SCREENING OF ENDOPHYTIC FUNGI FOR CHOLESTESTEROL OXIDASE PRODUCER(S)

A
Thesis submitted
in partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

By

SHRUTI BINDAL
Roll no. 300901012

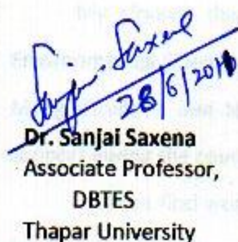
Under the supervision of

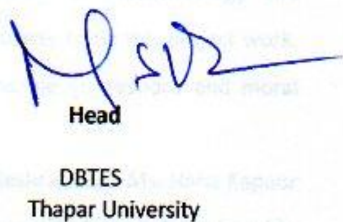
Dr. Sanjai Saxena

Department Of Biotechnology and Environmental Sciences
THAPAR UNIVERSITY, PATIALA
JUNE, 2011

Certificate

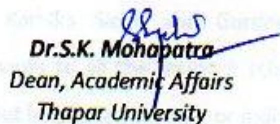
This is to certify that the thesis entitled **"Screening of Endophytic Fungi for Cholesterol oxidase producer(s)"** being submitted by **Shruti Bindal (Roll no. 300901012)** in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.





Dr. Sanjai Saxena
Associate Professor,
DBTES
Thapar University

Head
DBTES
Thapar University



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Thapar University

Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled **"Screening of Endophytic Fungi for Cholesterol oxidase producer(s)"** in partial fulfilment of the requirements for the award of degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of Jan 2011 to Jun 2011, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Place: Patiala

Date: 29/6/11

Shruti Bindal

(Roll No. 300901012)

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

Dr. Sanjai Saxena

Associate Professor / Supervisor

Head

(DBTES)

Department of Biotechnology & Environmental Sciences

Thapar University, Patiala 147004

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Date:

Place: **Shruti Bindal**

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ABBREVIATIONS

LDL Low Density Lipoproteins

VLDL Very Low Density Lipoproteins

HDL High Density Lipoproteins

ChOx Cholesterol Oxidase

FAD Flavin Adenine Dinucleotide

GMC Glucose/Methanol/Choline

VAO Vanillyl-Alcohol Oxidase

POD Peroxidase

BSA Bovine Serum Albumin

NBT Nitro blue tetrazolium

PDA Potato Dextrose Agar

VCA Vogel's Cholesterol Agar

VCSPA Vogel's Cholesterol Sucrose Potato dextrose Agar

PDB Potato Dextrose Broth

VCB Vogel's Cholesterol Broth

GAA Glacial Acetic acid

PAGE Poly-acrylamide Gel Electrophoresis

EXECUTIVE SUMMARY

Increasing concern for cholesterol related disorders, like atherosclerosis, cardiovascular disease and myocardial infarctions, has made Cholesterol oxidase the second largest diagnostic enzyme in demand, after Glucose oxidase, in clinical industry. Moreover, ChOx has been used in various other applications, such as, in insect resistant GM-crops, monitoring and regulation of cholesterol levels in food stuffs and many more such uses in food, agriculture and clinical industries. Investigators have been searching novel Cholesterol oxidase sources, with high level of production capacity, so that this high demand of ChOx in the market can be met efficiently.

In the current study, we explored a new group of microorganisms, the endophytic fungi, for the production of cholesterol oxidase. The potential of endophytic fungi to degrade/utilize cholesterol was assessed by growing fungi on a solid medium containing cholesterol as a sole carbon source. Isolates those are capable of growing on such medium showed either the filamentous growth or a clear zone around the colony, indicating utilization of cholesterol. Such isolates were then raised in production medium (Vogel's Cholesterol Broth supplemented with 0.02% cholesterol).

Four of the culture filtrate viz. #1022 AMSTITYEL, #1048 AMSTITYEL, #1082 AMSTITYEL, and #23(a) RSSTNEY showed acceptable activity against cholesterol, wherein, #23(a) RSSTNEY showed maximum potential.

#23(a) RSSTNEY is an endophytic fungus isolated from *Rawolfia serpentina*, a medicinal plant. Enzyme activity in crude protein precipitate obtained from cell free filtrate of this isolate was assessed by an enzyme assay, designed on the basis of the method described by Waybenga *et al.* (1970), and the enzyme activity staining.

Further studies on protein purification, characterization, kinetics would open up possibilities of protein engineering for commercial exploitation of this enzyme.

Chapter 1

INTRODUCTION

1.0 INTRODUCTION

Cholesterol is a metabolically important biomolecule. It is essential for maintaining membrane structure of cells in the eukaryotes as well as acts as a precursor for the synthesis of bile in liver, vitamin D, and hormones like cortisol, aldosterone, progesterone, estrogens and testosterone and their derivatives (Fig 1). Cholesterol is transported through lipoproteins in the blood as it is

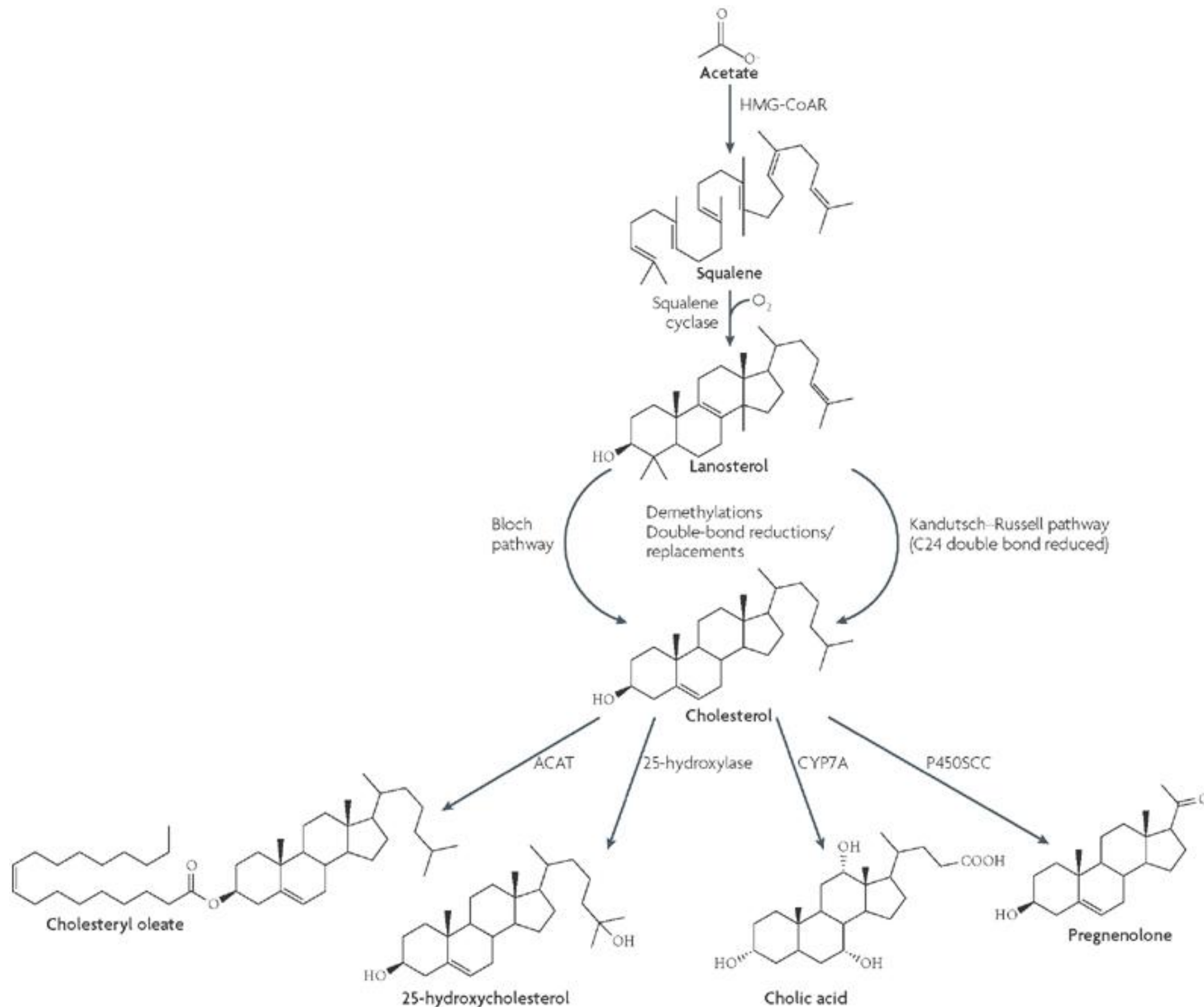

Figure 1 Cholesterol Metabolism

insoluble in water. These lipoproteins have been classified according to their density as HDL [high density lipoproteins], LDL [low density lipoproteins], VLDL [very low density lipoproteins] and chylomicrons. LDL molecules are the major carriers of cholesterol in the blood, and each one contains approximately 1,500 molecules of cholesterol ester. Abnormal cholesterol levels also known as Hypercholesterolemia are characterized by presence of higher concentrations of

LDL as compared to HDL's and are associated with cardiovascular diseases since it leads to atheroma development in arteries known as atherosclerosis . Atherosclerosis in turn is responsible for myocardial infarction (aka heart attack) and peripheral vascular disease. Elevated cholesterol levels are treated with a strict diet consisting of low-saturated fat, trans-fat free, low cholesterol foods, often followed by one of various hypolipidemic agents such as statins, fibrates, cholesterol absorption inhibitors, nicotinic acid derivatives or bile acid sequestrants.

The determination of serum cholesterol is a method to assess diseases like atherosclerosis, coronary heart disease and other lipid disorders, and for determining the risk of heart attack and thrombosis. Cholesterol oxidase (ChOx), a 3β-hydroxysterol oxidase (EC1.1.3.6), is a flavin adenine dinucleotide (FAD)-dependent enzyme that catalyzes two reactions in one active site: the initial oxidation of a Δ^5-ene-3β-hydroxysterol to Δ^5-ene-3β-ketosteroid, with the reduction of molecular oxygen to hydrogen peroxide, followed by isomerization to yield a Δ^4-ene-3β-ketosteroid as the final product. In bacteria, ChOx is the first enzyme in the catalytic pathway that yields propionate and acetate as final products. Importantly, there is no mammalian homolog of ChOx. ChOx has been used in clinical chemistry to measure serum cholesterol since 1973.

Cholesterol and its oxides have been detected in a variety of foods and foodstuffs, especially eggs, milks, meats, seafoods and their processed products (Paniangvait *et al.* 1995). Around 74 cholesterol oxidation products have been identified, but many more remain unidentified thus far. The bacterial degradation of cholesterol by cholesterol oxidase have largely been reported from bacteria like *Brevibacterium* (Uwajima *et al.* 1973), *Corynebacterium* (Shirokane *et al.* 1977), *Pseudomonas* (Aono *et al.* 1994), Rhodococcus (Watanabe *et al.* 1986; Kreit *et al.* 1994; Sojo *et al.* 1997). Although, there are many reports on steroids degradation by fungi, but cholesterol oxidase production, from this eukaryotic microorganism, has been reported for some species in the genera *basidiomycetes*, for example *Schizophyllum commune* (Fukuyama and Miyake 1979). Endophytic microorganisms are the microbes which colonize in the internal lining tissue of plants without exhibiting any signal of their existence within the system (Bacon and White, 2001). The most frequently isolated endophytes are fungi, and it

seems obvious that they are a rich source of genetic diversity and a pool of novel secondary metabolites (Strobel, 2003). Endophytes produce various enzymes necessary to colonize plant tissue. Most of the investigated endophytes utilize xylan and pectin, show lipolytic activity and produce non-specific peroxidases and laccases, chitinase and glucanase (Firakova, 2007). In the present proposal study we propose to screen the potential of cholesterol utilization by endophytic fungi, assuming that they have the capacity to undertake the bioconversion of phytosterols.

Chapter 2

REVIEW OF LITERATURE

2.1. Cholesterol and Cholesterol oxidase

Cholesterol is a major constituent of most eukaryotic plasma membrane. It performs an array of functions which begin from regulating the membrane fluidity, increases membrane thickness, establishes the permeability barrier of the membrane, modulates the activity of various membrane proteins, and is the precursor for steroid hormones and bile acids (Yokoyama, 2000). The total demand for cholesterol of an organism is fulfilled by its system itself. Therefore, all the cholesterol taken up from external sources is surplus and results in improper maintenance of cholesterol in the body, which can further leads to various physiological disorders. Role of cholesterol in atherosclerosis, cardiovascular diseases, and cerebrovascular diseases has long been at the forefront of medical sciences. More recently, cholesterol also thought to encroach strongly upon the field of dementia and neurological diseases, in particular its possible role in development of Alzheimer's disease (Harris and Milton, 2010).

Importance of cholesterol level monitoring during diagnosis of various disease have resulted in the emergence of cholesterol oxidase enzyme in clinical industry. Richmond, in 1973, isolated cholesterol oxidase from *Nocardia sp*., and then utilized the enzyme for the determination of serum cholesterol, that ousted the traditional methods like Libermann-Burchard reaction. More specific and easy determination of serum cholesterol has made ChOx the second most demanded enzyme in clinical diagnosis, following glucose oxidase. By then, ChOx has been exploited in various other fields, like agriculture, biotechnology, etc.

Cholesterol oxidase (ChOx, EC 1.1.3.6), or more precisely, 3β-hydroxysterol oxidase catalyzes oxidation of the C3-OH group of cholesterol (and other sterols) to give the corresponding Δ^5-3-ketone (cholest-5-en-3-one) and its isomerization to Δ^4-3-ketone (cholest-4-en-3-one) (Fig. 2). In bacteria, ChOx is the first enzyme in the catalytic pathway that yields propionate and acetate as final products. Importantly, there is no mammalian homolog of ChOx.

There are two forms of cholesterol oxidase, one containing the FAD cofactor non-covalently bound to the enzyme (class I) and another containing the cofactor covalently

linked to the enzyme (class II; Sampson and Vrielink 2003). It has been found that there is no sequence homology between the two and they belong to two different protein families. The class I enzyme belongs to the GMC (glucose/methanol/choline) oxidoreductase family, while the class II enzyme belongs to the VAO (vanillyl-alcohol oxidase) family, a group of enzymes which contain a fold proposed to favor covalent flavinilation (Doukyu, 2009).

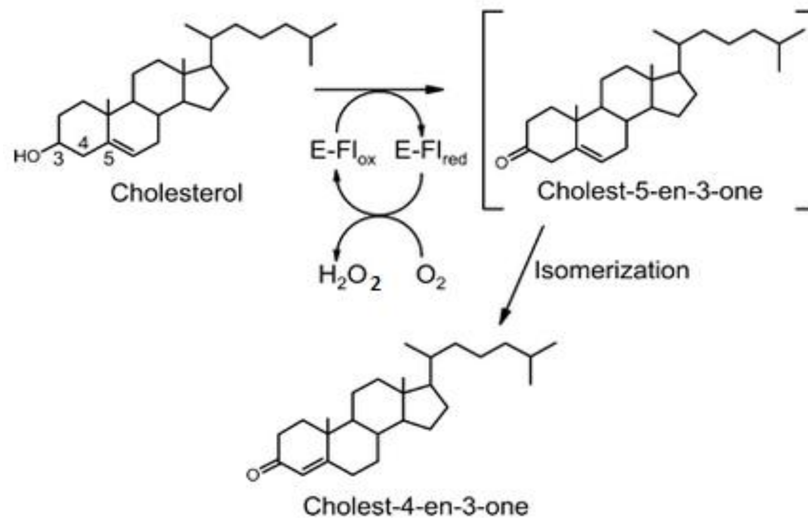

Figure 2 Mechanism of Cholesterol Oxidase Action

2.2 Sources of ChOx and screening of ChOx producers

ChOx was first isolated and characterized from *Rhodococcus erythropolis* by Turfitt (1948) and since then it has been found to be produced by a vast array of gram positive and gram negative microorganisms. Actinomycetes however are the most prolific producers of cholesterol oxidase. The production of ChOx is both intracellular as well as extracellular. Subsequently production of cholesterol oxidase was reported in soil *Mycobacterium* (Schatz *et al.*, 1949). Stadtman *et al.* (1954) first isolated the product 4-cholesten-3-one from the incubation with a cell free extract of the soil Mycobacterium. ChOx has been isolated from various microorganisms such as *Arthrobacter* (Arima et al; 1969), *Brevibacterium*, *Corynebacterium, Mycobacterium, Nocardia* (Buckland et.al, 1976)*, Rhodococcus, Streptomyces*, and *Streptoverticillium* (Doukyu, 2009). The enzymes have also been isolated from several gram-negative bacteria such as *Burkholderia* (Doukyu and Aono 1998), *Chromobacterium* (Doukyu *et al.* 2008), and *Pseudomonas* (Lee *et al.* 1989). A cholesterol oxidase from a eukaryotic microorganism identified as *basidiomycetes*,

Schizophyllum, has also been reported (Fukuyama and Miyake 1979). Among the ChOx-producing bacteria, *R. equi* (previously identified as *B. sterolicum* ATCC 21387) exhibits high levels of ChOx activity, but commercial use of this microorganism is limited because of its highly pathogenic nature (Pollegioni, 2009).

Generally, for screening microorganisms for ChOx production, they are supplemented by cholesterol in the medium to induce the production of ChOx. Sometimes, cholesterol is supplemented as a sole carbon source in the medium (Kanchana *et al.*, 2011). γ-Proteobacterium (Isobe *et al.* 2003) and Pseudomonas sp. (Lee *et al.* 1989) have been isolated using a ChOx screening method similar to that of colony staining which is based on the principle of Allain's method. In this method, filter papers that were dipped into a solution containing color developing reagents were placed on colonies grown on an agar medium. Cholesterol oxidase activity of the test colonies was indicated by a red color due to the formation of quinoneimine dye (Doukyu, 2009). The production of 4-cholesten-3-one can be indicated by halo formation on the agar medium (MacLachlan, 2000). In case of fungi, both filamentous growth and clear-zone formation have been used to indicate the utilization of cholesterol by the fungi when used as a sole carbon source.

2.3 Applications of ChOx

ChOx is a diagnostic enzyme since it helps in determining the cholesterol content of serum from the patients, in the cell membrane of erythrocytes (Patzer *et al.* 1978), in human bile and gall stones. Traditional methods like Libermann Burchard's reactions were replaced by the enzymatic method. More specificity could be achieved by this method based on the amount of hydrogen peroxide produced during the reaction. This is a three step reaction in which the cholesterol is de-esterified by the enzyme cholesterol esterase, followed by ChOx converting it into Cholest- 4-en-3-one and

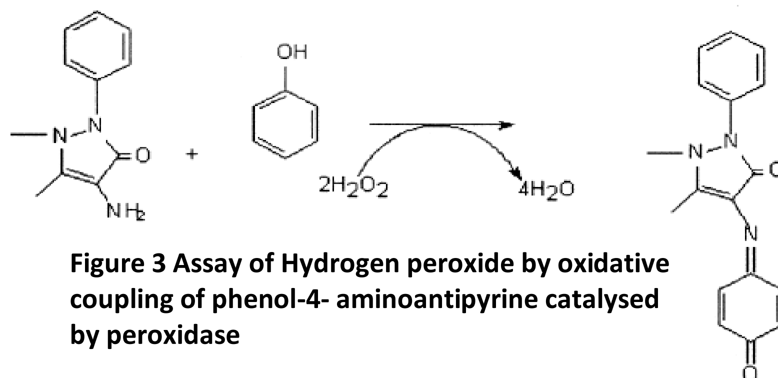

Figure 3 Assay of Hydrogen peroxide by oxidative coupling of phenol-4- aminoantipyrine catalysed by peroxidase

finally oxidative coupling of hydrogen peroxide formed with 4-aminoantipyrine and phenol to form a red quinoneimine dye which can be measured spectrophotometrically.

Determining the concentration of serum cholesterol is fundamental in the assessment of a variety of diseases (e.g. in atherosclerosis and other lipid disorders) and for estimating the risk of thrombosis, myocardial infarction, etc. More recently it has been proved that the β–amyloid formed during Alzheimer's disease combines with Cu^{2+} ions and converts the brain cholesterol into cholest-4-en-3-one, thus mimicking the activity of ChOx. It has been observed in clinical studies that the cholest-4-en-3-one content in brain is two folds higher in patients suffering from Alzheimer disease compared to control (Puglielli et al, 2005).

As cholesterol oxidase possess a broad range of substrate specificity and therefore could be exploited for biotransformation of 3β- hydroxysteroids for the synthesis of steroids and pharmaceutically important steroidal molecules in presence of organic solvents in an aqueous medium containing modified cyclodextrin (Guo *et al.* 2003; Alexander and Fisher 1995). It can also be used for optical resolution of allylic alcohols (Biellmann, 2001).

In a screening program in search of a suitable insecticide the researchers at Monsanto Inc, (USA) found a actinomycete culture filtrate possessing strong larvicidal activity of the Boll weevil (*Anthonomus grandis grandis* Boheman). Subsequent isolation and characterization of the active larvicidal protein lead to Cholesterol oxidase. Purified ChOx is active against boll weevil larvae at a 50% lethal concentration (LC_{50}) of 20.9 μg/ml, which is comparable to the bioactivity of *B. thuringiensis* proteins against other insect pests (Purcell et al, 1993).

Cholesterol oxidase also finds applications in the food industry for reducing the cholesterol content in the non-vegetarian foods. Pimaricin is a macrolide antifungal antibiotic widely used in the food industry. The antifungal activity of pimaricin is involved in its interaction with membrane sterols, causing the alteration of membrane structure and leading to the leakage of cellular materials. *S. natalensis* cholesterol oxidase has been described as a key enzyme in the biosynthesis of the polyene macrolide pimaricin (Mendes *et al.* 2007; Aparicio and Martín 2008). Pimaricin is used as an antibiotic preservative in the manufacture of cheese and non-sterile food and also for treatment of fungal keratitis wherein it causes the cell leakage thereby killing the pathogenic fungus.

2.4 Endophytic fungi as a source of enzymes

As fungal endophytes reside within the plant system they have evolved mechanisms to colonize within the host plants by production of enzymes which help them in directly or indirectly procuring plant nutrients and cellular components to grow and flourish. One hypothesis prevails that the enzymes assays can provide a clue that fungi can change their mode of life from an endophyte, to a saprobe or pathogen. The commonly tested enzymes are cellulases, amylases, pectinases, laccases, ligninases and xylanases (Choi et al, 2005). A novel Sago starch degrading glucoamylase has been isolated from endophytic *Acremonium* sp. The enzyme possesses a strong amylopectin degrading activity with optimum temperature and pH for enzyme activity around 55°C and 5.5, respectively. The enzyme was stable in a pH range of 3.0–7.0 and temperatures up to 60°C (Marlida et al, 2000). *Monotospora* sp. was the first endophytic fungus producing the enzyme laccase (Wang et al, 2006). *Periconia* sp. (BCC2871), an endophytic fungus belongs to the phylum Ascomycota was found to produce a novel thermostable β-glucosidase which was later cloned and expressed in the methylotropic yeast *Pichia pastoris* (Harnpicharnchai et al 2009). However there are no reports of endophytic fungi producing Cholesterol oxidase.

2.5 Novel ChOx sources: the need

Wide and varied uses of ChOx in clinical, agricultural and biotechnology industry display the ever growing demand of this enzyme. Various strategies have been applied to get higher yields of ChOx from diverse microbial sources. *Rhodococcus equi* exhibits the highest levels of cholesterol oxidase activity of any of the known ChOx producing bacteria, but its highly pathogenic nature has ruled out its use for commercial production (MacLachlan, 1999). ChOx genes from several sources have been cloned and expressed as recombinant proteins, for example, expression of the gene encoding *Rhodococcus equi* cholesterol oxidase in *Streptomyces lividans* that resulted in a yield of protein production approximately 85-fold higher than that from the natural organism (Ohta *et al.* 1992). Down-streaming problems associated with intra-cellular and membrane bound forms of enzyme demand more and more sources of extracellular form. The highest production levels have been reported in the non-

pathogenic *Streptomyces* sp., reaching up to 2500 U/L in fermentation broth in terms of purified protein (Pollegioni, 2009). The current sources of cholesterol oxidases are primarily prokaryotic exception being *Schizophyllum commune* a basidiomycetous fungus. For applications in agriculture and for human diagnostic purposes a eukaryotic source of cholesterol oxidase would be more appropriate. It could be easier for cloning and expression of gene responsible for ChOx production, into the plant system, make the plant resistant to many insects, for example, boll weevil (*Anthonomus grandis grandis* Boheman) larvae, by destroying their mid-gut epithelial cell membrane. Gene from a eukaryotic system is easier to express in plant, as there are several factors inside the cell which regulates the expression of genes.

2.6 Preliminary Screening: ChOx producers

Solid media based enzyme assay is a cheaper and faster alternative as comprared to individually assessing enzymatic activity from the fungal fermentation broths of endophytic fungi. Thus solid media based assays can provide a rapid, large scale screening to ascertain extracellular expression level of specific enzymes (Hankin, 1975). The cholesterol oxidase producing microbes have largely been screened on a solid based medium having cholesterol as the sole carbon source for example Arima Medium (Arima et al, 1969). Takagi *et al.* (1982) examined the effect of the adsorption of oleic acid onto the cell surface of *Schizopyllum commune* upon cholesterol oxidase production. Thus, instead of cholesterol various other media supplements have been examined to enhance the ChOx production by different microorganisms. Potato starch and malt extract were also used for the production of ChOx from *Streptomyces lavendulae* NCIM 2421 (Verma and Nene, 2003).

2.7 Enzyme Assay

The objective of measuring enzyme activity is normally to determine the amount of enzyme present under defined conditions, so that activity can be compared between one sample and another. Study of enzyme activity can be done by either following the loss of the substrate or the formation of one or more products. Also, it can be performed directly with the enzyme and the substrate in study, or can be coupled with any other enzymatic reaction whose monitoring

is easier. Usually, two methods are employed for enzyme activity assays, the stop-time assay and the real-time assay. Optimal pH, saturating substrate concentrations, and the temperature convenient to control, are some of the important conditions during an assay, which has to be maintained properly for reproducible results. Coupled reactions are also utilized for assessing the cholesterol oxidase activity. Horseradish peroxidase isoenzyme C (POD; hydrogen peroxide oxidoreductase donor(EC 1.11.1.7) is a classic heme enzyme containing a ferric protoporphyrin IX prosthetic group (Chakrabarti and Basak, 1996) and the prototypic class III plant peroxidase (Welinder *et al.*, 1992). ChOx and POD reduces the colour reagent prepared from 4-aminoantipyrine (4-AAP), Phenol and Sodium phosphate buffer which can be measured at 505nm spectrophotometrically (Torabi et al, 2007). The procedure described by Wybenga *et al.* (1970) for direct manual determination of residual cholesterol was employed. The assay is based on the reduction of cholesterol concentration in a mixture containing the fermentation filtrate or crude enzyme exhibiting cholesterol oxidase activity.

2.8 Protein Purification

Protein precipitates are aggregates of protein molecules large enough to be visible and collected by centrifugation. Precipitation can be promoted by agents such as neutral salts (e.g. Ammonium sulfate), organic solvents (e.g. Acetone), high molecular mass polymers (e.g. Polyethylene glycol), or by appropriate pH adjustments. Ammonium sulfate is likely the most common precipitant utilized. This neutral salt is popular due to its high solubility, inexpensiveness, lack of denaturing properties towards most of the proteins, and its stabilizing effect on many proteins (Walsh, 2002). Like most of steps in protein purification protocol, ammonium sulfate fractionation is a compromise between recovery and purity. Keeping the temperature low, to increase stability and decrease solubility, it is best to operate at a neutral pH between 6.5-7 (Rosenberg, 2005). Initially the addition of neutral salts often increases the solubility of proteins in a solution, known as 'salting-in' effect. However, on increasing salt concentration over a particular level leads to destabilization of proteins in the solution and eventually promotes their precipitation. At high concentration, such salts compete with the protein molecules for water of hydration, which promotes protein-protein interaction. This

phenomenon is called ‘salting-out’ effect. Ammonium sulphate fractionation/ precipitation method has been used for isolation of cholesterol oxidase from Pseudomonas sp. (Doukyu *et al.*, 1998); bacteria isolated from fermented flatfish (Rhee et al, 2002); *Streptomyces lavendulae* NCIM 2421 (Verma and Nene, 2003).

2.8 Total Protein estimation by Lowry’s Method

The Folin-Lowry’s method is one of the commonly used methods to determine the concentration of total proteins in a sample. It is based on the principle of formation of a colored compound which depends on the amount of protein present. In Folin-Lowry’s method, two color reactions take place. Firstly, the colored complex formed by –CONH group with copper ions in alkaline medium, and secondly, the subsequent reduction of Folin-Ciocalteau reagent (phosphomolybdicphosphotungstic acid) to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic amino acid residues present in the protein. The developed blue color absorbs strongly at 660nm. Bovine serum albumin (BSA) is used to prepare the standard stocks as it is rich in aromatic amino acids which respond to this reaction (Nigam, 2007). Cholesterol oxidase from *Pseudomonas sp*. was estimated by Rhee *et al.* in 1991 by employing Lowry’s method only. Also, referring to Kanchana *et al.*, 2011, wherein ChOx from *Micrococcus* sp. was estimated by this method.

2.9 Enzyme activity Staining

This technique is based on the principle that after the separation of enzymes on native polyacrylamide gel, localization/staining of a particular enzyme can be done based on its action on its substrate. The employed technique is somewhat similar to the Zymography technique (Leber, 1997). But instead of staining the substrate, here, the enzyme was stained. For activity staining of oxidases/dehydrogenases, whose action on their substrate results in the production of hydrogen peroxide, can be done by using Nitro blue tetrazolium (NBT). NBT reacts with H_2O_2 and reduces to a formazan dye, giving purple color at the site of action. Thus, if there is any oxidase/dehydrogenase activity on the gel containing substrate for the particular enzyme, then purple color bands can be obtained through such staining technique. Gadda *et al.* in 1997,

employed activity staining method for the characterization of ChOx from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum.* This method has also been used for other oxidases like alcohol oxidase from *Aspergillus* (Kumar and Goswami, 2008) and xanthine oxidase (Ozer *et al.*, 1998).

Chapter 3

AIM OF STUDY

3.0 Aim of the study

Aim of the current study is oriented towards the screening of endophytic fungi for the Cholesterol oxidase producers.

1. Screening endophytic fungus, an untapped source, for Cholesterol Oxidase (ChOx) enzyme producers.
2. Production and partial purification of the crude enzyme

Chapter 4

MATERIALS & METHODS

4.1 Maintenance of Pure Cultures of Endophytic Fungi

This involves preparation of Potato Dextrose Agar (PDA) plates, sub-culturing of the pure cultures from time to time so as to maintain them as pure isolates and the storage of pure cultures in PDA slants (Agarwal and Hasija, 1986).

4.1.1 Preparation of Potato Dextrose Agar (PDA) plates

39.0 g of PDA (Hi Media) was dispensed in one liter of distilled water and stirred thoroughly and autoclaved at 121 °C, 15 lbs for 15 min. Glass petri plates were sterilized at 121 °C, 15 lbs for 20 min. Then under sterile conditions 25 ml of autoclaved PDA was dispensed in sterile 90mm plates and allowed to solidify at room temperature. The plates were kept at 26 ± 2°C for quality check for one day.

4.1.2 Sub- culturing

The fungi grown on PDA plates were further sub cultured on fresh PDA plates to get pure isolates. The plates were incubated at 26 ± 2° C for maximum of 5 days or till fungal growth was seen. All procedures were carried out aseptically.

4.1.3 Maintenance of pure cultures

The pure cultures of the endophytic fungi were stored in PDA slants under optimal incubation conditions.

4.2 Screening of Cholesterol degradation/utilization

Vogel's Cholesterol Agar (VCA) media was prepared by adding cholesterol, dissolved in Triton-X-100 and isopropanol, to the Vogel's media (5 µl Triton-x-100 and 0.1 ml isopropanol per mg of cholesterol); pH was adjusted to 6.0±0.2. Cholesterol concentration of VCA media was 0.2 mg/ml. Media was autoclaved at 121 ^{o}C/15 lbs for 15 min. and the plates were poured when the temperature of the media reached around 45 ^{o}C. 5 mm mycelial plugs of 7-days old culture, grown on PDA plates, were transferred on to the VCA media petri-plate using an inoculation loop, aseptically. The plates were then incubated at 28 ^{o}C and 12 hours photoperiod. Growth of the fungi was monitored on daily basis for 15 days.

Similarly, screening in VCSPA medium, which contains sucrose (0.1%) and potato dextrose broth (PDB; 1%) in 5X Vogel's medium in addition to cholesterol (0.1%) was also done.

4.2 *In vitro* enzyme production

VCB broth was prepared having 0.2 mg/ml concentration of cholesterol; pH was adjusted to 6.0±0.2. Media was then autoclaved at 121 ^{o}C/15 lbs for 15 min. 5mm mycelial plug of 7-days old culture grown on PDA plates was transferred to 50 ml VCB media in 250 ml Erlenmeyer flask (in duplicate) under aseptic conditions. These were then incubated at 28 ^{o}C and 130 rpm for 15 days.

4.4 Protein Recovery from Cell free culture filtrate (Doukyu, 1998; Verma and Nene, 2003)

Total protein was precipitated from cell free culture filtrates by employing ammonium sulfate precipitation. Ammonium sulfate was added to the cell free culture filtrates and was kept overnight at 4 ^{o}C for proteins to get precipitated. The filtrate was then centrifuged at 15000 rpm for 15 min. at 4 ^{o}C to get a protein pellet which was then re-dissolved in phosphate buffer. Starting with 10% saturated solution of ammonium sulfate followed by centrifugation, saturation was carried till 60%, when no more precipitate was obtained.

4.5 Protein estimation by Folin-Lowry's method

Protein sample was thawed and dilutions of different concentrations of Bovine serum albumin (BSA) viz. 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, and 0.1 mg/ml were prepared for the standard curve preparation. Protein sample was diluted in double distilled water and thoroughly vortexed. 1 ml of the sample/standard dilutions was taken in respective test tubes and 1.4 ml of Lowry's reagent was added, vortexed briefly and incubated in dark for 20

	1	2	3	4	5	6	7	8	9	10	11	12
A	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
B	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
C	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
D	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
E	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
F	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
G	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T
H	E	R	S1	S2	S3	S4	S5	S6	E	E	E	T

Figure 4: Microtitre plate template for the Folin-Lowry assay to estimate total protein
Where, R= Reference; S= Standards; T= Test sample and E= Empty

min. After incubation, 0.2 ml of freshly prepared 1N Folin's reagent was added and again incubated for 30 min. in dark after a thorough mixing. Subsequently, 200 µl aliquot of each of the standard and sample reaction mixture was withdrawn and dispensed in a 96-well titer plate as per the template (Figure 4). Thereafter, the absorbance was read at 660 nm using a Biotek medium throughput reader Powerwave 340. Amount of protein in the sample was then determined by plotting the values on standard curve, prepared by using absorbance readings of BSA (Figure 5).

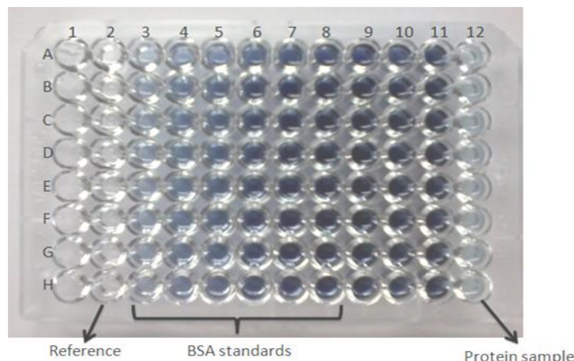

Figure 5: Folin-Lowry assay on Microplate

4.6 Enzyme Assay (as per Wybenga *et al.* 1970)

4.6.1 Standard Curve

Briefly 50 µl of different standard concentrations of cholesterol viz. 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 2.5 mg/ml and 3.0 mg/ml prepared in Glacial Acetic acid (GAA) were dispensed in 7 test tubes. Subsequently 5 ml cholesterol reagent was added in all the test tubes. The test tubes were then thoroughly vortexed and simultaneously placed in boiling water bath at 100 °C for 1.5 min. Thereafter they were cooled in tap water for 5 min followed by withdrawal of 200 µl aliquot from each test tube and then dispensed on to a 96-well titer plate as per the template given (Figure 6). Thereafter the absorption value of each well was recorded at 560 nm using a Biotek medium throughput reader Powerwave 340.

	1	2	3	4	5	6	7	8	9	10	11	12
A	E	R	S1	S4	E	E	E	E	E	E	E	E
B	E	R	S2	S4	E	E	E	E	E	E	E	E
C	E	R	S2	S5	E	E	E	E	E	E	E	E
D	E	R	S2	S5	E	E	E	E	E	E	E	E
E	E	R	S3	S5	E	E	E	E	E	E	E	E
F	E	R	S3	S6	E	E	E	E	E	E	E	E
G	E	S1	S3	S6	E	E	E	E	E	E	E	E
H	E	S1	S4	S6	E	E	E	E	E	E	E	E

Figure 6: Microtitre plate template for standard curve of cholesterol by method of Wybenga *et al.*(1970)
Where, E= Empty; R= Reference; S= Standards

4.6.2 Cholesterol oxidase activity of Culture filtrates

Cell free culture filtrates (2 ml) were taken in their respective test tubes and then 1 ml of Cholesterol solution (with initial concentration of 2 mg/ml) was added in each of the test tubes

simultaneously. These were then incubated at a required temperature for half an hour. After the incubation, 50 µl aliquot was withdrawn from each test tube and dispensed in fresh test tubes already containing 5 ml of Cholesterol reagent. The test tubes were then thoroughly vortexed and simultaneously placed in boiling water bath at 100 °C for 1.5 min. Then following exactly the same procedure as for standards, 200 µl aliquot from each test tube was withdrawn and dispensed on to a 96-well titer plate as per the template (Figure 7) and the absorbance was read at 560 nm using a Biotek medium throughput reader.

	1	2	3	4	5	6	7	8	9	10	11	12
A	E	R	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
B	E	R	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10
C	E	R	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10
D	E	R	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10
E	E	C	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10
F	E	C	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10
G	E	C	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10
H	E	C	T1	T2	T3	T4	T5	T6	T8	T8	T9	T10

Figure 7: 96 well microtitre plate template for cholesterol oxidase activity of fungal culture filtrates
Where, R=Reference; C=Control; T1=#1022AMSTITYEL; T2= #20CMBANEY; T3= #4 CMBANEY; T4= #26 CMBANEY; T5= #7 AMSTYEL; T6= #1088 AMSTITWLS; T7= #1048 AMSTITYEL; T8= #1003 AMSTITYEL; T9= #23(A) RSSTNEY; T10= #2(A) TMDSTNEY

4.6.3 Assay of crude protein

In the assay performed with crude protein, 1 ml of Cholesterol solution was incubated with 100 µl of crude protein of #23(a) RSSTNEY at four different temperatures viz. 20 °C, 26 °C , 30 °C, and 37 °C. Then, exactly as in above procedures, 50 µl aliquot of these incubated mixtures were withdrawn and dispensed in their respective test tubes that already contains 5 ml of Cholesterol reagent. Then following exactly the same procedure as for standards, 200 µl aliquot from each test tube was withdrawn and dispensed on to a 96-well titer plate as per the template (Figure 8 , Figure 9) and the absorbance was read at 560 nm using a Biotek medium throughput reader .

	1	2	3	4	5	6	7	8	9	10	11	12
A	E	R	T1	T2	T3	T4	E	E	E	E	E	E
B	E	R	T1	T2	T3	T4	E	E	E	E	E	E
C	E	R	T1	T2	T3	T4	E	E	E	E	E	E
D	E	R	T1	T2	T3	T4	E	E	E	E	E	E
E	E	S	T1	T2	T3	T4	E	E	E	E	E	E
F	E	S	T1	T2	T3	T4	E	E	E	E	E	E
G	E	S	T1	T2	T3	T4	E	E	E	E	E	E
H	E	S	T1	T2	T3	T4	E	E	E	E	E	E

Figure 9: 96 well microtitre plate template for cholesterol oxidase activity of crude protein isolated from fungal culture filtrate Where R= Reference; S= Standard; T1= Assay at 20 °C; T2= Assay at 26 °C; T3= Assay at 30 °C; T4= Assay at 37 °C

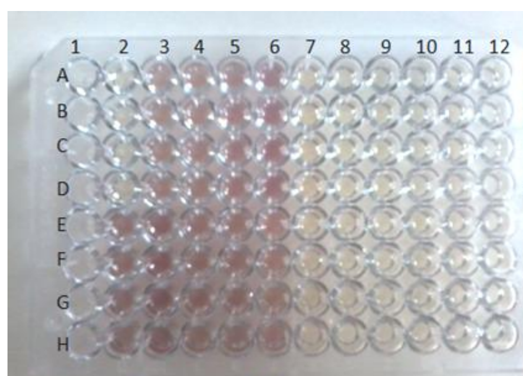

Figure 8: Cholesterol oxidase assay of crude protein on 96 well microtitre plate.

4.7 Native PAGE and Enzyme Activity Staining of Crude protein

The mixture of crude proteins obtained during ammonium sulfate precipitation was separated using 10% native poly-acrylamide gel which also incorporates cholesterol (2%; substrate for ChOx). Briefly the vertical electrophoresis apparatus was set up and then the gel was casted (Figure 10). On solidification the wells were loaded with 100 µl of sample which was prepared by adding equal volumes of protein solution and sample buffer, containing bromophenol blue (tracking dye) without reducing agents and heating. Gel was allowed to run for approximately 2hours or till the tracking dye reached the other end of the gel. After electrophoresis, gel was incubated in a 2mM solution of NBT for 24hours.

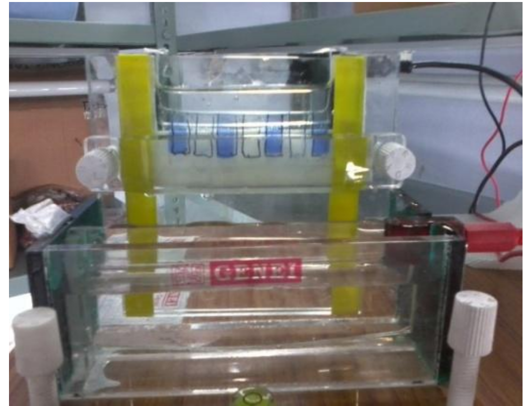

Figure 10 Vertical gel electrophoresis apparatus

Chapter 5

RESULTS AND DISCUSSION

5.1 Screening of Cholesterol degradation/utilization

Cultures of endophytic fungi, maintained on Potato Dextrose Agar (PDA), were screened for cholesterol degradation/utilization on two media viz. Vogel's Cholesterol Agar (VCA) and Vogel's Cholesterol Sucrose Potato dextrose Agar (VCSPA). 43 endophytic fungi were screened, to compare their efficiency of utilizing/degrading cholesterol in VCA as well as VCSPA.

Table 1: Screening results on VCSPA and VCS media					
S.no.	**Culture code**	**Name of Plant**	**Plant Part**	**VCSPA**	**VCA**
1	##8 AMLBRT	*Aegle marmelos*	Leaves	-	-
2	#9 AMLBRT	*Aegle marmelos*	Leaves	-	FG
3	#12 AMLBRT	*Aegle marmelos*	Leaves	-	-
4	#1016 AMLBRT	*Aegle marmelos*	Leaves	-	FG
5	#7 AMSTYEL	*Aegle marmelos*	Stem	-	FG
6	#18 AMSTYEL	*Aegle marmelos*	Stem	-	CZ
7	#23 AMSTYEL	*Aegle marmelos*	Stem	-	FG
8	#23(b) AMSTYEL	*Aegle marmelos*	Stem	-	CZ
9	#32 AMSTYEL	*Aegle marmelos*	Stem	-	FG
10	##10 AMSTITYEL	*Aegle marmelos*	Stem	-	-
11	##11 AMSTITYEL	*Aegle marmelos*	Stem	-	-
12	##22 AMSTITYEL	*Aegle marmelos*	Stem	-	FG
13	#1003 AMSTITYEL	*Aegle marmelos*	Stem	-	FG
14	#1022 AMSTITYEL	*Aegle marmelos*	Stem	FG	FG
15	#1048 AMSTITYEL	*Aegle marmelos*	Stem	-	FG
16	#1070 AMSTITYEL	*Aegle marmelos*	Stem	-	FG
17	#1082 AMSTITWAY	*Aegle marmelos*	Stem	FG	CZ
18	#20 AMSTWLS	*Aegle marmelos*	Stem	-	-
19	#12 AMBAWLS	*Aegle marmelos*	Bark	-	CZ
20	#1099 AMSTITWLS	*Aegle marmelos*	Stem	-	CZ
21	#1088 AMSTITWLS	*Aegle marmelos*	Stem	FG	FG
22	#4 CMBANEY	*Cinnamomum malabaricum*	Bark	FG	FG
23	#20 CMBANEY	*Cinnamomum malabaricum*	Bark	FG	FG
24	#21 CMBANEY	*Cinnamomum malabaricum*	Bark	-	FG
25	#26 CMBANEY	*Cinnamomum malabaricum*	Bark	FG	FG
26	#16 CMBABRT	*Cinnamomum malabaricum*	Bark	-	-
27	#1 CSSTOT	*Cimmelia sinensis*	Stem	-	CZ
28	#2 CSSTOT	*Cimmelia sinensis*	Stem	-	FG
29	#29/23 CZSTITBRT	*Cinnamomum zeylanicium*	Stem	-	CZ
30	#5 CZBAWLS	*Cinnamomum zeylanicium*	Bark	-	FG
31	#19 CZBAWLS	*Cinnamomum zeylanicium*	Bark	-	CZ
32	#23 JTLSVNP	*Jatropha curas*	Leaves	FG	FG
33	#31 JTLSVNP	*Jatropha curas*	Leaves	-	FG
34	#1 RSBANEY	*Rawolfia serpentina*	Bark	-	FG
35	#14 RSBANEY	*Rawolfia serpentina*	Bark	-	FG
36	#1 RSLBRT	*Rawolfia serpentina*	Leaves	-	FG
37	#16 RSLBRT	*Rawolfia serpentina*	Leaves	-	FG
38	#3 RSSTNEY	*Rawolfia serpentina*	Stem	-	FG
39	#23(a) RSSTNEY	*Rawolfia serpentina*	Stem	-	FG
40	#2(a) TMDSTNEY	*Tabarnaemontana divaricata*	Stem	-	FG
41	#4 TMDSTNEY	*Tabarnaemontana divaricata*	Stem	-	FG
42	#13(a) WGSTNEY	*Wild ginger*	Stem	-	FG
43	#15 WGSTNEY	*Wild ginger*	Stem	-	FG

*Filamentous growth (FG); Clear Zone (CZ); No growth (-)

Until now only *Schizophyllum commune* (a basidiomycetous fungus) has been reported to be the lone producer of the enzyme Cholesterol oxidase (Fukuyama and Miyake, 1979)

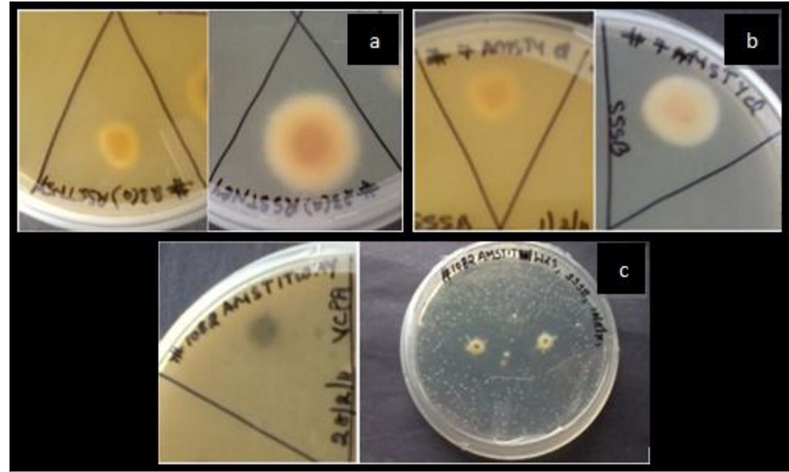

Figure 11 Cultures: (a) #23(a) RSSTNEY; (b) #7 AMSTYEL; and (c) #1082 AMSTITWLS on VCSPA (left) and VCA (right).

In VCSPA, sucrose and potato dextrose were supplemented as carbon sources, along with the cholesterol, in order to initiate the growth of the fungi on the media so that after the depletion of these carbon source fungi can switch over to cholesterol metabolism for the fulfillment of its energy requirement. Although for many cultures positive results were observed in both the media, however, for majority of the cultures best results were obtained in the medium containing cholesterol as sole carbon source with most of the cultures showing filamentous growth and some forming clear zone around the colony. Kanchana *et al.* (2011) have used medium containing 1.5% agar and 0.5% cholesterol as sole carbon source for enumeration of ChOx producing bacteria obtained from soil samples from Goa, India. On the basis of maximum growth on VCA plates after the incubation period of 14 days 13 cultures were selected for *in vitro* enzyme production in VCB broth (Table 2).

Table 2: Cultures selected for *in vitro* enzyme production

S.No.	Culture Code
1	#20 CMBANEY
2	#4 CMBANEY
3	#26 CMBANEY
4	#7 AMSTYEL
5	#1022 AMSTITYEL
6	#1088 AMSTITYEL
7	#1048 AMSTITYEL
8	#1003 AMSTITYEL
9	#2(a) TMDSTYEL
10	#23(a) RSSTNEY
11	#19 CZBAWLS
12	#29/23 CZBAWLS
13	#1099 AMSTITWLS

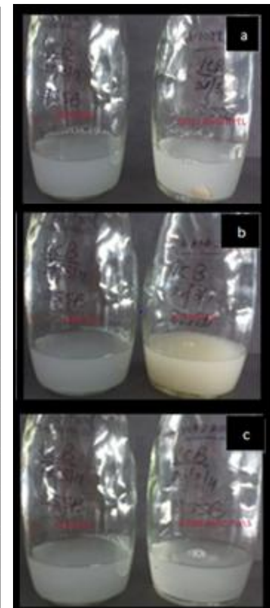

Figure 12 *In vitro* enzyme production: (a) #1022 AMSTITYEL; (b) #7 AMSTYEL; and (c) #1088 AMSTITYEL

5.2 Enzyme Assay with culture filtrates

Following the procedure described by Wybenga *et al.* (1970) standard curve of cholesterol was prepared (Figure 13) and subsequently residual cholesterol concentrations in a solution containing 2 mg/ml initial concentration of cholesterol, which was incubated with culture filtrates at 26 °C for half an hour, were determined (Figure 14). The standard curve equation was y=0.143x+0.022 with R^2= 0.979 exhibiting a high accuracy of the estimation method. Johnson et al. (1991) assayed *Rhodococcus equi* ATCC33706 cholesterol oxidase activity by estimating decrease in cholesterol concentration, using the colorimetric ferric chloride test.

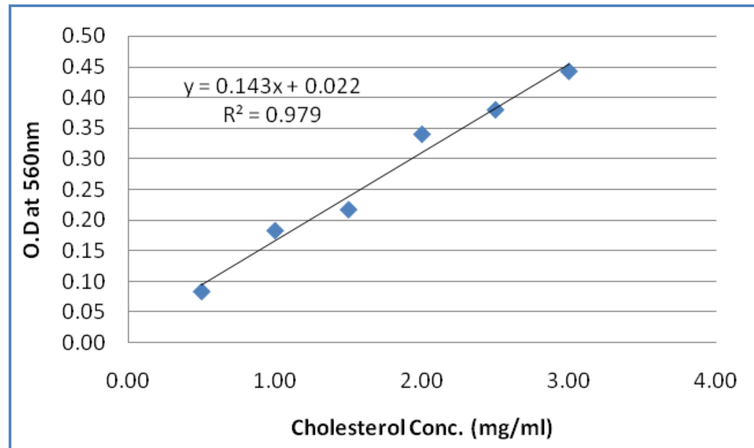

Figure 13 Standard Curve of Cholesterol by method of Wybenga *et al.* (1970) at 560 nm

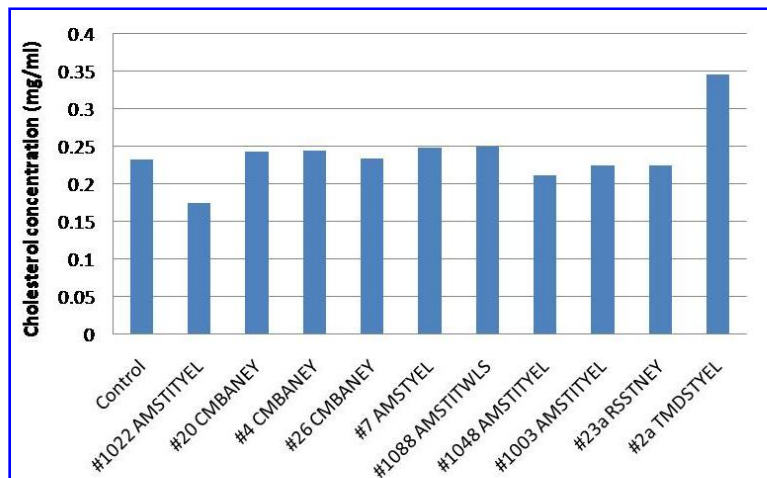

Figure 14 Comparative cholesterol reduction in different fungal culture filtrates by method of Wybenga et.al (1970) at 26 °C

Filtrates of four cultures viz. #1022 AMSTITYEL, #1048 AMSTITYEL, #1003 AMSTITYEL and #23(a) RSSTNEY demonstrated satisfactory enzyme activity at 26 °C (Figure 14).

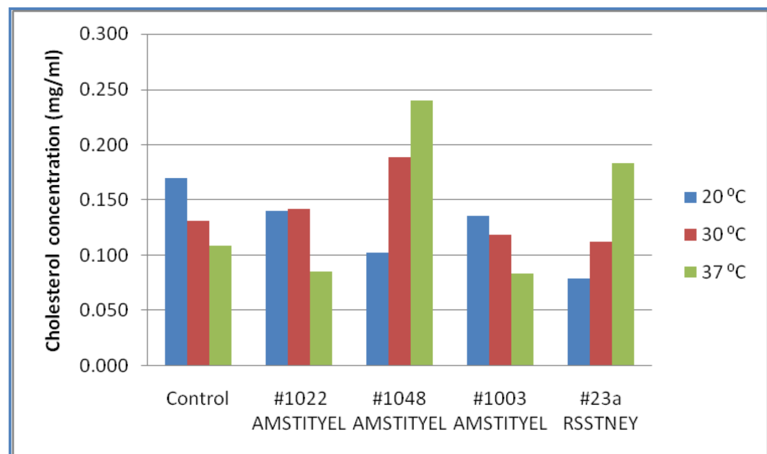

Figure 15 Comparative enzymatic activity of crude protein from different fungal isolates at different temperatures

Cell free culture filtrate have been used for the isolation of cellulases (Jahangeer *et al.*, 2005),

phytases from fungi (Marlida *et al.*, 2010). However this is the very first time when culture filtrate of endophytic fungi have been used for the screening of cholesterol oxidase activity. Then, in order to determine the optimal temperature of the enzyme for its maximum activity, these culture filtrates were then again assayed at three different temperatures of 20 ^{o}C, 30 ^{o}C, and 37 ^{o}C. It was observed that maximum cholesterol reduction occurred in the culture filtrate of #23(a) RSSTNEY at 20 ^{o}C as compared to 30 ^{o}C and 37 ^{o}C when compared to culture filtrate of other isolates. Hence #23(a) RSSTNEY was chosen for further studies (Figure 15). These results imply that the optimal temperature range for the prospective cholesterol oxidase (ChOx) enzyme produced by #23(a) RSSTNEY is 20-30 ^{o}C. Hiol *et al.* (1999) has also carried out incubation of the *Mucor himelis* culture filtrate at different temperatures to determine the optimal temperature for the lipase activity.

5.3 Production and estimation of crude protein from #23(a) RSSTNEY

Further production of enzyme by #23(a) RSSTNEY was done in a total volume of 1.2 L of VCB broth (Figure 16). Crude protein pellet was sufficiently recovered, approx. 0.304 mg/ml, from the cell free filtrate by ammonium sulfate precipitation. The total protein concentration was found to be 0.025 mg/mg of crude protein pellet, as calculated by the Bovine Serum Albumin standard curve by Folin-Lowry's method (Figure 17). Crude protein content of precipitates obtained from *Micrococcus* sp. and

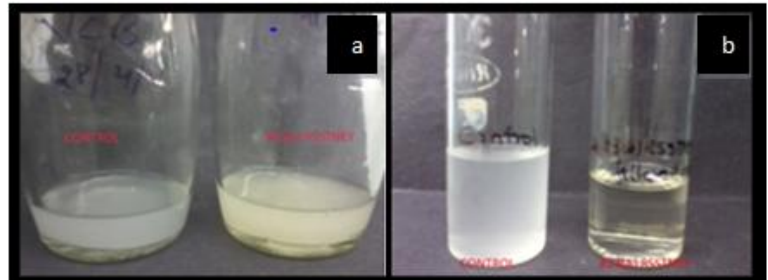

Figure 16 #23(a) RSSTNEY - (a) culture broth; and (b) culture filtrate

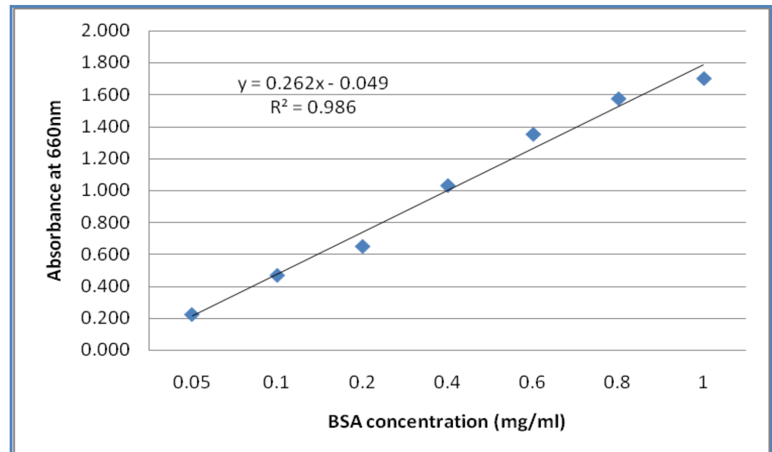

Figure 17 BSA standard curve using Lowary-Folin's

Pseudomonas sp. culture filtrates were also estimated by Folin-Lowry's method (Kanchana et al., 2011; Rhee et al., 1991).

5.4 Enzyme assay of crude protein

The crude protein obtained by precipitation was assayed at four different temperatures viz. 20 ^{o}C, 26 ^{o}C, 30 ^{o}C, and 37 ^{o}C; following the same procedure as above for culture filtrates given in section 5.2 Based on the observations it was found that maximal cholesterol oxidase activity existed at 30 °C (Figure 18). Crude alkaline protease isolated from *Aspergillus niger* exhibited a very good activity in the range of 27 °C to 37 °C. The enzyme exhibited optimal activity at 27 °C (Dubey et al, 2010).

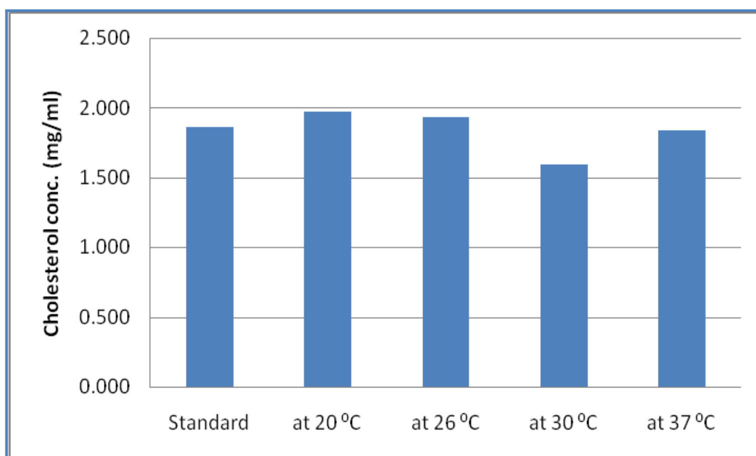

Figure 18 Enzyme assay of crude protein obtained from cell free filtrate of #23(a) RSSTNEY

5.5 Enzyme activity staining

Presence of cholesterol oxidase (ChOx) enzyme in the crude protein was assessed by the activity staining technique. On native polyacrylamide gel containing cholesterol, purple colored bands of the enzyme specific for the substrate were observed due accumulation of formazan at the site of enzyme action. As per the mechanism, oxidation of cholesterol by ChOx results in the simultaneous reduction of oxygen molecules to hydrogen peroxide. In principle, if such reactions are also supplied with a chemical species like nitroblue tetrazolium (NBT), which results into a purple

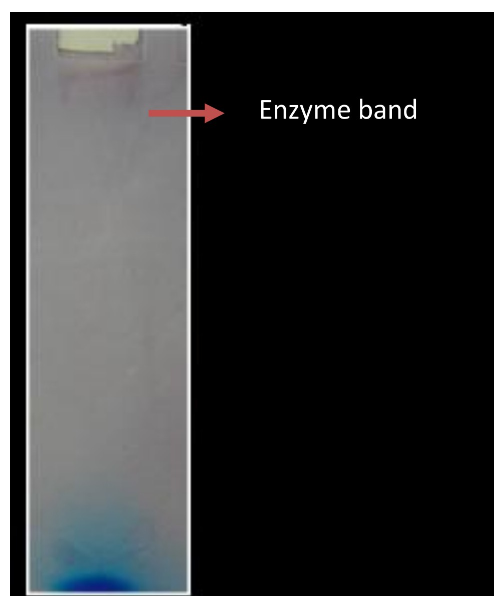

Figure 19 Activity of staining cholesterol oxidase produced by #23(a) RSSTNEY

colored formazan dye on reduction by hydrogen peroxide, then a visual analysis of such oxidation reactions can be carried out. As this result was reproduced, thus it could not be regarded as an artifact. Gadda et al. (1997) used 3β-hydroxy-5-androsten-17-one and pregnenolone as substrates in the presence of iodine tetrazolium salts for activity staining of cholesterol oxidases obtained from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum*.

Chapter 6

CONCLUSION

CONCLUSION

The current study revealed that, beside bacteria and actinomycetes endophytic fungi are capable of producing cholesterol oxidase. Almost all the endophytic fungal cultures, screened for utilization/degradation of cholesterol, showed positive results on a medium containing cholesterol as sole carbon source.

#1022AMSTITYEL, #1048AMSTITYEL, #1082AMSTITYEL (from *Aegle marmelos)* showed satisfactory enzyme activity at different temperatures while #23(a) RSSTNEY (from *Rawolfia serpentine)* demonstrated maximum potential of degrading/transforming cholesterol in an optimal temperature range of 20°C - 30 ^{o}C.

Presence of #23(a) RSSTNEY cholesterol oxidase was demonstrated partially by the enzyme assay and rest by enzyme activity staining method. But further work is required to study the enzyme kinetic and mode of action. Also decoding of the protein sequence would help in the comparison of this protein with all the other already sequenced and submitted cholesterol oxidases in the protein database. Such comparisons often tell about the novelty of the proteins.

Chapter 7

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