Screening and isolation of α-amylase inhibitors from endophytic fungi for their possible use in management of type 2 diabetes

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Of

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In

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Submitted By

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DECLARATION

I hereby declared that the work being presented in thesis entitled "**Screening and isolation of α-amylase inhibitors from endophytic fungi for their possible use in management of type 2 diabetes "** in the partial fulfilment of the requirements for the award of degree of Master of Science, Department of Biotechnology, Thapar Uuniversity , Patiala is my own laboratory work done during the period of January 2017 to July 2017 under the esteemed supervision and conception of **Dr. Sanjai Saxena** , Professor, Department of Biotechnology , Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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Date: 14th July'17

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

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CERTIFICATE

This is to certify that the thesis **entitled "Screening and isolation of α-amylase inhibitors from endophytic fungi for their possible use in management of type 2 diabetes"** being submitted by **Ms. Avneet Dhunna** (Roll No-301501001) in the partial fulfilment of the requirements for the award of the degree of master of science in Biotechnology , Thapar university, Patiala, is bonafide work carried out under the esteemed supervision and conception of **Dr. Sanjai Saxena** and the no part of this thesis has been submitted for the any other award of degree.

14/7/17

Dr. Sanjai Saxena

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ABBREVATIONS

S.No.	Abbreviation	Full form
1.	µg	Micro gram
2.	µl	Micro litre
3.	µM	Micro molar
4.	DNA	Deoxyribonucleic acid
5.	dNTP	Deoxynucleotide triphosphate
6.	DPPH	1,1-diphenyl-2-picrylhydrazyl
7.	EDTA	Ethylene diamine tetra acetic acid
8.	Et Br	Ethidium bromide
9.	HPLC	High performance liquid chromatography
10.	ITS	Internal transcribed spacer
11.	L	Litre
12.	MEA	Malt extract agar
13.	mg	Milli gram
14.	MHA	Muller hinton agar
15.	MHB	Muller hinton broth
16.	ml	Millilitre
17.	ng	Nano gram
18.	PCR	Polymerase chain reaction
19.	PDA	Potato dextrose agar
20.	PDB	Potato dextrose broth
21.	pH	Potential of hydrogen
22.	PLA	Pine leaf agar
23.	rpm	Revolutions per minute
24.	RT	Room temperature
25.	SNA	Synthetic nutrient deficient agar
26.	TAE	Tris acetate EDTA
27.	UV	Ultra violet
28.	WA	Water agar

EXECUTIVE SUMMARY

Diabetes mellitus is a chronic, metabolic disorder which is caused either due to insulin deficiency or decrease in insulin in the circulation. Diabetes is characterized by hyperglycaemic condition i.e increase blood sugar levels. In today's scenario, diabetes has said to affect about 382 million people globally and is responsible for over 5.1 million deaths. According to WHO report, diabetes have high economic burden globally. In 2014, the global economic burden was estimated to be US$612 billion.

Endophytic fungi are symbionants residing within the plant. The endophytic fungi are reservoirs of large number of bioactive compounds such as antimicrobial agents, anticancer agents etc. The present study aimed at exploring endophytic fungi isolated from *Aegle marmels, Cinnamomum malabaricum, Cinnamum zeylanicum* and *Vitis vinifera* for α-amylase inhibitors. α-amylase inhibitors are compounds which acts on the α-amylase -enzyme responsible for breakdown of carbohydrates inside the body for release of glucose. Thus, these inhibitors are responsible for maintain blood glucose level.

The culture filtrates of 105 isolates were obtained and screened for their potential activity against α-amylase inhibitors. The preliminary assay was agar disc diffusion served as both qualitative as well as quantitative method of screening in which 5 isolates showed positive result i.e. #19VVLPM, #13(P)VVLPM, #22(P)VVLPM, #19(P)VVLPM and #107VVLSWN. On basis of percentage of inhibition, isolate culture code #107VVLSWN was selected and subjected to mass production. The compound was extracted by different solvents by liquid –liquid solvent extraction method. The extracted compound was then subjected to qualitative screening by DNSA method. On qualitative screening it was found that DCM fraction exhibited maximum α-amylase inhibitor activity. The different extracted fraction was also subjected for the study of antimicrobial and antioxidant assay. The antimicrobial studies showed that maximum antimicrobial activity was seen in chloroform extract against *E.co*li. The TLC was also done for the DCM fraction of the isolate #107VVLSWN and 6 bands where observed in the solvent system Hexane: Toluene: Formic acid in ratio of 3:2:.01.

On performing morphological studies of the isolate, it was tentatively identified as *Alternaria* sp. The genomic DNA of the selected isolate was extracted, amplified and further send for sequencing for molecular identification.

Chapter – 1

INTRODUCTION

INTRODUCTION

Diabetes mellitus is the most common endocrine, chronic disorder which is caused either due to deficient insulin production (type 1 DM) or combined resistance to insulin action and the insulin secretary response (type 2 DM). The type 2 diabetes has more prevalence in the population than Type 1 diabetes (Esser et al., 2014). Diabetes is characteristically marked by Hyperglycaemia i.e. raised blood sugar level (Asmat et al., 2016). Other symptoms include excessive excretion of urine (polyuria) and thirst (polydipsia), constant hunger (polyphagia), weight loss, vision changes and fatigue. Prolonged hyperglycemia if not monitored properly can lead to complications such as atherosclerosis, cardiac dysfunction, retinopathy, neuropathy and nephropathy due to increased generation of reactive oxygen species (ROS) and impairment of endogenous antioxidants (Oboh et al., 2016)

The statistics have shown that global prevalence of diabetes in 2015 was 8.5%, affecting 422 million adults which are estimated to increase to 55% by 2035, reaching 592 million adults between 20 and 79 years of age (WHO report, 2016).

For treatment of type 1 diabetes, only therapy that is clinically accepted is substitution of insulin. But there are many approaches to treat Type 2 diabetes such as reduction of the demand for insulin, stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues and inhibition of degradation of oligo- and disaccharides. One such practical approach used nowadays is to decrease the postprandial glucose levels by inhibition of carbohydrate- hydrolyzing enzymes such as α-amylase and α-glucosidase leading to reduction in the absorption of glucose as these enzymes are responsible for breakdown of oligosaccharides and disaccharides into monosaccharides which are easily absorbed(Sales et al.,2012). Thus inhibitors of these enzymes can delay carbohydrate digestion by prolonging overall carbohydrate digestion time, reducing rate of glucose absorption, thereby reducing the postpradinal blood glucose level. Commercially available inhibitors for diabetes control are Acarbose, Migitol, Voglibose, Nojirimycin and 1-deoxynojirimycin.

However,the are many side effects associated with these drugs, most common being bloating, abdominal discomfort,diarrhea and flatulence. (Oboh et al., 2016). In some cases as irritable bowel syndrome or severe kidney or liver dysfunction α-glucosidase are contraindicate (Sales et al., 2012). Therefore, there is need to explore alternatives for these inhibitors. Plants like Vitis

vinifera have been reported to posses potent α-amylase inhibitory activity in their extracts. However resourcing large amount of plant material for isolation of drugs becomes a formidable task apart from having ecological constraints. Therefore an alternative approach is to use endophytic fungi as endophytes have been recognised as microbial factories for production of plant metabolites (Firáková et al., 2007).

Endophytes are organisms which live within the plant tissue for all or part of life cycle and causing no apparent infection (Chandrasekar et al., 2013). Endophytic fungi have ability to produce bioactive secondary metabolites which are industrially, pharmaceutically and agriculturally important. Hence exploring endophytic fungi for screening inhibitors of α-amylase appears as a possible alternative.

The present study is based on the hypothesis that endophytic fungi colonizes plants and produce certain enzymes which are important for plant metabolism as well as for the survival of endophytes. Endophytic fungi are thought to produce α-amylase inhibitor so that they act on amylase and prevent hydrolysis of carbohydrates as hydrolysis causes increase in free glucose inside the cell, leading to disbalance in osmolarity. So to maintain the osmotic pressure α-amylase inhibitory activity has to be there.

Chapter – 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1. DIABETES MELLITUS

Diabetes mellitus (DM) is one of the most ancient diseases known in human civilization with first report registered in Egyptian manuscript which is about 3000 years old (Ahmed et al.,2002).

Diabetes is a chronic disorder which either occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces .Diabetes is an endocrine related metabolic disorder characterized by Hyperglycaemic condition i.e. elevated blood sugar level. Diabetes is usually characterized by excessive urination, thirst, constant hunger, weight loss, vision changes and fatigue. The uncontrolled diabetes can lead to serious damage to the heart, blood vessels, kidneys and nerves.

Type 1 diabetes, Type 2 diabetes and gestational diabetes are 3 categories of diabetes that are known.

Type 1 Diabetes is also known as insulin dependent diabetes or juvenile diabetes or childhood – onset diabetes is usually diagnosed in children and adolescents. Type 1 diabetes is an autoimmune disorder which caused by destruction of pancreatic beta cells. It is characterized by deficient insulin production in the body.

Type 2 Diabetes is also known as non insulin dependent diabetes or adult onset diabetes. Diabetes 2 is caused due to resistance to insulin or reduced insulin secretion (DeFranzo, 1988). Genetic and metabolic factors together contribute as major factors of risk of type 2 diabetes.

2.1.1. MOLECULAR MECHANISM OF INSULIN SECRETION IN NORMAL CELL

In normal cells secretion of insulin in response to glucose is a complex process. This process can be divided into following steps:

a. Transport of glucose and its oxidation
b. electrophysiological changes
c. fusion of insulin-containing secretory granules with the beta-cell plasma membrane

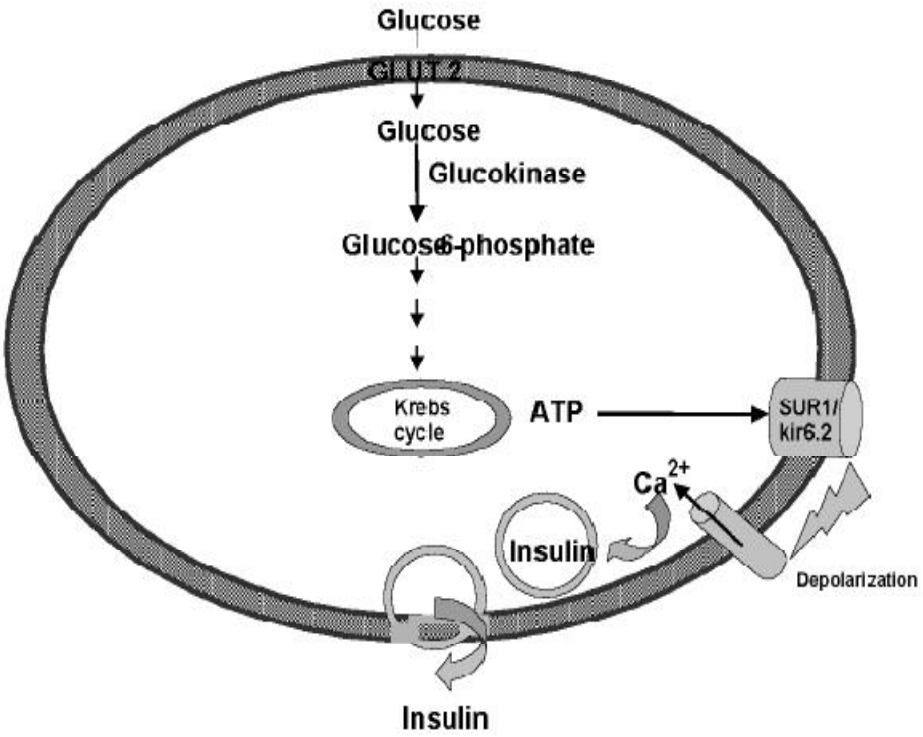


Figure 2.1: Basic mechanism of insulin secretion (Mejia, 2006)

Glucose enters the cell by GLUT 2 (glucose transport proteins) through facilitated diffusion (Joost et al., 2002) Inside the cell glucose is converted to glucose-6-phosphate by enzyme referred to as glucokinase(also known as glucosensor). Glucose oxidation leads to formation of ATP molecule which causes closure of ATP-sensitive K^+ channel viz. Hetro-octomer consisting of 4 subunits of sulphonylurea 1 receptor (SUR 1) and four subunits of the kir6.2. (Ashcroft *et al*., 1994). The closing of the channel causes depolarization of the plasma membrane and inward movement of the extracellular calcium causing fusion of secretory insulin granules, which increase in the insulin in the circulation (Rorsman *et al.*, 2003).

2.1.2.METABOLISM IN TYPE-2 DIABETES

Characteristic feature of type 2 diabetes impaired insulin action or abnormal insulin secretion. The earliest abnormality related to it is inability exert biological effect of insulin in circulation leading to insulin resistance. Insulin resistance further leads to complications such as dyslipidemia, hypertension and glucose intolerance.

Insulin resistance leads to rapid decline uptake of glucose by peripheral tissue (Cline et al., 1999; Bogardus et al., 1984). Insulin resistance also effects different metabolism inside the cell such

as impaired glycogen synthase and glucose breakdown, resistance to antilipolytic action. These factors together contribute to increase risk of various diseases inside the cell.

2.1.3. COMPLICATION RELATED TO DIABETES

Diabetes if left unmanaged can lead to series of complications which can be at times be life threatening. Abnormally high blood glucose can have a life threatening impact if it triggers diabetic ketoacidosis(DKA) and hyperosmolar coma in type 2 diabetes. Over time diabetes can damage the heart, blood vessels, eyes, kidneys and nerves, and increase the risk of heart disease and stroke. Such damage can result in reduced blood flow, which – combined with nerve damage (neuropathy) in the feet – increases the chance of foot ulcers, infection and the eventual need for limb amputation. Diabetic retinopathy is an important cause of blindness and occurs as a result of long term accumulated damage to the small blood vessels in the retina. Diabetes is among the leading causes of kidney failure (Pasquier, 2011).

2.1.4. ECONOMIC IMPACT OF DIABETES

Large economic burden is been imposed by Diabetes. The economic burden of diabetes is measured in 3 parameters i.e. direct medical cost, indirect costs associated with productivity loss, premature mortality and negative impact of diabetes on nation's GDP.

Figure 2.3: Various components leading to economic burden (WHO report, 2016)

In 2013 , the American Diabetes Association released a report in which it was estimated that the total cost of diabetes diagnosed have increased by 41% in the period of five years from $174 billion in 2007 to $245 billion in 2012. In 2014, the global economic burden estimated to be US$612 billion (WHO report, 2016).

2.1.5. GLOBAL BURDEN OF DIABETES

In 2012, there were 1.5 million deaths worldwide directly caused by diabetes. Diabetes has been identified as eighth leading cause of death among both sexes and the fifth leading cause of death in women in 2012. The total burden of deaths from high blood glucose in 2012 has been estimated to amount to 3.7 million. This number includes 1.5 million diabetes deaths, and an additional 2.2 million deaths from cardiovascular diseases, chronic kidney disease, and tuberculosis related to higher-than-optimal blood glucose. The largest number of deaths resulting from high blood glucose occur in upper-middle income countries (1.5 million) and the lowest number in low-income countries (0.3 million). (WHO report, 2016)

After the age of 50, middle-income countries have the highest proportion of deaths attributed to high blood glucose, for both men and women. Except in high-income countries, the proportion of deaths attributable diabetes to high blood glucose for both men and women are highest in the age group 60–69 years (Singh et al., 2013; Danaei et al., 2006)

Forty-three per cent of all deaths attributable to high blood glucose occur prematurely, before the age of 70 years – an estimated 1.6 million deaths worldwide. Globally, high blood glucose causes about 7% of deaths among men aged 20–69 and 8% among women aged 20–69.

According to WHO, Eastern Mediterranean, South-East Asia, and African Regions have highest rate of death due to diabetes. In the WHO European and South-East Asia Regions and the Region of the Americas, high blood glucose mortality rates are considerably higher for men than women.

2.1.6. PREVALENCE OF DIABETES AND ASSOCIATE RISK FACTORS

WHO estimates that, globally, 422 million adults aged over 18 years were living with diabetes in 2014 . The number of people with diabetes (defined in surveys as those having a fasting plasma glucose value of greater than or equal to 7.0 mmol/L or on medication for diabetes/raised blood glucose) has steadily risen over the past few decades, due to population growth, the increase in

the average age of the population, and the rise in prevalence of diabetes at each age. Worldwide, the number of people with diabetes has substantially increased between 1980 and 2014, rising from 108 million to current numbers that are around four times higher.

Forty per cent of this increase is estimated to result from population growth and ageing, 28% from a rise in age-specific prevalence, and 32% from the interaction of the two. In the past 3 decades the prevalence (age-standardized) of diabetes has risen substantially in countries at all income levels, mirroring the global increase in the number of people who are overweight or obese. The global prevalence of diabetes has grown from 4.7% in 1980 to 8.5% in 2014, during which time prevalence has increased or at best remained unchanged in every country. Over the past decade, diabetes prevalence has risen faster in low- and middle-income countries than in high-income countries (WHO report, 2016).

2.1.7. TREATMENT FOR DIABETES

Diabetes cannot be cured but can be managed. Management of diabetes can be done at various stages which include:

- diet and exercise
- oral hypoglycaemic therapy
- insulin therapy

Oral hypoglycaemic therapy and insulin therapy posses many side effects. Therefore, there is need to find alternative medication to treat diabetes (Pasquier et al., 2010)

2.2. α-AMYLASE INHIBITORS POTENTIAL SOURCE FOR TREATMENT OF DIABETES

α-amylase inhibitors are also referred as starch blockers because they prevent dietary starch from being absorbed in the body. The main attribute of alpha amylase inhibitor is to block α-amylase present in the body, which is responsible for breakdown of complex carbohydrates which are then easily absorbed in the body. The alpha amylase inhibitors have been used in treating health condition such as obesity, Type 1 Diabetes, Type 2 Diabetes.

α-amylase inhibitors have been classified into 3 categories based on composition. α-amylase inhibitors have been also classified on the basis of source of extraction.

2.2.1. . α-AMYLASE INHIBITOR ON BASIS COMPOSITION

α- amylase inhibitors on basis of composition are classified as non – proteinaceous inhibitors, proteinaceous inhibitor and bifunctional inhibitors.

2.2.1.1. NON – PROTEINACEOUS INHIBITORS

This category contains organic compounds such as acarbose, isoacarbose, acarviosine-glucose, hibiscus acid and the cyclodextrins. The two hibiscus acid forms, purified from Roselle tea (*Hibiscus sabdariffa*), the acarviosine-glucose, the isoacarbose and α-, β- and γ-cyclodextrins are highly active against porcine and human pancreatic α-amylase (PPA and HPA) (Kim et al., 1999). The inhibitory activity of these compounds is based on the competitive inhibition as their cyclic structure resembles α-amylase substrates and bind to catalytic structure

The cyclodextrin mechanism of PPA inhibition is pH-, temperature- and substrate-dependent. In case of amylase is substrate, the inhibition is of competitive type but in case of maltopentose as substrate the inhibition becomes noncompetitive (Koukiekoulo et al., 2001).

PPA inhibition by acarbose is by noncompetitive (Koukiekoulo et al., 2001). The valienamine ring of acarbose is considered to be crucial in the inhibition mechanism of α-glucosidases, α-amylases and other amylolytic enzymes (Wilox et al., 1984; Kadziola et al. 1998).

2.2.1.2. PROTEINACEOUS INHIBITORS

Proteinaceous α-amylase inhibitors are found in microorganisms, plants and animals (Ryan ,1990;Silano , 1987 ;Franco et al.,2000;Lulek at al.,2000). In plants, proteinaceous inhibitors are mainly present in cereals such as wheat *Triticum aestivum (*Silano , 1987 ;Franco et al.,2000;Lulek at al.,2000), barley *Hordeum vulgareum*(Abe et al.,1993), sorghum *Sorghum bicolour*(Bloch et al.,1991), rye *Secale cereal(*Lulek et al.,2000;Garcia-Casado et al.,1994*)* and rice *Oryza sativa* (Yamagata et al.,1998) but also in leguminosae such as pigeonpea *Cajanus cajan* (Giri et al.,1998), cowpea *Vigna unguiculata* (Melo et al.,1999) and -bean *P. Vulgaris (*Grossi de Sá et al. ,1997).

Table 2.1. Activity of amylase inhibitors from different plant sources against mammalian and insect α-amylases.

Inhibitors	Source	Inhibitory Activites		References
		Mammalian	Insect	
α-AI1	*P. vulgaris*	PPA	*Callosobruchus maculatus* *Callosobruchus chinensis* *Diabrotica virgifera virgifera* *Hypothenemus hampei* *Tenebrio molitor*	(Ishimoto et al.,1989; Titarenko et al., 2000; Nahoum et al.,2000;Feng et al. ,1996)
α-AI2	*P. vulgaris*	None activity	*Zabrotes subfasciatus*	(Grossi et al. ,1997; Silva et al. ,2001)
Wheat	*T. aestivum*	PPA and HSA	*Diabrotica virgifera virgifera*	(Franco et al.,2000; Zeng et al. ,2000)
0.19	*T. aestivum*	PPA and HSA	*Diabrotica virgifera virgifera* *Callosobruchus maculatus* *Zabrotes subfasciatus* *Acanthoscelides obtectus* *Tenebrio molitor* *Sitophilus oryzae* *Tribolium castaneum*	(Feng et al.,1996)
0.53	*T. aestivum*	HSA and PPA (low)	*Tenebrio molitor* *Callosobruchus maculatus* *Zabrotes subfasciatus* *Acanthoscelides obtectus*	(Maeda et al.,1983)
0.28	*T. aestivum*	PPA and HSA	*Tenebrio molitor*	(Maeda et al.,1983)
WRP25	*T. aestivum*	None	*Sitophilus oryzae* *Tribolium castaneum* *Tenebrio molitor* *Callosobruchus*	(Franco et al.,2000;Feng et al.,1996)

			maculatus Zabrotes subfasciatus	
WRP26	*T. aestivum*	None	*Tenebrio molitor Sitophilus oryzae Tribolium castaneum Callosobruchus maculatus*	(Franco et al.,2000;Feng et al.,1996)
WRP27	*T. aestivum*	None	*Tenebrio molitor* (low) *Sitophilus oryzae*	(Feng et al.,1996)
1,2 and 3	*S. cereale*	HSA	*Tenebrio molitor*	(Garcia-Casado et al.,1994)
BIII	*S. cereale*	HSA and PPA	*Zabrotes subfasciatus Acanthoscelides obtectus*	(Lulel et al., 2000)
AAI	*A. hypochondriacus*	None activity	*Tenebrio molitor Hypothenemus hampei Prostephanus truncatus*	(Chagolla-López et al., 1994; Pereira et al., 1999; Valência et al., 2000)
CAI	*V.unguiculata*	None	*Callosobruchus maculatus* (low)	(Melo et al,. 1999)
PAI	*C. cajan*	HSA and PPA	*Helicoverpa armigera* (low)	(Giri et al.,1989)
Zeamatin	*Z mays*	None activity	*Tribolium castaneum Sitophilus zeamais Rhyzoperta dominica*	(Blanco-Labra et al.,1980; Schimoler-O'Rourke et al.,2001)
SIα1, SIα2	*S. bicolor*	HSA (low)	*Locusta migratoria*	(Bloch et al., 1991)
and SIα3			*Periplaneta americana*	

α -amylase inhibitors have been also categorised on the basis of different sources i.e. plants, algae, bacteria and fungi.

2.2.2. DIFFERENT SOURCES OF ALPHA AMYLASE INHIBITORS

2.2.2.1. α- AMYLASE INHIBITORS FROM PLANTS

Plants mainly consist of proteinaceous inhibitors. There are six distinct types of alpha amylase inhibitors that are found in higher plants, based on their tertiary structure. Classes are as follow:

1. Knotiins

First reported in Amaranthus hypochondriacus seed (Mexican crop plant). It is smallest know alpha amylase inhibitor with 32 residues and three disulfide bonds. The NMR structure of inhibitor shows that inhibitor contains a knottin fold, three antiparallel β strands and a characteristic disulfide topology.

2. γ-Thionins

Three isoforms of this type has been identified and isolated from sorghum (Sorghum bicolor). Isoforms are SIα1, SIα2, SIα3 which are small proteins of 47 – 48 amino acid residues containing four disulfide bridges, with 42-87 % sequence identity. These inhibitors strongly inhibit insect a-amylases (Bloch et al., 1991) .

3. Ceral type (CM proteins)

These types of inhibitors are derived from cereal family. They are composed of 120-160 amino acids forming five disulphide bonds. There are large number of inhibitor that belongs to this category. These inhibitors act on insect as well as mammalian amylase. These inhibitors are known as sensitizing agent in humans because upon repeated exposure it causes allergy, dermatitis and baker's asthma.

The most studied cereal α-amylase inhibitor includes 0.19 (exogenous wheat α-amylase inhibitor) and the bifunctional inhibitor from Indian finger millet. The other examples are 0.28, WRP25, WRP26 and WRP27 (Sialno, 1987).

4. Kuntiz like alpha amylase inhibitor

These inhibitors are reported in cereals such as barley,wheat and rice and said to contain around 180 residues and four cystines. Barley α-amylase/subtilisin inhibitor (BASI) is the best characterized inhibitor from this class (Chagolla-López et al.,1994).

5. Thaumatin like inhibitors

These are protein inhibitor with molecular mass of nearly 22 KDa. Zeamatin isolated from maize is best characterized bifunctional inhibitor.

6. Lectin like inhibitor

These inhibitors are extracted from white, red and black kidney beans. Common inhibitors belonging to this class are αAI1 and αAI2 (Kasahara et al.,1996;Marshall et al. ,1975;Wilox et al. ,1984)

Table 2. 2. Different structural classes of α-amylase inhibitors, based on a classification by Richardson

Structural class	Name	Source	References
Legume lectin type	α-A11 & α-A12	Beans	(Nahoum et al.,2000; Grossi de Sá et al.,1997)
Knottin type	AA1	Amaranth	(Lu et al.,1999;Martins et al.,2001)
Ceral type	0.19, 0.53, 0.28 WRP25, WRP26, WRP27 & RB	Wheat , barley & Indian finger millet	(Franco et al., 2000; Richardson, 1990;Campos et al.,1983)
Kunitz type	BASI, WASI & RASI	Barley , wheat & rice	(Ohtsubo et al., 1992; Vallé et al. ,1998)
Thaumatin type	Zeamatin	Maize	(Blanco-Labra et al.,1980; Schimoler-O'Rourke et al.,2001; Richardson et al.,1987)
γ-Purothionin type	SIα1, SIα2 & SIα3	Sorgum	(Bloch et al.,1991)

2.2.2.2. α- AMYLASE INHIBITORS FROM ALGAE

Reports have shown that Red algae Palmaria sp. Brown algae Alaria sp, Brown algae Ascophyllum sp.(Nwosu et al. ,2011), Brown algae Eisenia bicyclis(Okada et al.,2004), brown alga Spatoglossum asperum posses alpha amylase inhibitory effects.

Polysaccharide compound Fucodian isolated from *Ascophyllum nodosumis also said to posses alpha amylase inhibitory activity (*Kim et al*.,* 2014). pentagalloylglucose 3-O-digalloyl-1,2,6-trigalloylglucose derived from green algae Spirogyra varians *also posses inhibitory activity*.(Cannell et al., 1988).

2.2.2.3.α-AMYLASE INHIBITORS FROM BACTERIA

Streptomyces is known for producing alpha amylase inhibitors. Compound such as Tebdamistat, Haim,Paim are said to posses the activity.

2.2.2.4. α-AMYLASE INHIBITOR FROM ENDOPHYTIC FUNGI

Till now endophytic fungi have yet not been exploited to its potential for screening of inhibitors. But there has been a report of

2.2.3. WHY NEED OF ALTERNATIVE SOURCE FOR A-AMYLASE INHIBITORS?

Clinically used α-amylase inhibitors are acarbose and miglitol which prevent the digestion of carbohydrates and provide short-term glycemic control. The drawback of such inhibitors is their non-specificity in targeting different glycosidases (Cheng et al., 2005). These inhibitors are known to produce serious side effects that limit their use as a therapeutic drug.

Therefore there is need to look for the alternative. One such alternative is used of endophytic fungi.

2.3. ENDOPHYTIC FUNGI

The term “endophyte” refers to as microorganism which inhabit inside the plant for at least one life cycle (Chanway, 1996). In 1809 Heinrich Friedrich, German botanist ,was the first to describe endophytes. Endophytes are usually found in the internal plant tissues beneath the epidermal cell layers where they live symbiotically (Strobel, 2003).

2.3.1. ENDOPHYTIC FUNGI AS SOURCE OF ENZYME INHIBITORS

Due to many side effects from the synthetic sources, there have been a constant demand for natural bioactive products which are easily obtainable, renewable, ecofriendly for their application in many fields such as pharmaceutical, agriculture and industrial(Liu *et al*.,2001). There are about 200,000 natural metabolites of microbial origin which have been reported to posses therapeutic activity (Bérdy, 2005). Due to success of lot of medicinal drugs from microbial origin, focus of drug discovery has shifted from plants to microorganisms.

The endophytic fungi have been seen as potential source of pharmaceutical application from a very long time. Presence of novel bioactive compounds such as antimicrobial, anticancer and antiviral agents have been reported from endophytic fungi (Selim *et al*, 2012) Due to the ability to produce enzymes, endophytes have been widely exploited for numerous applications in the field of biotechnology. Endophytes products have been used for environmental application, medical applications etc. (Firáková *et al.,* 2007, Pimentel *et al*. 2011). For the colonization in the plant tissue, endophytic fungi produce necessary enzymes. These enzymes can be isolated, purified and can be used for wide range of applications. It has been observed that endophytic fungi produce similar bioactive compounds to that of plants.

Therefore , endophytic fungi can be explored as source of inhibitor.

Chapter – 3

AIM OF THE STUDY

AIM AND OBJECTIVES

α-amylase inhibitor has great potential in treatement of diabetes. Therefore main aim is to find potential producers of α-amylase inhibitor.

- To screen endophytic fungi for α-amylase inhibitors
- Characterization of the α-amylase inhibitors producing fungi

Chapter – 4

MATERIALS AND METHODS

MATERIALS AND METHODS

4.1. PROCUREMENT, MAINTENANCE AND PRESERVATION OF THE ENDOPHYTIC FUNGI

Total 105 cultures were procured from the isolates of *Aegle marmelos*, *Cinnamomum* and *Vitis vinifera* from the pre-existing repository library of endophytic fungi isolates present at Dr. Sanjai Saxena Lab, Thapar University, Patiala. The endophytic fungi were grown on 90 mm petriplates of Potato Dextrose Agar (PDA) (pH 5.6± 0.2, Himedia) supplemented with 1 mg/ml of antibiotic for which 5 mm mycelia plug was taken from the mother culture and placed on freshly prepared PDA plates. Subsequently the plates are incubated at 27± 2 ˚C for 5-6 days (Freire et al., 2016).These plates were then used for preparation of glycerol stock as well as inoculum for liquid culture.

For preparation of glycerol stock, 5 mm mycelia plug was taken and placed in the sterile vials containing 20% glycerol and PDA (Lalaymia et al., 2014). For each isolate three slants were prepared which were then stored at 4˚C till further use.

4.2. PRODUCTION OF CULTURE FILTRATE

Production of all 105 culture filtrate was carried out for induction of secondary metabolites. For production, 5 mm mycelia disc of 7-10 days old culture were inoculated into 250 ml of flask containing 50 ml of autoclaved potato dextrose broth (PDB) (pH 5.1± 0.2,Himedia). The flasks were incubated in incubator shaker at 28˚C, 120 rpm. After 7 days of incubation, the mycelia were separated by filtration through whatman filter paper 4 (GE healthcare,USA). For getting cell free cultures, the filtrate was centrifuged (Hitachi,Japan) at 1200 rpm for 10 minutes. The pellet was discarded and supernatant was transferred to fresh falcon tubes and stored at -20˚C until further use **(**Rodrigues et al., 2000; Vicente et al., 2001).

The cell free culture filtrate obtained was then further used for screening of α-amylase inhibitor.

4.3. LIQUID-LIQUID EXTRACTION FOR ISOLATION OF A-AMYLASE INHIBITORS

Liquid -liquid extraction method with slight modification in Yamauchi et al. 2011 was adopted to partially purify the α-amylase inhibitors. The cell free filtrate of the selected culture was subjected to extraction by different solvents in order of their increasing polarity from Hexane, Dichloromethane, Chloroform, Ethyl acetate to Methanol as shown in figure 5.1. Extraction of filtrates was carried out three times with each solvent and solvent layers were pooled. The organic layer containing the compounds of interest was collected and the remaining solvent was evaporated in Rota evaporator for dehydration of extract. The extract obtained was reconstituted with methanol and stored at - 20˚C until further use (Yamauchi et al., 2011).

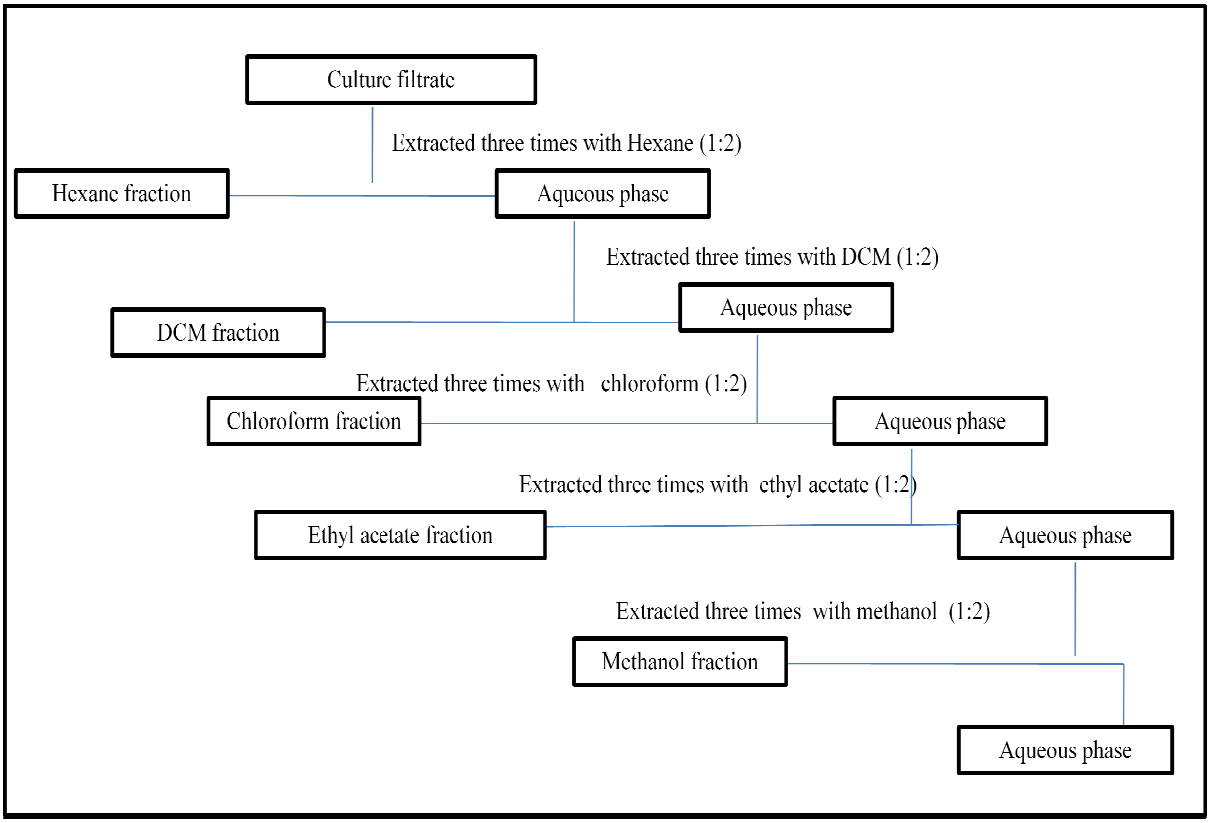


Figure 4.1: Schematic representation of the solvent extraction of the culture filtrate of selected endophytic fungi #107VVLSWN

4.4. SCREENING OF A-AMYLASE AND A-AMYLASE INHIBITORS PRODUCING ENDOPHYTIC FUNGI

The screening of the producers was carried by two method, qualitative screening by agar well diffusion assay and quantitative screening by DNSA method.

4.4.1. PRELIMINARY SCREENING BY AGAR WELL DIFFUSION ASSAY

This assay served as both qualitative as well as quantitative method for screening of inhibitors. The main principle behind this assay is based on the hydrolysis of starch. For the assay 1% starch agar plates were prepared by taking 1gm of starch, 2 gm of agar in 100 ml of distil water , autoclaved and poured in sterile petri plates. 5 mm diameter wells were made on the agar plates by using sterile cork borer. For quantitative screening direct filtrates were used. The filtrates were thawed and used subsequently.

For screening of α-amylase producers, 30 µl of filtrate was pipette in each well and plates were sealed with parafilm and incubated at 37˚C for 12-14 h in incubator (Metrex Instruments, New Delhi). After incubation, zone of hydrolysis was observed by flooding the plates with 1%iodine solution (5Mm iodine in 1% potasium iodine solution), excess was drained and zone diameter was measured. For the screening standard α-amylase (α-amyase from fungus,Himedia) was used as positive control and PDB was used as negative control.

For the inhibition assay, 50µl of 0.312 mg/ml of standard enzyme i.e 18 U of α-amylase was mixed with 50 µl of filtrate in a clean, autoclaved eppendoff and incubated for 10 min at 37˚C, then 30 µl of the mixture was then pipette into the wells, plates were then incubated for 12-14h at 37˚C. The diameter was measured, after flooding the plates with the iodine. Decrease in the diameter as compared to the standard enzyme served as indicator for the presences of the enzyme.

Results are reported as the Amylase Inhibition Percent by using formula:

$$\text{Amylase Inhibition Percent} = \frac{(\text{control} - \text{sample})}{\text{control}} \times 100$$

The screening process for all the filtrates was done in triplicates and data was represented as mean ± SD (Randhir et al., 2007).

Before screening of the inhibitors, standard enzyme activity was optimized by varying parameters for maximum enzyme activity. The parameters that were optimized include concentration of enzyme and incubation time (kulkarni et al., 2013).

4.4.1.1. OPTIMIZATION OF ENZYME CONCENTRATION

For the optimization of enzyme concentration, different concentration of the enzyme was taken i.e. 10 mg/ml , 5 mg/ml ,2.5 mg/ml, 1.25 mg/ml , 0.625 mg/ml , 0.312 mg/ml , 0.156 mg/ml . 30 µl of the enzyme was loaded into the wells bored in the 1% starch agar plates by sterile cork borer. The plates were then wrapped with parafilm and incubated for 12-14 hrs.

After incubation, the plates were flooded with iodine. Zone of hydrolysis was measured and the graph was plotted between zone diameter v/s concentration of enzyme.

Readings for each concentration was taken in triplicates. The data taken was represented at mean ± S.D.

4.4.1.2. OPTIMIZATION OF TIME OF INCUBATION

For the optimization of time, 0.312 mg/ml of enzyme (parameter optimized before) was loaded into the wells on 1% starch agar plates. The plates were incubated at 37 ˚ C and diameter of zone of hydrolysis was measured after every 30 minutes. The graph was plotted between diameters of zone v/s time.

Readings for each concentration was taken in triplicates. The data taken was represented at mean ± S.D.

4.4.2. QUANTITATIVE SCREENING –DNSA METHOD

For quantitative screening, first, kinetic study was carried out for standard α-amylase enzyme. For establishing the effect of inhibitor, first, optimization of substrate concentration, enzyme concentration and incubation time was done.

Standard curve using maltose was also prepared.

4.4.2.1. OPTIMIZATION OF SUBSTRATE CONCENTRATION

For reaction, stock solution of 1% starch was prepared in 20 mM of phosphate buffer. Different dilution of the starch was prepared. Dilutions of substrate taken were 0.2, 0.4, 0.6, 0.8 %. 0.5 mg/ml of alpha amylase enzyme was used.

In a clean, autoclaved eppendoff tubes, total 800 µl of the reaction mixture was prepared. For preparation of the reaction mixture, 200 µl of enzyme was taken with 200 µl of phosphate buffer and 200 µl of different concentration of the substrate was added. The mixture was then incubated at 37˚C for 30 minutes. After incubation, 200 µl of stopping agent DNSA was added and the tubes were incubated in boiling water bath for 20 minutes. The reaction mixture was then cooled at room temperature and O.D. was measured at 540 nm.

Readings for each concentration was taken in triplicates. The data taken was represented at mean ± S.D.

4.4.2.2. OPTIMIZATION OF THE INCUBATION TIME

Total reaction mixture of 800 µl was prepared by taking 200 µl of enzyme in 200 µl 0.02 M phosphate buffer with 200 µl of 1% starch. The reaction was carried out for different time period. The reaction was stopped by DNSA and O.D. was measured at 540 nm.

Different incubation time taken was 30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 4.5 h, 5 h, 5.5 h, 6h.

Readings for each concentration was taken in triplicates. The data taken was represented at mean ± S.D.

4.4.2.3. OPTIMIZATION OF ENZYME CONCENTRATION

For enzyme concentration optimization, the reaction mixture was kept same as above. But enzyme concentration was varied as 0.2, 0.4, 0.6, 0.8, 1 mg/ml.

Readings for each concentration was taken in triplicates. The data taken was represented at mean ± S.D.

4.4.2.4. MICROTITER PLATE METHOD FOR QUANTITATIVE ESTIMATION OF A-AMYLASE INHIBITOR

For quantitative screening DNSA method was used. In a fresh tube , reaction mixture was made by adding of 100 µl of α-amylase enzyme, 100 µl of phosphate buffer and 100 µl of 1 mg/ml of extracted fraction of culture filtrate. The tubes were then incubated at room temperature for 10 minutes. After incubation, 100 µl of 1%starch was added and tubes were incubated at 37˚C for 2 h. After 2h 100 µl of DNSA was added and tubes were incubated in boiling water bath for 20 minutes. O.D. was then measured at 540 nm. For control, reaction mixture remained same but instead of extract fraction buffer was added.

Percentage of inhibition was calculated as:

$$\text{Amylase Inhibition Percent } = \frac{(\text{control} - \text{ sample})}{\text{control}} \times 100$$

Reading of each sample was taken in triplicates.

4.5. IDENTIFICATION OF ENDOPHYTIC FUNGI

The most potent α-amylase inhibitor producing endophytic fungi were identified by morphological taxonomy and molecular taxonomy (Paul et al., 2012)

4.5.1. MORPHOTAXONOMY

The identified potential producer of inhibitor was grown on different media plates i.e. Potato dextrose agar (PDA) , Water agar (WA), Synthetic nutrient deficient agar (SNA), MEA, Czapek dox agar (CDA) for 4-5 weeks at 28˚C for their morphological identification.

Grown cultures were microscopically examined for their identification. The glass slide was cleaned and drop of water was put on a clean glass slide. The mycelia was then put on the glass slide microscopic features, a drop of water was put on clean glass slide and teased properly with the help of needle. The mycelia was then stained with Lactophenol cotton blue, covered with cover slip and observed under the microscope at 10 X, 40 X and 100 X using microscope (Nikon,Japan)

4.5.2. MOLECULAR IDENTIFICATION OF ENDOPHYTIC FUNGI

4.5.2.1. DNA ISOLATION

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

4.5.2.2 AGAROSE GEL ELECTROPHORESIS

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

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

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

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

4.5.2.3 PCR AMPLIFICATION

ITS1-5.8S-ITS2 rDNA sequence was amplified using universal primer pair i.e. ITS1 and ITS 4, synthesized by integrated DNA technologies (IDT), USA, in a thermocycler (My cycler, Bio-Rad Laboratories, Inc.). Primer ITS 1(5'TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT TGA TAT GC 3') (White et al, 1990)was used for the amplificatopn. Amplification was performed in 25µl reaction mixture containing 25ng of extracted fungal DNA, 0.8µM of each primers , 2.5mM of dNTP , 1.5mM MgCl2, 1.5 U of Taq DNA polymerase in 10 X Taq buffer. The thermal cycling conditions consisted of initial denaturation at 96° C for 5 min followed by 39 cycles of 95° C for 1 min , 58° C for 1.30 min , 72° C for 1 min followed by final extension at 72° C for 5 min. The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio-Rad documentation system.

4.6. INVITRO ANTIMICROBIAL ASSAY

The extracts of the selected culture was subjected to anti-microbial test against four pathogenic bacteria cultures i.e. *e Bacillus, E.coli , MTCC 96 and MTCC 737*. For the test, MHA plates were prepared. The bacteria cultures were revived and 12-16 hour old cultures of bacteria were taken. The bacterial count was adjusted to 10^6 cfu/ml using 0.5 McFarland solution. For adjusting the count, 1 ml of culture broth was added to 9ml of saline. The culture was swabbed on the MHA plates and disc was placed on it. 25 µl fungal extract was pippetted on to the disc and plates were sealed. The plates were then incubated at 37°C for 24 hr. After incubation, diameter of zone of clearance was measured to know the inhibitory activity (Sheikh et *al*., 2010).

4.7. INVITRO ANTIOXIDANT ASSAY

According to Kitts et al. Assay along with slight modification, the antioxidant activity is the ability to scavenge free radicals was performed using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. 20 µl of the test sample (1 mg/ml) was added to 230 µl of DPPH (100 µM in methanol)

and mixed thoroughly. The mixture was incubated for 30 min at room temperature in dark. After incubation, the absorbance was measured at 517 nm. Quercetin(10-50 µg/ml) was used as standard and working DPPH used as control. The DPPH radical scavenging capacity was expressed as microgram of quercetin equivalents per milligram of extract.

Free radical scavenging activity of the fungal extract was expressed as percentage, calculated by formula:

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

4.8. TLC analysis

The crude bioactive fraction of selected culture exhibiting α-amylase inhibitor activity was fractionated by subjecting to preparative thin layer chromatography (TLC). The TLC plate of 0.5 mm thickness was prepared by coating silica gel on to 20 x 15 x 5mm clean glass plates and was activated by incubating at 100° C for 2 h prior to use. The sample was spotted 1 cm above the edge of activated TLC plate with help of capillary. Simultaneously, the TLC chamber was saturated with different solvent system consisting of mixture of solvents of different polarties and ratios for 30 min. The TLC plate kept in TLC chamber in such way that sample spot should be above the solvent level. When the solvent reaches to desired level, plate was taken out and viewed under UV light.

Retention factor is calculated as the distance moved by the solute to the distance moved by the solvent.

$$Retention\ Factor(RF) = \frac{Distance\ moved\ by\ the\ compund}{Distance\ moved\ by\ the\ solvent\ front}$$

Chapter – 5

RESULTS

RESULTS

5.1. PROCUREMENT, MAINTANCE AND PRESERVATION OF THE CULTURES

About 105 isolates were procured and sub-cultured. The isolates were from medicinally important plants belonging to families Vitaceae (*Vitis venifera),*Rutaceae *(Aegle marmelos)* , ,Lauaracaeae (*Cinnamomum malabaricum* and *Cinnamum zeylanicum*). These isolates were collected from different locations in India representing the biodiversity. In past, plants have been exploited to the large extend for their medicinal values. It has been found that endophytes live in symbiotic relationship with plants and mimic the properties of the plant. Literature has shown that endophytic fungi produces large amount of bioactive compound such as immunosuppressant, anticancer agent etc. which posses medicinal properties. Hence the present studies is undertaken to exploit the endophytic fungi for isolation of the α-amylase inhibitor for the treatment of diabetes.

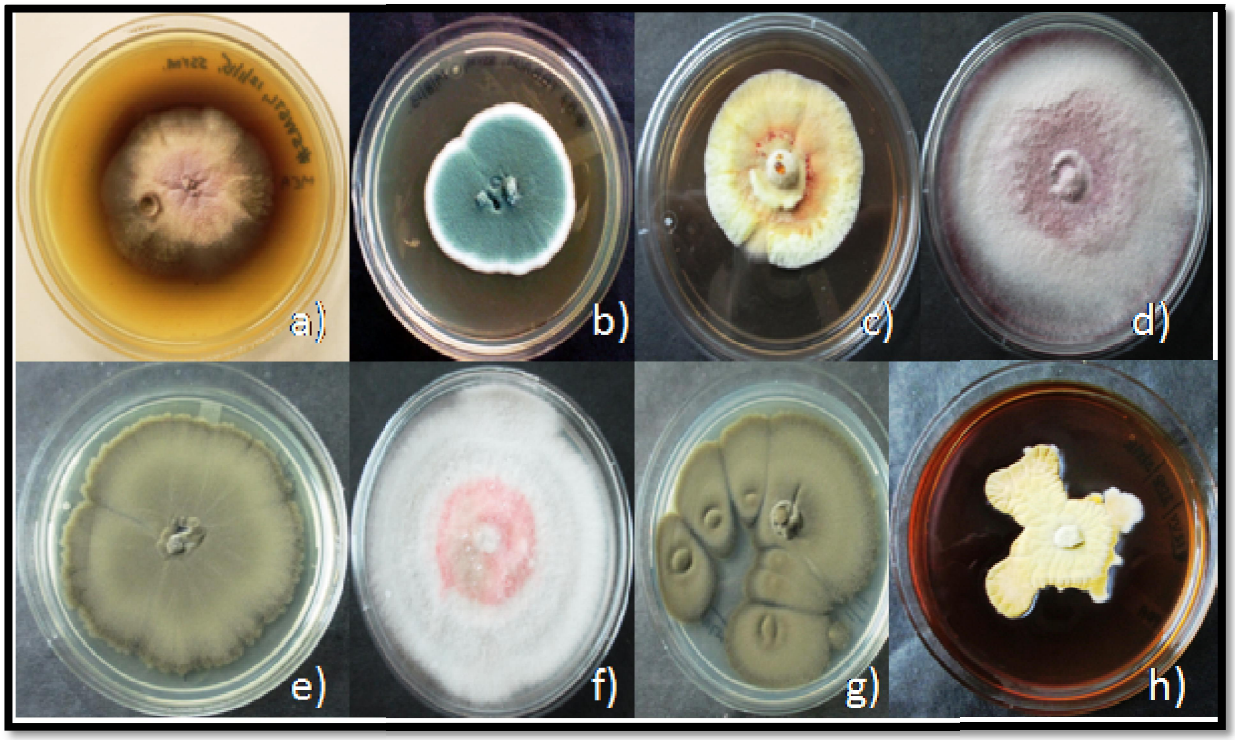


Figure 5.1: Different endophytic fungi procured from lab a) *Quambuleria* sp. ,b)*Aspergillus* sp. ,c)*Cheatomium*, d&e)*Fusarium* sp., f)&g) *Aspergillus sp.*and h) unidentified

The isolates were also regularly maintained. The isolates were also grown on the glycerol for their preservation and long term storage as glycerol serves as cryoprotectant.

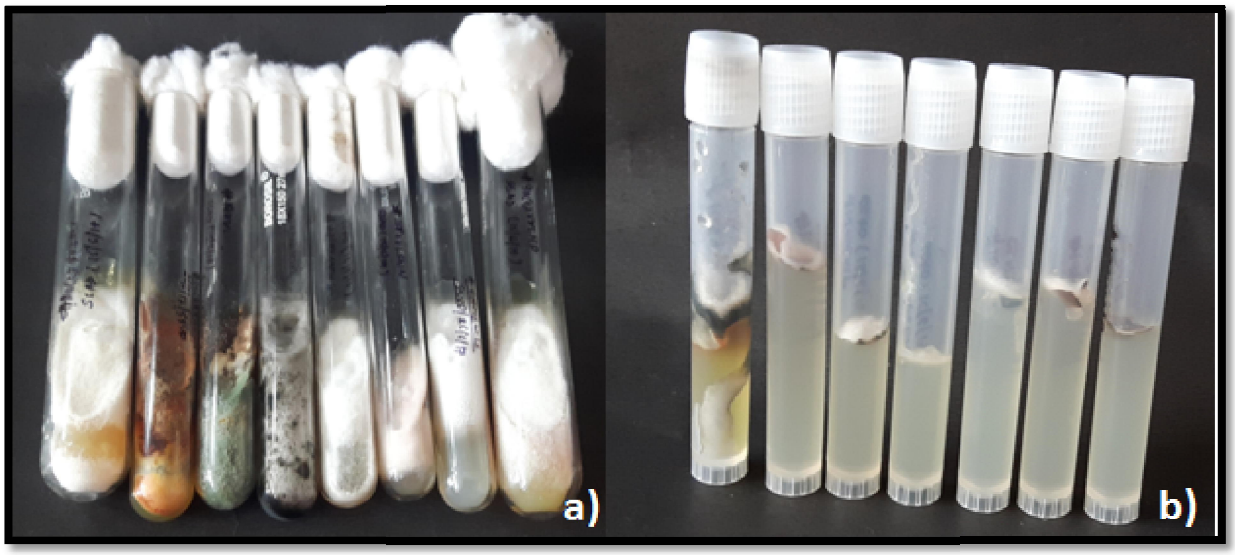


Figure 5.2: Maintained stocks of endophytic fungi a) in glycerol slants b) in vials

Out of 105 endophytic fungi, 8 isolates were from family Rutaceae. 9 isolates were from Lauaracaeae family out of which 2 were isolated from leafs, 1 were from bark and 6 from stem. 88 isolates were from Vitaceae out of which 3 were from stem and 85 from leaves.

The table 1 shows the list of endophytic fungi cultures screened for α-amylase and α-inhibitors.

Table 5.1: List of preliminary screened endophytic fungi for α- amylase and α- amylase inhibitors

S.No.	Culture Code	Host Plant	Plant Part	Sampling location	α-Amylase	α-Amylase inhibitor
1	#23(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
2	#51(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
3	#129(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	+	-
4	#14(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	+	-
5	#57(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
6	#140(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
7	#117(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
8	#11VSMP	*Vitis vinifera*	stem	Pune, Maharashtra	-	-
9	#43(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
10	#9VVGSTL	*Vitis vinifera*	stem	Rajendra nager, Lucknow	-	-
11	#131(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
12	#133(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
13	#113(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
14	#5VV	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
15	#12VV	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
16	#20VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
17	#19(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	+++
18	#22(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	+++
19	#13(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	+++
20	#15(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	+++
21	#41(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
22	#33(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
23	#71(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
24	#55(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
25	#30(b)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-

26	#103(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
27	#101(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
28	#68(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
29	#107(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
30	#122(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
31	#104(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
32	#25VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
33	#71(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
34	#19VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
35	#107VVLSWN	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	+++
36	#21VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
37	#8(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
38	#35VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
39	#83VVLNM	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	-
40	#17(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
41	#10(a)VVLNM	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	
42	#29(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
43	#27VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
44	#31VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
45	#20(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
46	#5(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
47	#24VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
48	#27(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
49	#12(a)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
50	#9VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
51	#18VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
52	#12(b)VLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-

53	#42VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
54	#25VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
55	#50(d)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
56	#671VVLSWN	*Vitis vinifera*	Leaf	Nasik,Mahaeashtra	-	-
57	#10(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
58	#15VVLSWN	*Vitis vinifera*	Leaf	Nasik,Maharashtra	-	-
59	#103VVLSWN	*Vitis vinifera*	Leaf	Nasik,Maharashtra	-	-
60	#31VVLSWN	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
61	#16(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	+	-
62	#18VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	
63	#8VVGSTL	*Vitis vinifera*	stem	Luckhnow,Uttar Pardesh	-	-
64	#10(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
65	#139(P)VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
66	#21VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
67	#105VVLNM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
68	#12(a)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
69	#79VVLPM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
70	#20(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
71	#50(d)VVLLK	*Vitis vinifera*	Leaf	Alambagh, Lucknow	-	-
72	#104VVLNM	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	-
73	#9(b)VVLLK	*Vitis vinifera*	Leaf	Alambagh, Lucknow	-	-
74	#50(d)VVLLK	*Vitis vinifera*	Leaf	Alambagh, Lucknow	-	-
75	#14VVRLLK	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	-
76	#105VVLNM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
77	#90(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
78	#43(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
79	#10VVLNM	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-

80	#35(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
81	#79(b)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
82	#27(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
83	#73VVLNM	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	-
84	#18(d)VVLLK	*Vitis vinifera*	Leaf	Alambagh, Lucknow	-	-
85	#35(c)VVLMP	*Vitis vinifera*	Leaf	Pune, Maharashtra	-	-
86	#1(d)VVLLK	*Vitis vinifera*	Leaf	Alambagh, Lucknow	-	-
87	#109VVLNM	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	-
88	#77VVLNM	*Vitis vinifera*	Leaf	Nashik, Maharashtra	-	-
89	#4CMBABRT	*Cinnamomum malabaricum*	Bark	BRT Wildlife Sanctuary, Karnataka	-	-
90	#23CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar, Kerala	-	-
91	#54CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
92	#43CMSTITBRT	*Cinnamomum malabaricum*	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
93	#13CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
94	#96CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
95	#11CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-	-
96	#40CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT Wildlife Sanctuary, Karnataka	-	-
97	#1CMSTBRT	*Cinnamomum malabaricum*	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
98	#1032AMSTITYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
99	#1078AMSTITYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	-	-
100	#1013AMSTITYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	+++	-
101	#9AMBLBRT	*Aegle marmelos*	Bark	BRT wildlife sanctuary	++	-
102	#1069AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	++	-
103	#1032AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	+++	-
104	#1058AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	++	-
105	#1003AMSTYEL	*Aegle marmelos*	Stem	Yelundur, Karnataka	++	-

*Note: (+) and (-) indicates the severity of α-amylase and α-amylase inhibitor production. Where (+++) indicates very good activity, (++) indicates good activity and (+) indicates fair activity.

5.2. PRODUCTION OF CULTURE FILTERATE

The isolates for screening were subjected to secondary metabolites production in PDB medium. The growth of the endophytic fungi was determined by increase in the biomass. Most of the cultures were acidic in nature. The table 5.2, list cultures with their biomass weight and pH.

Table 5.2: List of isolates along with their biomass, volume of filtrate and pH

S.No.	Culture Code	Weight Of Biomass(gm)	Volume Of Filtrate(ml)	Ph
1	#23(P)VVLPM	6.38	18	5.22
2	#51(P)VVLPM	4.5	25	5.2
3	#129(P)VVLPM	2.1	26	4.8
4	#14(P)VVLPM	1.2	27	6.22
5	#57(P)VVLPM	2.3	30	5.4
6	#140(P)VVLPM	3.5	26	4.56
7	#117(P)VVLPM	8.83	20	6.23
8	#11VSMP	4.56	36	5.6
9	#43(P)VVLPM	7.51	23	6.36
10	#9VVGSTL	6.62	25	5.85
11	#131(P)VVLPM	3.4	28	4.13
12	#133(P)VVLPM	2.12	32	5.1
13	#113(P)VVLPM	1.2	25	6.64
14	#5VV	4.26	37	5.86
15	#12VV	2.93	33	3.21
16	#20VVLMP	4.2	25	7.25
17	#19(P)VVLPM	1.1	20	6.68
18	#22(P)VVLPM	2.46	25	5.32
19	#13(P)VVLPM	1.2	22.5	6.92
20	#15(P)VVLPM	1.72	25	6.54
21	#41(P)VVLPM	5.82	15	6.57
22	#33(P)VVLPM	1.53	25	6.55
23	#71(P)VVLPM	2.1	36	4.23
24	#55(P)VVLPM	1.32	38	6.29
25	#30(b)VVLPM	0.42	20	6.07
26	#103(P)VVLPM	4.41	35	6.3
27	#101(P)VVLPM	3.82	25	5.43
28	#68(P)VVLPM	5.29	40	6.08
29	#107(P)VVLPM	0.72	38	5.13
30	#122(P)VVLPM	1.5	26	5.6
31	#104(P)VVLPM	0.22	23	5.69
32	#25VVLMP	2.4	38	4.31
33	#71(P)VVLPM	3.5	26	5.31
34	#19VVLPM+	1	20	6.34
35	#107VVLSWN	3.69	10	6.6
36	#21VVLPM	2.8	28	5.4
37	#8(b)VVLMP	3.9	27	4.6

38	#35VVLMP	2.1	35	6.34
39	#83VVLNM	1.7	38	5.46
40	#17(b)VVLMP	2.8	26	5.73
41	#10(a)VVLNM	1.2	32	4.9
42	#29(b)VVLMP	2.3	45	3.8
43	#27VVLMP	1.8	29	7.1
44	#31VVLMP	1.9	20	6.4
45	#20(b)VVLMP	0.87	32	5.38
46	#5(b)VVLMP	3.9	29	4.6
47	#24VVLMP	2.1	32	5.42
48	#27(c)VVLMP	1.0	30	6.8
49	#12(a)VVLMP	2.2	26	5.1
50	#9VVLMP	1.89	19	6.32
51	#18VVLMP	4.28	27	6.31
52	#12(b)VLMP	2.56	29	5.12
53	#42VVLMP	1.23	18	4.7
54	#25VVLMP	1.65	23	6.47
55	#50(d)VVLMP	2.1	21	5.8
56	#671VVLSWN	2.63	23	6.85
57	#10(b)VVLMP	2.93	30	5.50
58	#15VVLSWN	2.93	12	6.59
59	#103VVLSWN	1.16	25	6.78
60	#31VVLSWN	2.77	30	3.65
61	#16(b)VVLMP	3.56	28	6.51
62	#18VVLMP	1.89	24	5.5
63	#8VVGSTL	2.3	22	6.5
64	#10(P)VVLPM	0.98	24	5.8
65	#139(P)VVLPM	2.4	16	5.98
66	#21VVLPM	1.89	22	4.7
67	#105VVLNM	2.48	33	3.5
68	#12(a)VVLMP	2.8	22	4.8
69	#79VVLPM	1.21	35	5.67
70	#20(c)VVLMP	2.1	24	5.12
71	#50(d)VVLLK	3.1	22	5.41
72	#104VVLNM	1.31	19	4.56
73	#9(b)VVLLK	2.31	25	6.8
74	#50(d)VVLLK	1.9	17	6.31
75	#14VVRLLK	1.4	14	4.7
76	#105VVLNM	2.1	27	5.4
77	#90(c)VVLMP	1.23	29	4.8
78	#43(c)VVLMP	2.01	34	4.9
79	#10VVLNM	2.39	26	5.3
80	#35(c)VVLMP	2.78	28	3.8
81	#79(b)VVLMP	1.9	20	6.4
82	#27(c)VVLMP	0.87	32	5.38
83	#73VVLNM	3.9	29	4.6
84	#18(d)VVLLK	2.1	32	5.42

85	#35(c)VVLMP	6.38	18	5.22
86	#1(d)VVLLK	4.5	25	5.2
87	#109VVLNM	2.1	26	4.8
88	#77VVLNM	1.2	27	6.22
89	#4CMBABRT	2.3	30	5.4
90	#23CMLNEY	4.26	37	5.86
91	#54CMSTNEY	2.93	33	3.21
92	#43CMSTITBRT	4.2	25	7.25
93	#13CMSTNEY	1.1	20	6.68
94	#96CMSTNEY	2.46	25	5.32
95	#11CMSTNEY	1.89	22	4.7
96	#40CMLBRT	2.48	33	3.5
97	#1CMSTBRT	2.8	22	4.8
98	#1032AMSTITYEL	1.21	35	5.67
99	#1078AMSTITYEL	2.1	24	5.12
100	#1013AMSTITYEL	2.1	35	6.34
101	#9AMBLBRT	1.7	38	5.46
102	#1069AMSTYEL	2.8	26	5.73
103	#1032AMSTYEL	1.2	32	4.9
104	#1058AMSTYEL	4.5	25	5.2
105	#1003AMSTYEL	0.98	24	5.8

Figure 5.3: Production of different isolates

5.3. SOLVENT EXTRACTION

The cell free filtrate of #107VVLSWN was subjected to liquid-liquid extraction by different solvents in order as Hexane, Dichloromethane, chloroform, Ethyl acetate and Methanol. The maximum bioactive residue was observed in case of methanol and minimum yield was obtained in DCM extract.

Table 5.3: Yield of bioactive compounds recovered from different cultures under study

Different extracts of #107VVLSWN	Yield of crude compound(mg)
Hexane extract	22
Dichloromethane extract	6
Chloroform extract	7
Ethylacetate extract	7
Methanol	25

5.4. SCREENING OF α-AMYLASE AND α-AMYLASE INHIBITORS PRODUCING ENDOPHYTIC FUNGI

5.4.1. PRELIMINARY SCREENING

The preliminary screening was carried out using agar disc diffusion assay. Total 105 cultures were screened using it for both for α-amylase as well as α-amylase inhibitors. But before screening standard enzyme parameter i.e. enzyme concentration and time were optimized.

5.4.1.1. OPTIMIZATION OF ENZYME CONCENTRATION

On enzyme optimization, the concentration of the enzyme for screening was fixed as 0.3125 mg/ml which is equivalent to 18 units of enzyme. The enzyme concentration was chosen in the basis of diameter zone.

Table 5.4: Zone of diameter measured at different concentration

Conc.(mg/ml)	Zone diameter in mm*
0.15	16.33 ± 0.5
0.31	18.33 ± 0.5
0.62	21.6 ± 0.5
1.25	24.67 ± 0.5
2.5	27.33 ± 0.5

5	29.33 ± 0.5
10	31.67 ± 0.5

*Data represented as mean ± S.D.

The graph was plotted between zone diameter in mm and enzyme concentration. The linear equation y=mx+c was obtained.

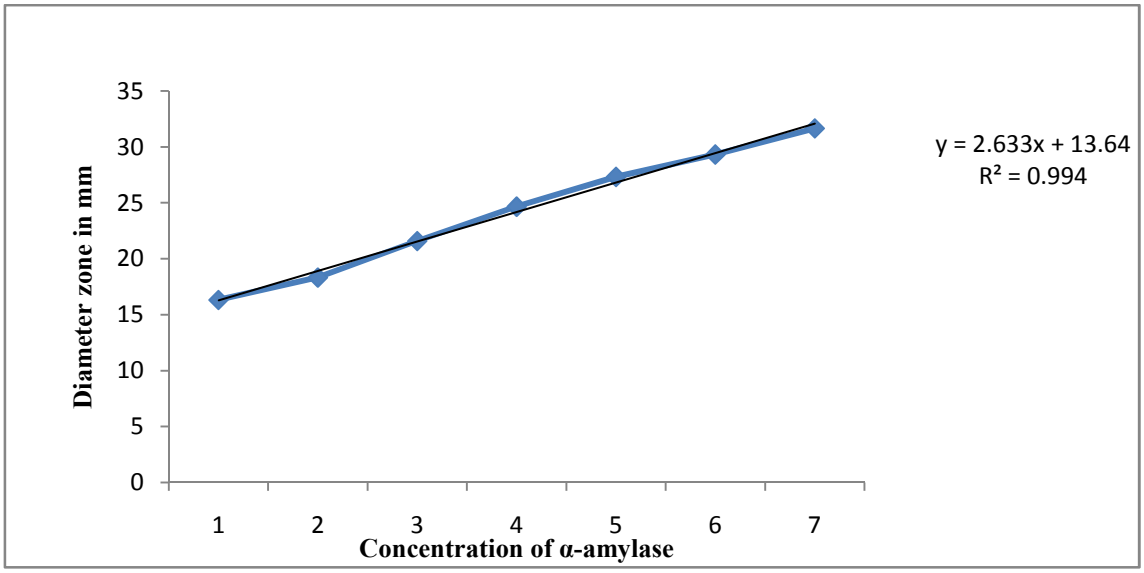


Figure 5.4: Graph showing zone diameter v/s enzyme concentration

5.4.1.2. OPTIMIZATION OF INCUBATION TIME

Once the concentration was fixed, the incubation time was optimized. The graph was plotted between diameter of zone and incubation time as shown in figure .The graph shows increase in diameter till 12 h of incubation. There diameter of the zone remained constant after that and there was no noticeable increase in the diameter. Therefore, incubation time was fixed as 12 h.

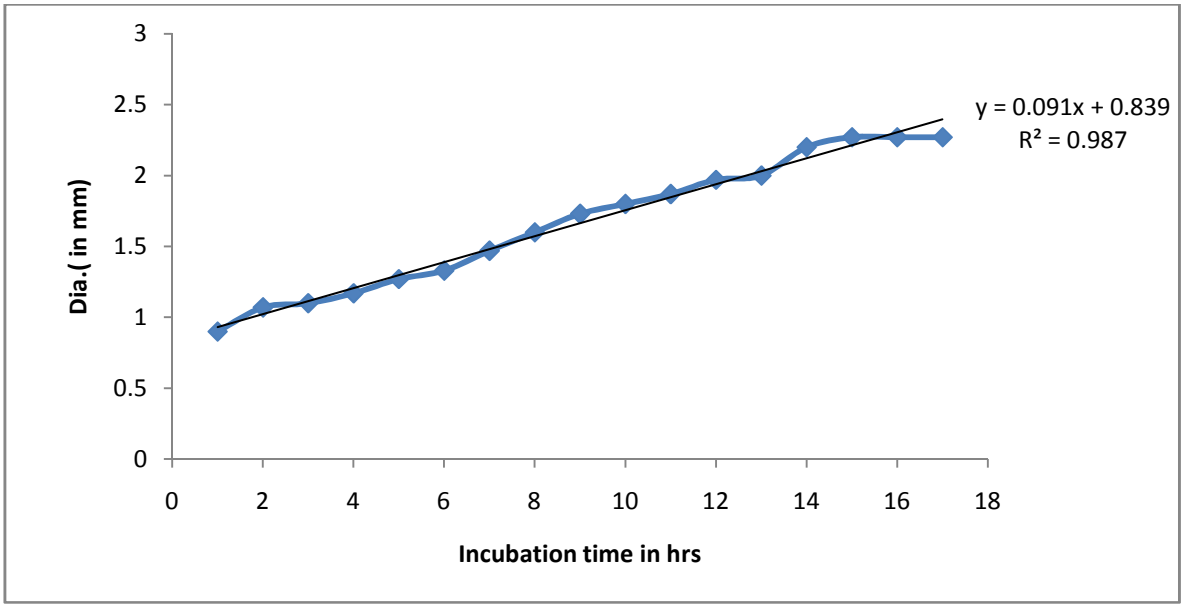


Figure 5.5 : Graph showing zone diameter v/s time

Out of total cultures screened, it was found that 9 isolates were α-amylase producers and 5 isolates were of α-amylase inhibitor producers. The table 5.4 gives the list of producers.

Table 5.5: List of the α-amylase and α-amylase inhibitor producers

S.No.	α-amylase	α-amylase inhibitor
1	#1013AMSTITYEL	#13(P)VVLPM
2	#9AMBLBRT	#19(P)VVLPM
3	#1069AMSTYEL	#19VVLPM
4	#1032AMSTYEL	#22(P)VVLPM
5	#1058AMSTYEL	#107VVLSWN
6	#1003AMSTYEL	
7	#129(P)VVLPM	
8	#14(P)VVLPM	
9	#16(b)VVLPM	

The inhibitor activity was also calculated and plotted. Out of all the inhibitors isolated #107VVLSWN was chosen for further studies as it exhibited maximum enzyme activity.

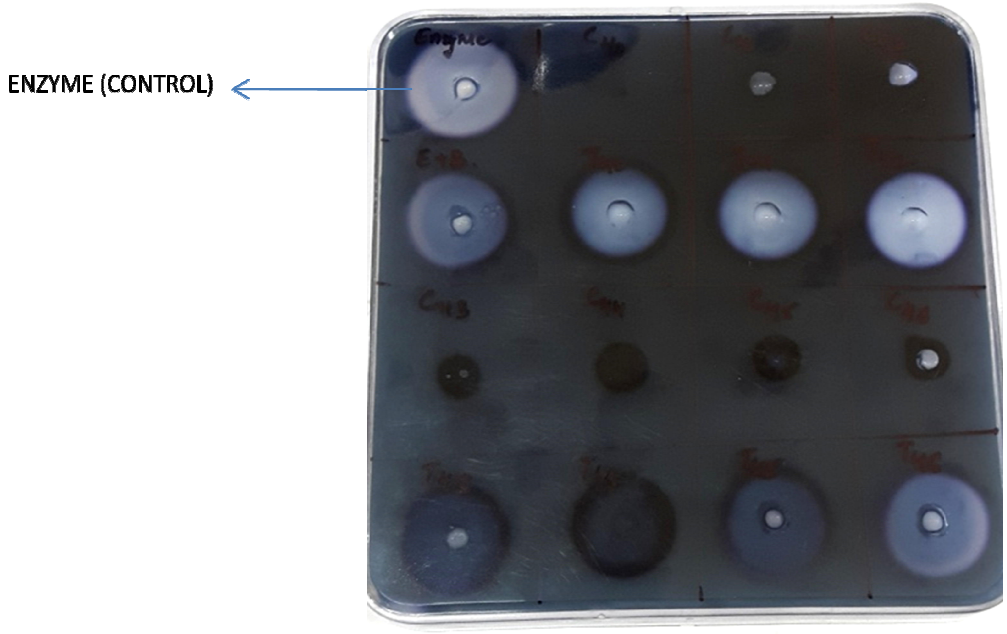


Figure 5.6: Preliminary screening of α-amylase and α-amylase inhibitor

Table 5.6 : Percentage of inhibition along with culture code

Culture Code	% Inhibition
#19(P)VVLPM	13.24±1.5
#22(P)VVLPM	16.98±0.5
#13(P)VVLPM	18.9±0.5
#15(P)VVLPM	16.98±0.5
#19VVLPM	16.98±0.5
#107VVLSWN	18.9±0.5

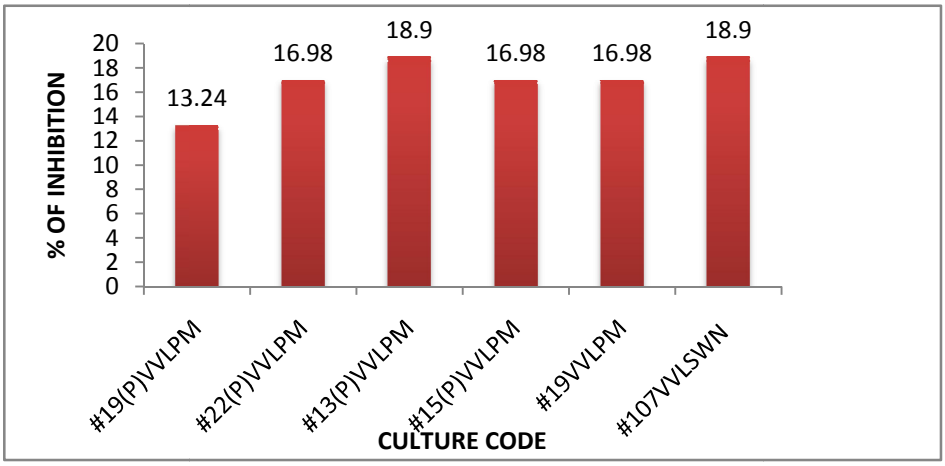


Figure 5.7 : Bar graph depicting percentage of inhibition of selected isolates

5.4.2. QUANTITATIVE SCREENING FOR INHIBITORS

Before quantitative screening, the parameters of the standard enzyme were optimized. The optimized parameters were substrate concentration, enzyme concentration and time optimization. These parameters were optimized so as to get standard condition of working of enzyme so as to get maximum activity of the enzyme.

5.4.2.1. OPTIMIZATION OF SUBSTRATE CONCENTRATION, ENZYME CONCENTRATION AND TIME OPTIMIZATION

The substrate concentration was the first parameter to be optimized. The substrate (starch) concentration was taken in percentage as 0.1, 0.2, 0.4, 0.6, 0.8 and 1. On measuring optical density, the saturation was observed after 1% concentration of the substrate. Therefore 1% starch concentration was finalised.

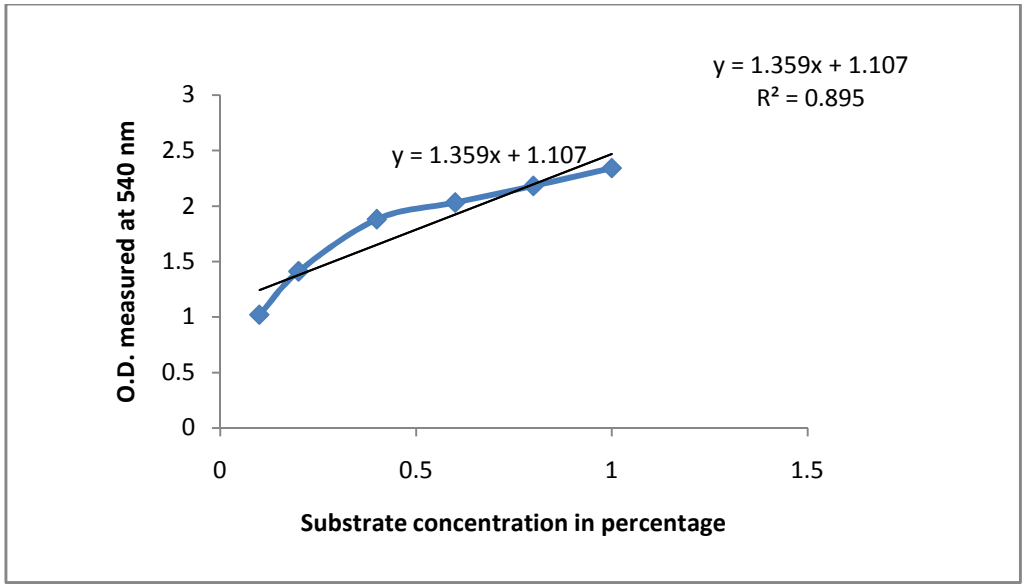


Figure 5.8: Substrate concentration optimization of standard enzyme

Once the substrate concentration was optimized, the next parameter that was optimized was time. For time optimization, the reaction of enzyme-substrate was carried out for different time period. During time optimization it was found that the best result was shown by enzyme at 2 hrs. After 2 hrs, enzyme started to degrade and there was decline in the enzyme activity.

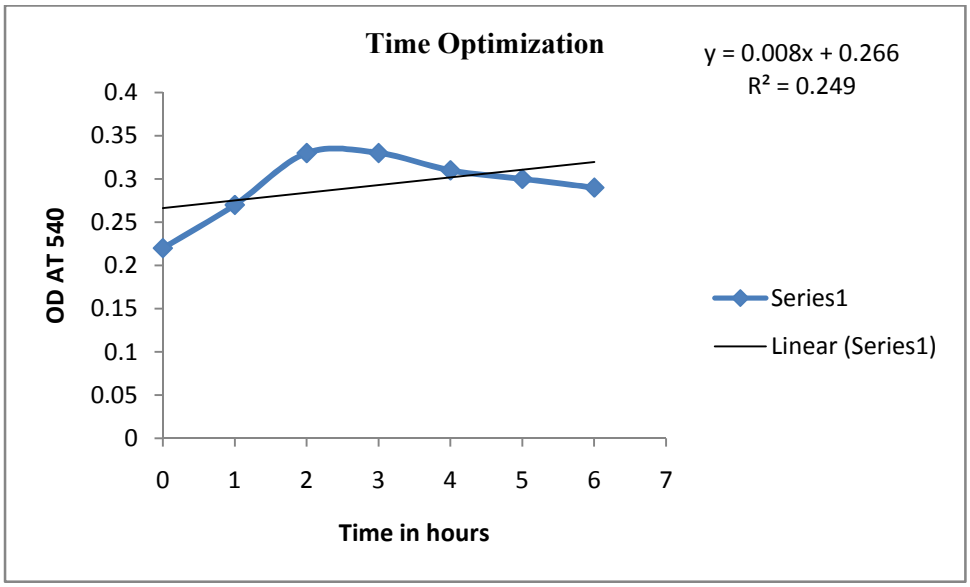


Figure 5.9 : Time optimization of standard enzyme

At the optimization of the enzyme was carried out. The enzyme concentration chosen was 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. The best activity was seen in the 0.8 mg/ml concentration of the enzyme.

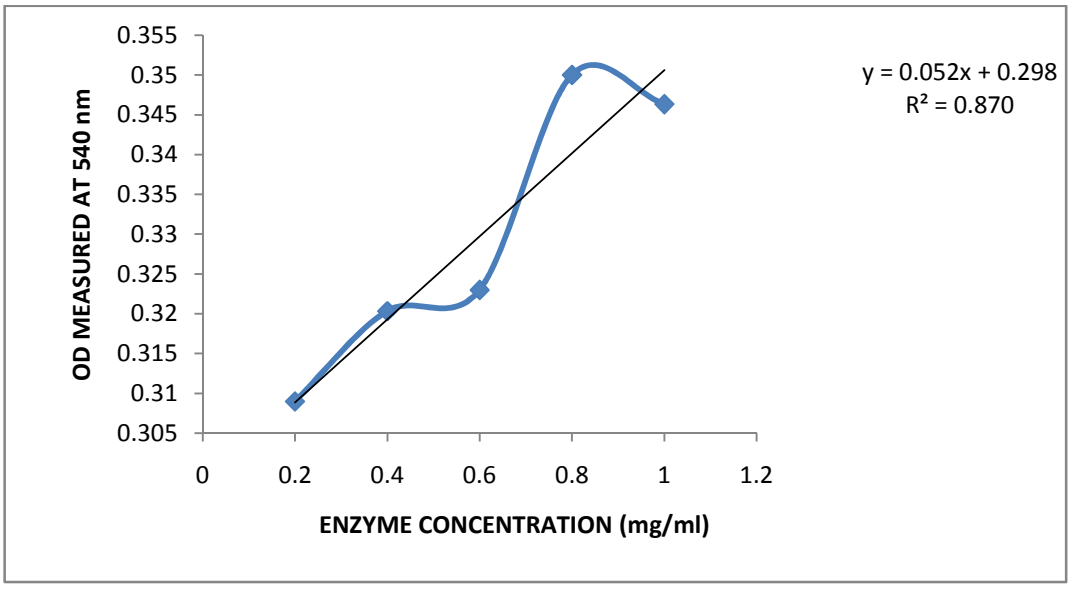


Figure 5.10: Concentration optimization of standard enzyme

On qualitative screening it was found that DCM extract exhibited maximum percentage of α-amylase inhibition. Minimum inhibition was seen in case of ethyl acetate extract. The table shows different extraction of #107VVLSWN along with their percentage of inhibition.

Table 5.7 : Percentage of inhibition as inhibited by different fractions of filtrate

Extract	% of Inhibition
Hexane extract	25.6
Dichloromethane extract	70.8
Chloroform extract	23.6
Ethyl acetate extract	43
Methanol extract	63.2

*data represented as mean ± S.D.

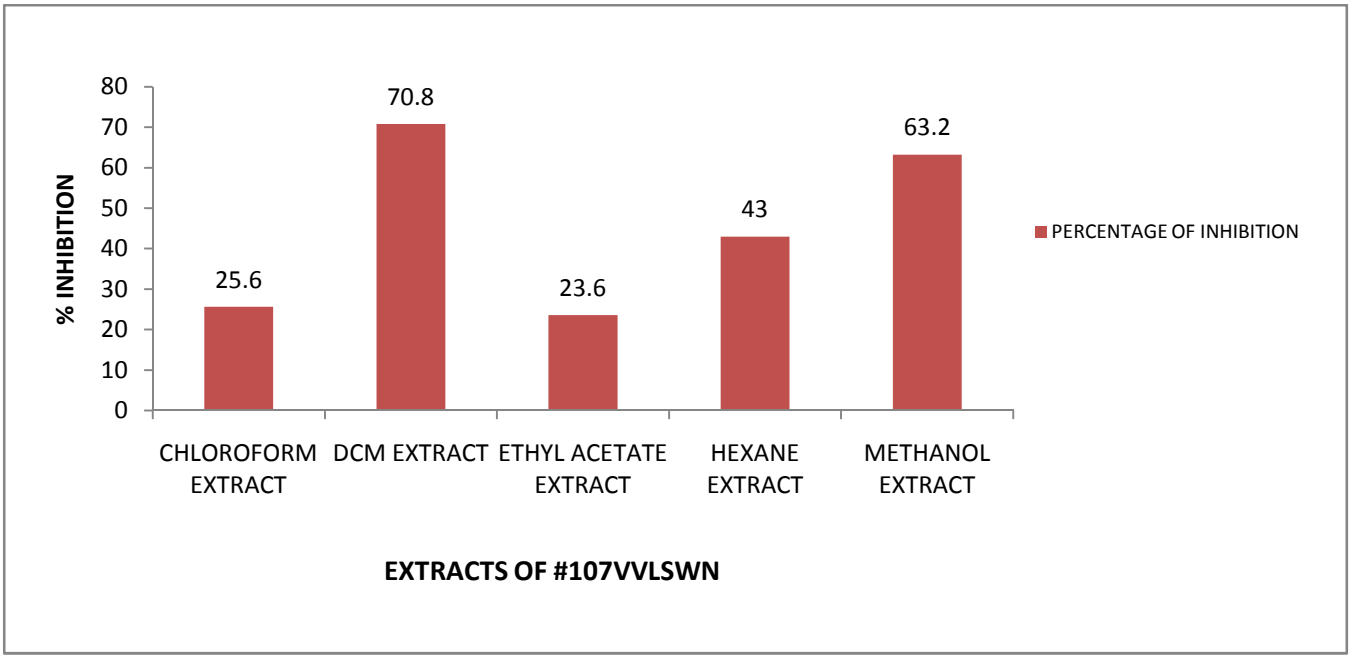
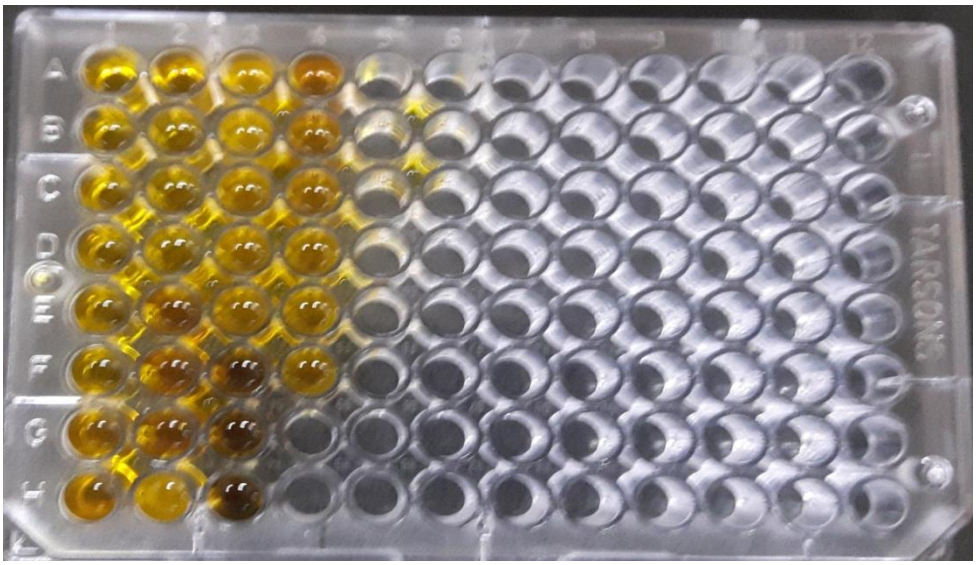


Figure 5.11 : Bar graph depicting percentage of inhibition of different extracts of #107VVLSWN



(a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C3	C.E.	E.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
B	C1	C4	C.E.	E.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
C	C1	C4	H.E.	E.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
D	C2	C4	H.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
E	C2	M.E.	H.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
F	C2	M.E	D.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
G	C3	M.E	D.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
H	C3	C.E	D.E.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

(b)

Figure 5.10: a) ELISA Titre plate for quantitative test of α-amylase inhibitors b)template for quantitative screening. Where C1, C2, C3, C4 are control; M.E-methanol extract; C.E.-chloroform extract; H.E-hexane extract ; D.E.-DCM extract; E.E.-ethyl acetate extract

5.5. IDENTIFICATION OF ENDOPHYTIC FUNGI

5.5.1. MORPHOTAXONOMY

Colony of #107VVLSWN is rapidly growing. Over PDA and MEA medium they appeared grey in colour from front and light brown in color from back, with flat margin, downy to mealy and is covered with greyish aerial hyphae (Figure 5.6, a,b)

Over PLA and SNA, colony was brown in colour from front and reverse side with flat margin. Its appearance was downy to mealy, the fungus produce dark brown soluble pigment without any odour (figure 5.6, c, d)

Among microscopic characterstic hyphae were thick,septate ,multinucleate and branched. Conidia were brown in chain upto 10 or more on conodia sphere (Figure 5

Hence on basis of morphological and microscopic characterstic the potential fungi #107VVLSWN, was tentatively identified as *Alternaria* sp.

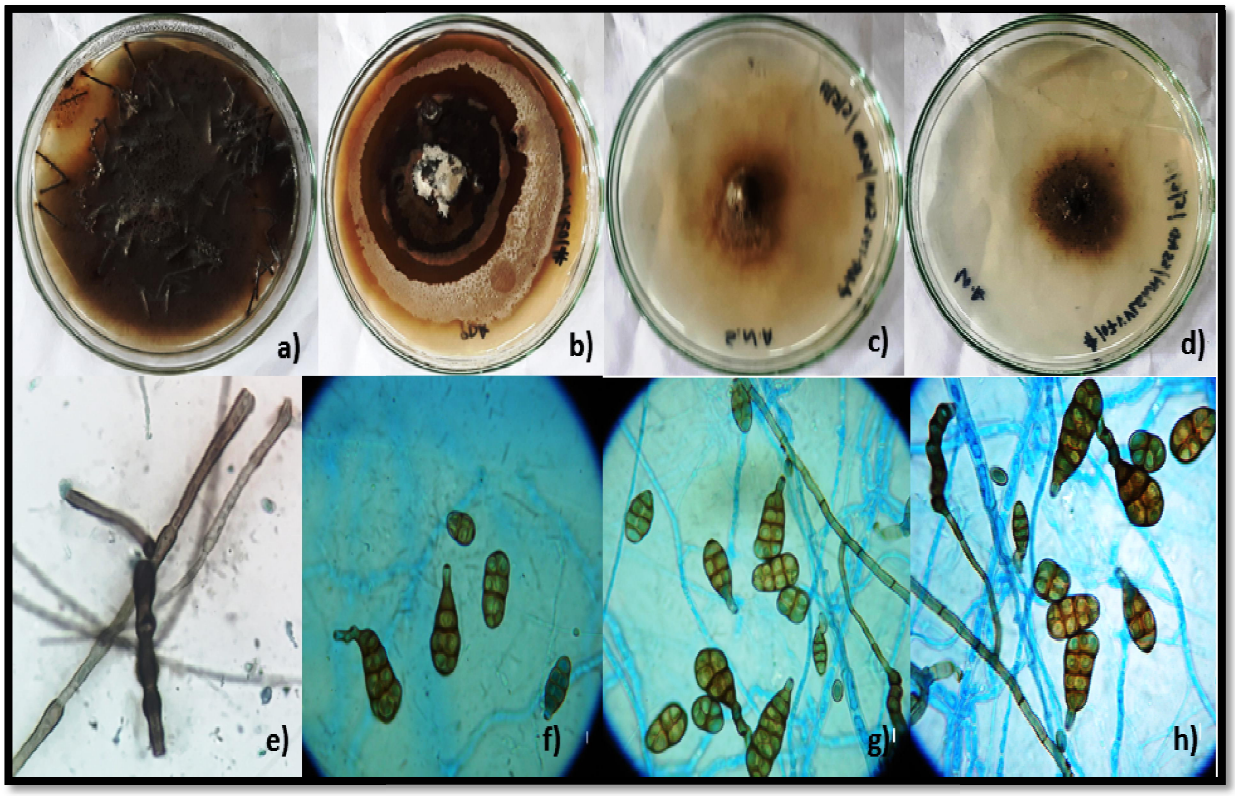


Figure 5.13: Colony morphology of #107VVLSWN on a) PLA, b) PDA, c) SNA, d) PLA. Microscopic feature of #107VVLSWN over GLA and SNA e) hyphae, g to h) brown color conidia arrange in chain.

5.5.2. MOLECULAR IDENTIFICATION

The concentration of isolated genomic DNA was determined approximately to be 35 ng/µl. On microscopic examination, #107VVLSWN was tentatively identified as *Alternaria* sp. For further specification, the purified gemonic DNA was further amplified. 1.5 % gel was used to resolve PCR product. The size was determined on the basis of its mobility along side of 5000 bp ladder. The approx. estimated size determined to be between 550 to 600 bp. The size was comparable to amplified ITS region. The amplified DNA has been send for sequencing to amplicon at Eurofins Genomics India Pvt. Ltd., Bangalore

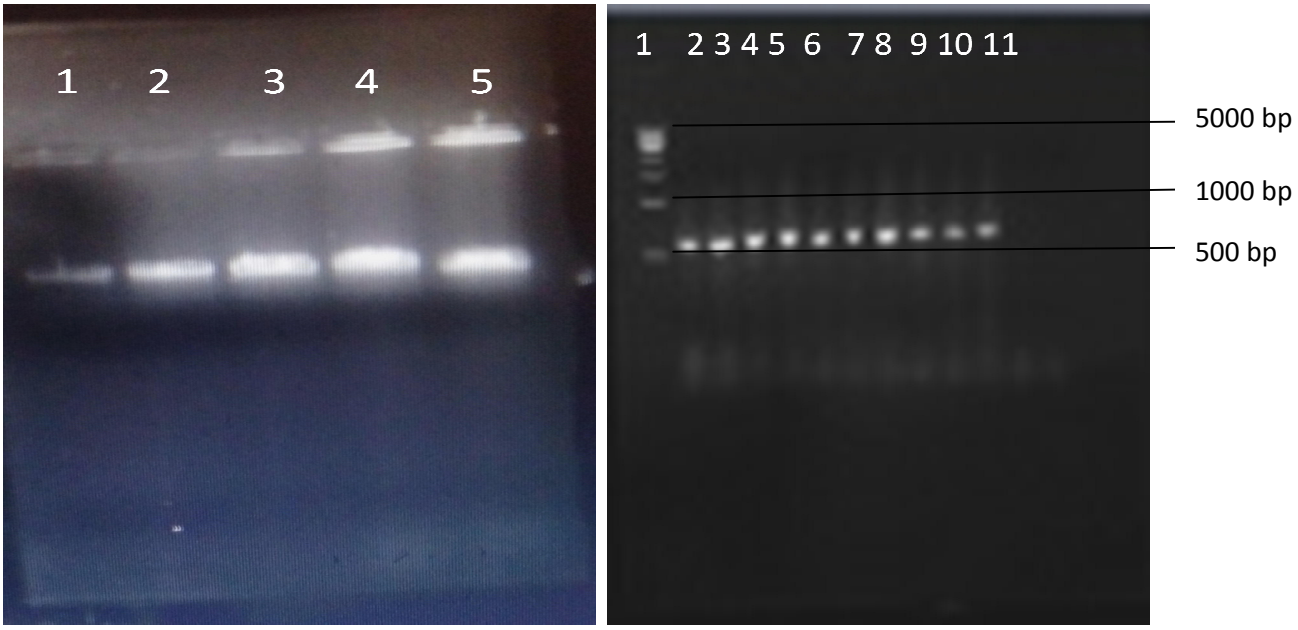


Figure 5.14: Agarose gel electrophoresis of (a) genomic isolation of #107VVLSWN,where lane 1-5 are bands of genomic DNA (b) PCR product of isolated genomic DNA, where lane 1 is DNA ladder of 5000 bp ,lane 2to 11 PCR product

5.6. INVITRO ANTIMICROBIAL ASSAY

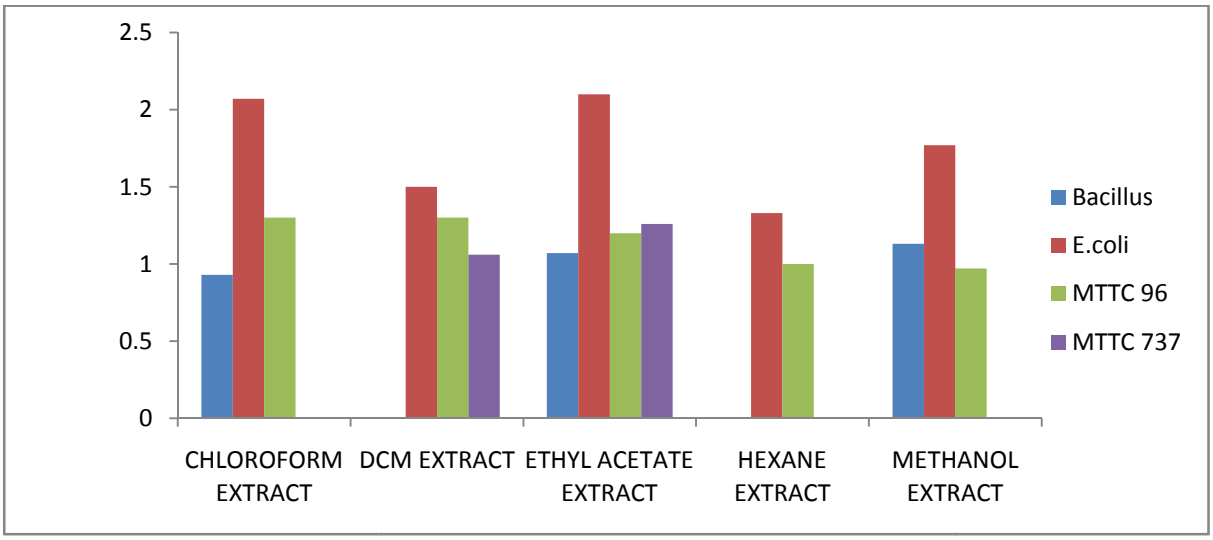
Antimicrobial assay for different extract of culture #107VVLSWN was performed against four pathogenic namely *Bacillus subtilis, E.coli,* MTCC 96 and MTCC 737. The antimicrobial test aims at testing the sensitivity of the extracted compound for the pathogenic bacteria. This test helps us to determine the effect of the compound on microbial flora when administrated into the body.

In case of *Bacillus*, the inhibition was seen in case of chloroform extract, ethyl acetate extract and methane extract. The highest activity was see/n against methane extract. In case of *E.coli*,

the inhibition was seen against all the extract. The highest activity was seen against chloroform extract and minimum activity was seen against Hexane extract. In case of MTCC 96, the inhibition was seen against all the extract. The highest activity was seen against DCM extract and minimum activity was seen against methane extract. In case of MTCC 737, the inhibition was seen against only DCM and ethyl acetate extract.

Table 5.8 : Results of antimicrobial activity of different fractions of #107VVLSWN against Bacillus subtitles, E.coli and Staphylococcus aureus (MTCC 96 and MTTC 797)

EXTRACT FRACTION OF #107VVLSWN	Zone diameter in cm			
	Bacillus	***E.coli***	**MTTC 96**	**MTTC 737**
CHLOROFORM FRACTION	0.93	2.07	1.3	0
DCM FRACTION	0	1.5	1.3	1.06
ETHYL ACETATE FRACTION	1.07	2.1	1.2	1.26
HEXANE FRACTION	0	1.33	1	0
METHANOL FRACTION	1.13	1.77	0.97	0



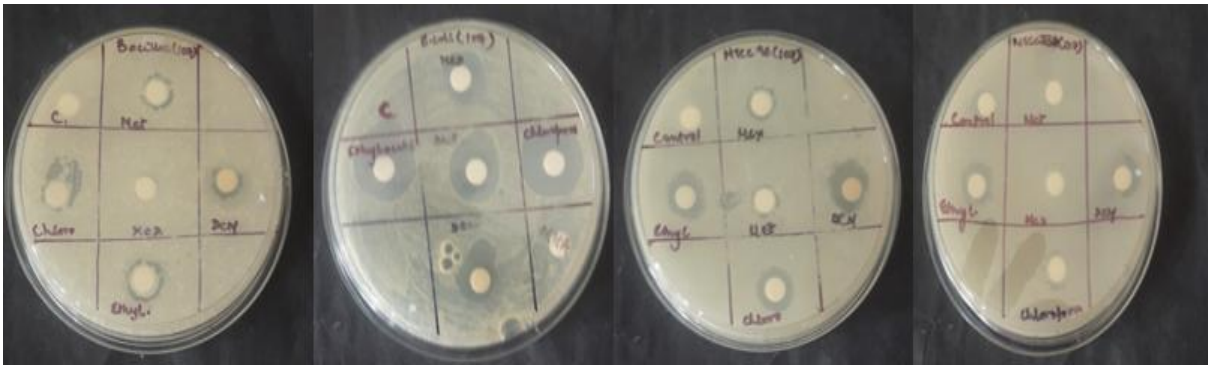


Figure 5.15 :Graphical representation and results of invitro antimicrobical activity

5.7. INVITRO ANTI OXIDANT ASSAY

Solvent fractions of the α-amylase inhibitor producing culture filtrate #107VVLSWN were evaluated for their capacity of antioxidant activity using the DPPH free radical scavenging system. After the incubation, results depicted that scavenging activity of ethyl acetate fraction with 72.63% followed by chloroform fraction with 66.2% whereas in Quercetin which was used as standard, the scavenging activity was 82.58 ± 0.61% (50µg/ml). These activity were also related to quercetin equivalent with maximum being to ethyl acetate fraction followed by chloroform fraction.

Table 5.9:Anti-Oxidant activity of different extracts of #107VVLSWN

S.No.	Solvent fraction	% free radical scavenging*(%)	Equivalent Quercetin activity (µg Quercetin/mg extract)
1.	Chloroform	66.2 ± 0.3	35.9
2.	DCM	63.23 ± 0.4	33.9
3.	EA	72.63 ± 0.3	40.5
4.	Hexane	55.17 ± 0.3	28.1
5.	Methanol	38.41 ± 1.3	16.3

5.8. TLC ANALYSIS FOR ALPHA AMYLASE INHIBITOR

The qualitative analysis of α-amylase inhibitor producing culture #107VVLSWN was carried out through TLC. DCM fraction of #107VVLSWN was subjected to TLC by using different combination of solvents for achieving a good separation. The optimized solvent combination at which best separation was achieved was with Hexane: toluene: formic acid. The DCM extract was resolved into 6 bands.

Table 5.10: Different solvent systems used for separation of #107VVLSWN

S.No.	SOLVENT SYSTEM	RATIO USED	RESULT
1	Hexane: Ethyl acetate	2:1	No separation
2	Hexane:Ethyl acetate	1:2	No separation
3.	Hexane:Chloroform	2:2	2 bands
4	Hexane:Chloroform	2:1	3 bands
5.	Toulene:Chloroform	1:1	No separation
6.	Toulene:DCM	1:2	3 bands
7.	DCM: Chloroform	2:1	No separation
8.	Toluene:DCM	3:1	4 bands
9.	Hexane: Toluene: Formic acid	3:2:.01	6 bands

The retention factor was calculated for all the 6 bands.

Table 5.11: Calculated retention factor DCM extract fraction of #107VVLSWN

S.NO.	Distance covered by solute(cm)	Distance covered by solvent(cm)	Retention factor (Rf)
1.	2.5	12	0.20
2.	5	12	0.41
3.	6	12	0.5
4.	8	12	0.66
5.	10	12	0.83
6.	11	12	0.91

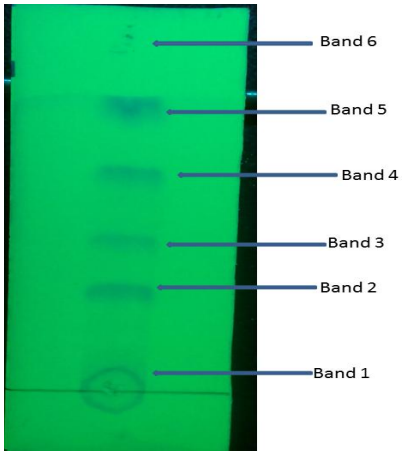


Figure 5.16: TLC analysis of #107VVLSWN

Chapter – 6

DISCUSSION

DISCUSSION

Diabetes mellitus is a chronic, metabolic disorder which is caused either due to insulin deficiency or decrease in insulin in the circulation. In today's scenario, diabetes has said to affect about 382 million people globally and is responsible for over 5.1 million deaths. According to WHO report 2016, diabetes have high economic burden globally.

One of the prime modalities of T2D management is lowering and maintenance of blood glucose level by inhibiting the enzyme α-amylase (α-1, 4-glucan-4-glucanphydrolase, EC 3.2.1.). α-amylase is a major secretary product of pancreas (5-6%) and breaks down starch into absorbable glucose in small intestine. Diabetes suffers from post prandial hyperglycaemia i.e. elevated blood sugar after dietary starch is broken down by α amylase subsequent to eating meals. Thus α-amylase serves as valid and safe target for development of new drugs for the management of T2D. USFDA approved commercial drug by inhibiting α- amylase. Voglibose®(Acarbose) inhibits α-amylase in a non-selective manner and has severe side effects on prolonged use such as abdominal pain, flatulence and diarrhoea.

However, there are many side effects associated with these drugs, most common being bloating, abdominal discomfort,diarrhoea and flatulence. (Oboh et al., 2016). In some cases as irritable bowel syndrome or severe kidney or liver dysfunction α-glucosidase are contraindicate (Sales et al., 2012). Therefore, there is need to explore alternatives for these inhibitors. Thus there is need of selective α-amylase inhibitors which has lesser side effects. Plants like *Vitis vinifera* and *Cinnamomum verum* have been reported to possess potent α-amylase inhibitory activity in their extracts. However resourcing large amount of plant material for isolation of drugs becomes a formidable task apart from having ecological issues. (Sales et al., 2012).

Endophytes are the microorganisms that live within the plant tissue atleast for one life cycle without causing any injury to the plants and they are symbiotically associated. (Silva et al., 2011; Strobel, 2002; Wang et al., 2015). Endophytic fungi have the capability to produce bioactive compounds such as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones (Fráková al., 2007)exhibiting potent inhibitory action against various metabolic enzymes such as lipase, tyrosinase , α-amylase (Gupta et al., 2014).There is possibility endophytic fungi might produce α-amylase inhibitors as endophyte share the therapeutic properties of the host plant (Zhao et al 2010).

In present study, 105 isolates were taken and subjected to preliminary screening of α-amylase producers and α-amylase inhibitor by agar well diffusion assay. The assay was based on starch hydrolysis. In case of inhibitors there was decrease in the zone of hydrolysis as compared to Control. (Randhir et al., 2007).Out of 105 cultures screened, only 5 cultures showed potent activity of α-amylase inhibitor. On the basis of percentage of inhibition #107VVLSWN was chosen for further studies.

α-amylase activity using microtiter plate based spectrophotometric assay was carried out which comprised of optimal substrate concentration, enzyme concentration, pH and incubation time. In earlier studies Sudha et al. (2011), screened 126 extracts in which 21 extracts showed positive result. The maximum inhibition of 60.5 % was observed for isopropanol extract of *M. alba f*ollowed by *L. Usitatisumum,*which showed inhibition of 55.7%, where as in our study DCM fraction of #107VVLSWN exhibited the 70% of inhibitory activity againstTyrosinase.

Thus, the endophytic fungi exhibiting potential α-amylase inhibitory activity was #107VVLSW.The organism was identified using morphological as well as molecular tools. Morphologically, the endophyte belongs to genus *Alternaria*. The morphology was carried out by following the book by Barnett et al 1998 and the sequencing is under way.

There are many reports about antimicrobial compounds produced by endophytes in cultures that were active against plant and human pathogenic microorganisms. Chareprasert et al. (2006) reported an antimicrobial activity exhibited by endophytic fungi isolated from teak and rain trees. These fungi were found to produce some metabolites active against bacteria and yeast. From 67 endophytic fungi isolated from Q. variabilis, 19.4% (Aspergillus sp., Penicillium sp., and Alternaria sp.) showed significant antimicrobial activity (Wang et al. 2007). The isolate #107VVLSWN which is tentivtely identified as Alternaria sp. possess best invitro-antimicrobial activity against *E.Coli*, *S.aureus* (MTCC 96, MTCC 737), and *Bacillus subtilis*. In our study Chloroform fraction of #107VVLSWN exhibited the maximum anti-microbial activity against *E.coli* and Methanol fraction against E.Coli &*B.subtilis*.

An enormous variety of plants have been studied for new sources of natural antioxidants.Phenols and flavonoids are well-known bioactive metabolite responsible for the antioxidant potential of the natural products (Baghiani et .,al 2010). There are many reports on contradictory results about the correlation between the total phenolic content and antioxidant potential of the extract. Yadav et al. (2014) have described about the positive correlation

between the antioxidant capacities of the extract to the phenolic constituents, in our study of the fungal endophyte Ethyl acetate fraction of #107VVLSWN exhibited maximum 72.63% while minimum 38.41% of free radical scavenging activity in Methanol fraction as compared to standard Quercitin 82.58 ± 0.006% (50 µg/ml).

TLC analysis of DCM fraction of #107VVLSWN was performed according to Sonkamble et al. , 2014. They have observed nine bands in case of chloroform extract and ten bands in case of ethanol extract. Whereas in our study DCM fraction of #107VVLSWN separated into 6 bands in mobile phase of Hexane: Toluene: Formic acid in 3:2:0.1 ratio.

The present results may lead to the conclusion that endophytes are considered to be a potential source for novel bioactive products (Strobel 2003). The data presented in this study demonstrated that extracts of endophytic fungus #107VVLSWN (*Alternaria* sp.) could be a novel source for α-amylase inhibitors and also have antimicrobial and antioxidant properties.

Chapter – 7

CONCLUSION

CONCLUSION

In the present study it can be concluded that #107VVLSWN,isolated from *vitis vinifera* is a potential producer of α-amylase inhibitor.

On quantitative estimation DCM fraction of #107VVVLSWN exhibited best activity. extracted fraction was also subjected for the study of antimicrobial and antioxidant assay. The antimicrobial studies showed that maximum antimicrobial activity was seen in chloroform extract against *E.co*li. The TLC was also done for the DCM fraction of the isolate #107VVLSWN and 6 bands where observed in the solvent system Hexane: Toluene: Formic acid in ratio of 3:2:.01.

On performing morphological studies of the isolate, it was tentatively identified as *Alternaria* sp. The genomic DNA of the selected isolate was extracted, amplified and further send for sequencing for molecular identification.

Chapter – 8

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BIBLIOGRAPHY

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APPENDIX

Media

1. **Potato dextrose agar**

   Potato dextrose agar – 39gm

   Distilled water – 1L

   Final pH (at 26°C)- 5.6±0.2

   Autoclave at 121ºC for 15 min

2. **Malt extract agar**

   Malt extract – 30gm

   Mycological peptone – 5gm

   Agar – 15gm

   Distilled water – 1L

   Final pH - 7.6 ± 0.2 at 37°C

   Autoclave at 121ºC for 15 min

3. **Synthetic nutrient deficient agar**

   Glucose – 0.2gm

   Sucrose – 0.2gm

   Pot. dihydrogen phosphate – 1gm

   Potassium nitrate – 1gm

   Magnesium sulphate – 0.25gm

   Potassium chloride – 0.5gm

   Agar – 15gm

   Distilled water – 1L

   Final pH – 5.4 ± 0.2 at 26°C

   Autoclave at 121ºC for 15 min

4. **Water agar**

   Agar – 15gm

   Distilled water – 1L

   Autoclave at 121ºC for 15 min

5. **Pine leaf agar**

   Pine leaves

   Agar – 15gm

   Distilled water – 1L

   Autoclave at 121ºC for 15 min

Buffers

1. **50XTAE**

   Tris base – 242g

   Glacial acetic acid – 57.1ml

   0.5M EDTA – 10ml

   Distilled water – 1L

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

   10 mM EDTA - 0.1 mM

   Distilled water - 100ml

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