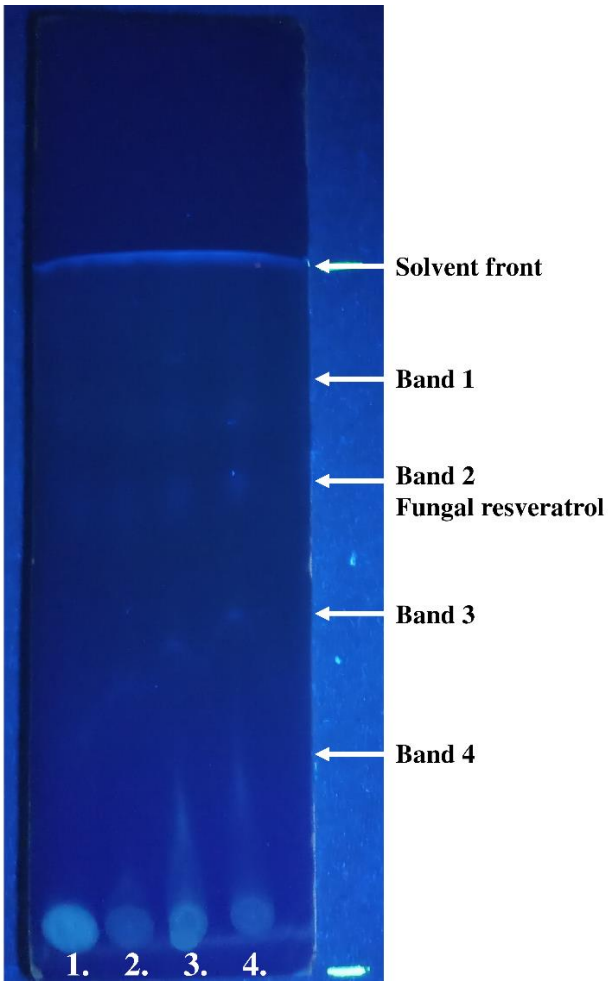

Figure 5.7: TLC profile of crude extract of #12VVLPM at different AZA concentrations using solvent system dichloromethane: chloroform in 1:1 ratio for mobile phase (Well 1: 1 µM, well 2: 3 µM, well 3: 5 µM and well 4: 10 µM).

5.7 Biological test

5.7.1 Anti-oxidant test

The culture filtrates of *Arcopilus aureus* were evaluated for their capacity of antioxidant activity using DPPH assay. The maximum free radical scavenging activity was seen in the cell free extract

of the 10 µM AZA fungal variant i.e., 50.311 ± 1.271%. A significant increase in free radical scavenging was observed as compared to wild strain whose free radical scavenging activity was 17.074 ± 2.415% (Figure 5.9). The increase in the FRS% is gradual from 1 µM (14.14 ± 1.114%), 3 µM (20.845 ± 0.918%), 5 µM (30.162 ± 1.374%) up to 10 µM (50.311± 1.271%) and then gradual decline until 100 µM. The DPPH assay exhibited the highest anti-oxidant property being displayed by the 10 µM AZA concentration.

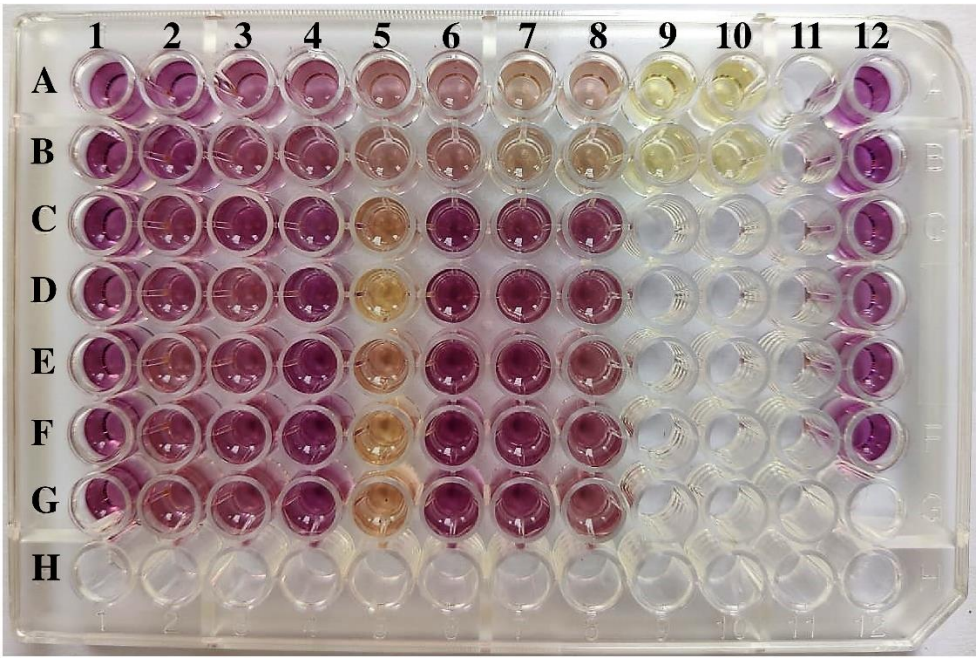

Figure 5.8: ELISA plate showing DPPH scavenging assay (A, B 1-10: Standard 10-50 µg/mL; C-G 1-8: Replicates of test concentrations; A-F 12: Positive control DPPH, A-F 11: Methanol)

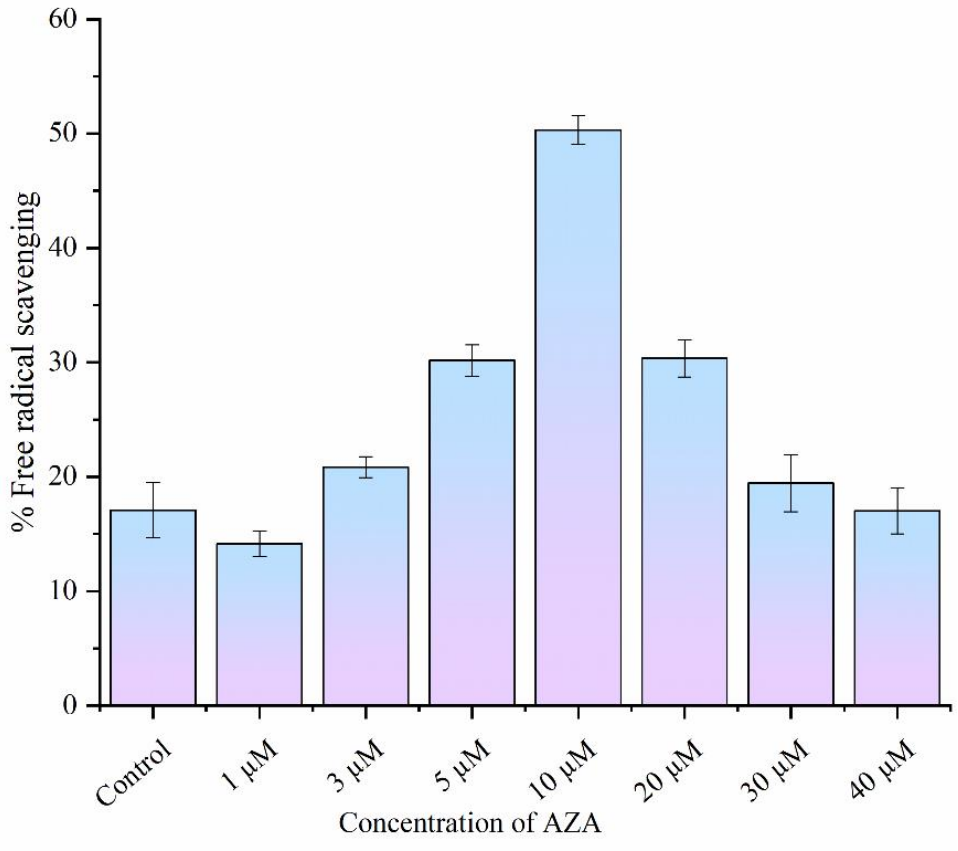

Figure 5.9: FRS% of different concentrations of AZA calculated after DPPH scavenging test

5.7.2 Total Flavonoid test

Total flavonoid content was calculated by taking Quercetin as standard. The concentration of flavonoid content in sample was calculated as Quercetin equivalent per mg of sample. The concentration was highest as observed in 10 µM AZA treated fungus extract was 448.416 ± 5.346 µg equivalent to Quercetin equivalent per mg of sample (Figure 5.10).

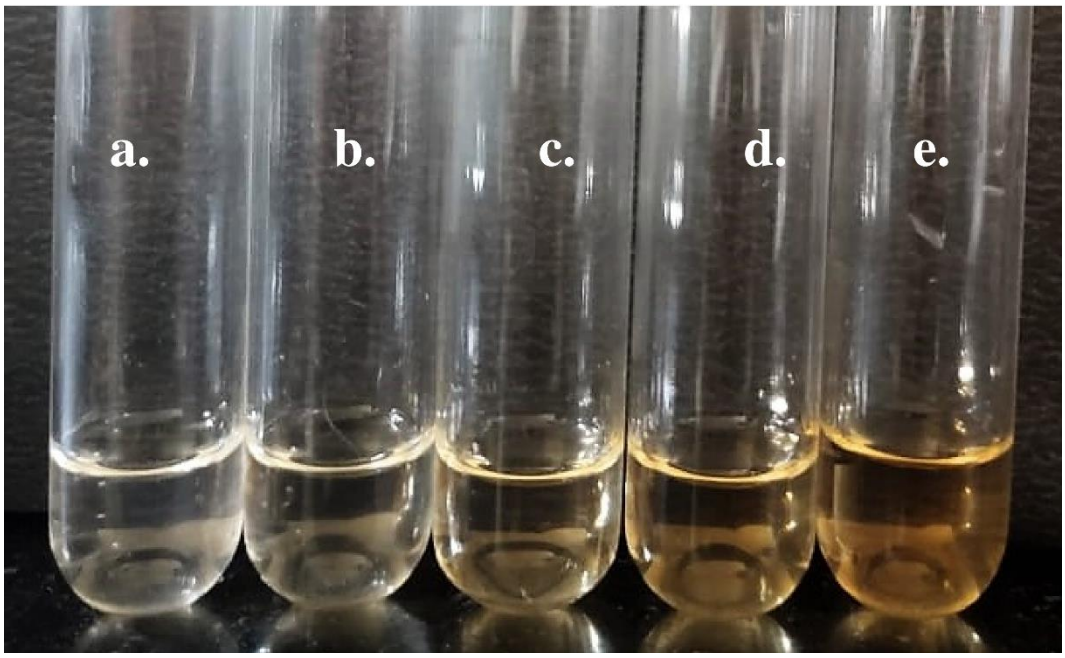

Figure 5.10: Total Flavonoid Content of different concentrations of AZA **a.** Control; **b.** 1 µM; **c.** 3 µM; **d.** 5 µM; **e.** 10 µM

5.7.3 Total Phenolic test

By using gallic acid as a reference point, the total phenolic content of the various concentrations was estimated. Gallic acid equivalent per mg of sample was used to calculate the amount of phenol in the sample. The maximum concentration was seen in fungal extract treated with AZA between 1 and 10 µM, and this was followed by a downward trend in concentration as AZA concentrations in fungus culture increased. The concentration of 10 µM AZA treated fungus extract was 217.84 ± 1.258 µg Gallic acid equivalent per mg of sample (Figure 5.11).

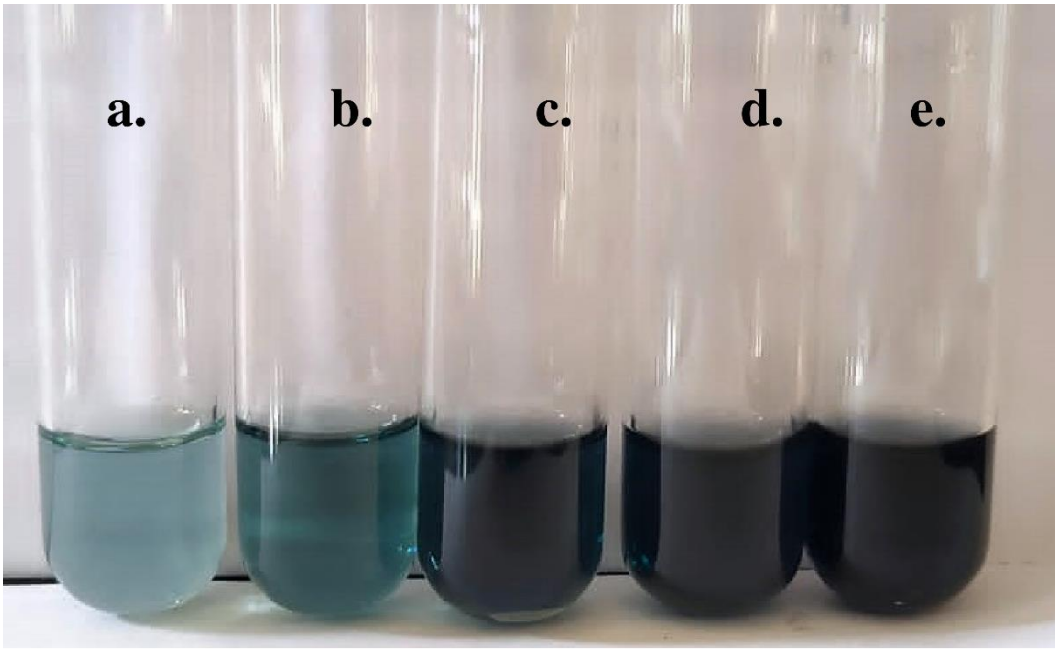

Figure 5.11: Total Phenolic Content of different concentrations of AZA **a.** Control; **b.** 1 µM; **c.** 3 µM; **d.** 5 µM; **e.** 10 µM

Table 5.2: TPC and TFC of different concentrations of AZA

Sr. No.	Concentration (µM)	TFC (µg equivalent to Quercetin/mg of fungal extract)	TPC (µg equivalent to gallic acid/mg of fungal extract)
1.	Control	140.63 ± 5.86 ^{c}	72.60 ± 0.28^{d}
2.	1 µM	194.96 ± 4.52^{b}	97.57 ± 0.44^{b}
3.	3 µM	178.50 ± 5.31^{b}	82.36 ± 0.30^{c}
4.	5 µM	169.00 ± 3.69^{b}	82.47 ± 0.36^{c}
5.	**10 µM**	**448.42 ± 5.35^{a}**	**217.84 ± 1.26^{a}**

*The data represents mean ± SD; values with different superscript letters are different by Tukey's post-hoc test ($p<0.05$).

CHAPTER 6

DISCUSSION

6. Discussion

Resveratrol is a natural polyphenolic stilbene that was first recognized as the major active compounds of the French paradox potent antioxidant potential. It is now known to have a wide range of applications in treating diseases like cardiovascular disease and neurodegenerative disorders (Jayaprakash *et al.*, 2020, Vibhute *et al.*, 2022). These factors have led to the widespread usage of resveratrol in nutraceuticals and cosmetics today.

The fungi that proliferate within plant tissue without causing any apparent harm to the host are known as endophytic fungus. Additionally, after colonizing a host plant, endophytes create a variety of secondary metabolites that can protect the host plant from stress situations, and the host plant in turn provides food and a habitat for endophytic fungi (Ogbe *et al.*, 2020). As a result, endophytes not only interact with their hosts but also exhibit characteristics because of close co-evolution, thereby producing analogous bioactive chemicals, some of which have medical significance (Olanrewaju & Babalola, 2019). It is now known that microorganisms have cryptic genes (also known as silent genes), some of which can be expressed when exposed to epigenetic modifiers like AZA (a chemical elicitor), which can modulate the production of secondary metabolites (Dwibedi *et al.*, 2019). Exploration of this intrinsic feature is crucial for the pharmaceutical, agrochemical, and cosmetic industries.

Through the use of various chemical elicitor that modulate the production of secondary metabolites, a comparative study was conducted to increase the production of resveratrol. Although *Xylaria sp*. had previously been identified from *Vitis vinifera*, *Xylaria sp*. #22(P) VVLPM is a member of the Xylariales family (Dwibedi *et al.*, 2019). The identification and quantification of resveratrol was confirmed by HPLC analysis. It was discovered that the concentration of resveratrol was increased and at its highest when treated with 5 µM SAHA and 10 µM AZA, followed by 10 µM SAHA and 5 µM AZA (Izquierdo-Torres *et al.*, 2019), whereas in this study the concentration of resveratrol was increased in the *Arcopilus aureus* by the addition of 10 µM AZA, the increase was observed to be gradual up to 10 µM starting from 1 µM and subsequently decreased after 10 µM till 100 µM. Previous studies on the endophytic fungus *Colletotrichum gloeosporioides* have noted an increase in cryptic chemicals and their antibacterial activity when employing epigenetic modifiers like grape extract and turmeric extract (Nishad *et al.*, 2019).

Epigenetic modifiers, like DMNTs (AZA) inhibitors, are intriguing pharmacological tools for expressing cryptic or quiet genes that are not expressed under typical laboratory conditions (Pinedo-Rivilla *et al.*, 2022). Earlier *Hypoxylon* sp. (CI-4), an endophytic fungus that produces myco diesel and a variety of volatile organic compounds, including cyclohexane, 1,2,4-tris(methylene) and 1,8-cineole, 1-methyl-1,4-cyclohexadiene (Chowdhary & Kaushik, 2019) was evaluated. This fungus was chosen as a candidate for the modulation of volatile organic compound (VOC) production after a successful attempt at epigenetic modulation was made using 5-azacytidine. In a co-culturing experiment with the endophytic fungus *Camporesia sambuci* (FT1061) and *Epicoccum sorghinum* (FT1062). Chunshun *et al.* (2017) also identified four other known compounds as well as a new N-methoxypyridone analogue. The results proclaim that fungi possess a large number of genes that are inactive under typical laboratory conditions and that these microbes require epigenetic modifiers to activate these genes and increase the production of novel secondary metabolites (Tomm *et al.*, 2019).

For confirmation of various bioactive compounds techniques such as thin layer chromatography is frequently employed for the separation and identification of chemicals in a mixture. According to our research, the standard (plant-derived) resveratrol had R_f value of 0.650 and our fungus-derived resveratrol produced by #12VVLPM at 10 µM AZA concentration had R_f value 0.625 which are quite close to that of standard resveratrol, therefore confirming the presence of resveratrol in the AZA treated #12VVLPM culture.

The antibacterial and antioxidant activity of endophytic fungus is well known. In our investigation, the endophytic fungus #12VVLPM displayed significant antioxidant activity. A considerable increase in antioxidant potential was attained following treatment with various dosages of chemical elicitor AZA. Increased scavenging potential was observed for DPPH when compared to the wild strain. When compared to the wild strain, treatment with 10 µM AZA demonstrated substantial antioxidant capacity among all epigenetic variations. In their investigation, Khanduja and Bhardwaj discovered that resveratrol had greater antioxidant capacity than catechin, myricetin, and fisetin (Tavsan, & Kayali, 2019). Due to the presence of resveratrol in grapes and jamun, Shrikanta *et al.* also discovered antioxidant properties. When compared to #12VVLPM control, a rise in TPC and TFC was seen in fungal treated samples with 10 µM AZA.

CHAPTER 7

CONCLUSION

7. Conclusion

The endophytic fungus *Arcopilus aureus* (#12VVLPM) was treated with different concentrations of the AZA epigenetic modifier, as a result 10 µM AZA was the most effective concentration for inducing the cryptic gene and increasing the production of resveratrol as well as antioxidant potential. According to the current study, resveratrol content and antioxidant potential are positively correlated. At 10 µM AZA concentration maximum FRS, TPC and TFC content was observed, making it a potent candidate for the medicinal use in the pharmaceutical sector. #12VVLPM is therefore a potent candidate for strain improvement and further study for industrial scale production is warranted.

CHAPTER 8

References

8. References:

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Analyzed document	ankita_for plagarism.pdf (D141503233)
Submitted	6/30/2022 12:31:00 PM
Submitted by	Shreya Gupta
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J	**Effect of precursor feeding, dietary supplementation, chemical elicitors and co-culturing on resveratrol production by Arcopilus aureus** URL: 5a37ba7c-f09e-4066-9fb2-069571b37182 Fetched: 5/3/2022 12:16:27 PM	6
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in Master of Science in Biotechnology from the Thapar Institute of Engineering and Technology in Patiala, Punjab, is a genuine work that was created with the guidance and inspiration of Dr. Sanjai Saxena.
iii | P a g e DECLARATION By submitting this statement, I affirm that the work described in the thesis entitled "Effect Of Azacytidine on Resveratrol Production by Arcopilus aureus"
I have not submitted the matter embodied in this thesis for the award of any other degree. Ankita Roll no-302001002 Place – Patiala Date – This is to verify that, to the best of my knowledge, the candidate's above statement is accurate and true.
iv | P a g e ACKNOWLEDGEMENT This project needed a lot of direction and help from many people in order to succeed and produce the desired results. I would like to take this opportunity to express my sincere gratitude to Dr. Sanjai Saxena, Professor of the Department of Biotechnology at the Thapar Institute of Engineering and Technology in Patiala, Punjab, for his honorable project guidance, insightful suggestions, insightful comments, gracious behaviour, and friendly assistance throughout the course of the