SCREENING OF ENDOPHYTIC FUNGI FOR XANTHINE OXIDASE PRODUCER(S)

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

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

By
AVOM GUPTA
Roll no. 300901003

Under the supervision of

Dr. Sanjai Saxena

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

Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled " Screening of Endophytic fungi as a source of Xanthine Oxidase" in partial fulfilment of the requirements for the award of degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2011 to June 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.

Avom Gupta

Patiala
Date 29-06-11

Avom Gupta
(Roll No. 300901003)

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

28/6/2011

Dr. Sanjai Saxena
Associate Professor / Supervisor

Head of the Department

Department of Biotechnology & Environmental Sciences
Thapar University, Patiala 147004

Certificate

This is to certify that the thesis entitled **"Screening of Endophytic fungi as a source of Xanthine Oxidase"** being submitted by **Ms Avom Gupta (Roll No. 300901003)** 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.

28/6/2011

Dr. Sanjai Saxena
Associate Professor,
Deptt. of Biotech. & Environ. Sciences
Thapar University

Head
Deptt. Of Biotech. & Environ. Sciences
Thapar University

Dr. S.K. Mohapatra
Dean, Academic Affairs
Thapar University

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AVOM GUPTA

TABLE OF CONTENTS

ABBREVIATIONS

ABTS	2, 2'-azino-di (3-ethylbenzthiazoline-6-sulphonate)
ATP	Adenosine Tri Phosphate
AWD	Agar Well Diffusion
BSA	Bovine Serum Albumin
ddW	Double Distilled Water
dL	deciliters
DNA	Deoxy Ribonucleic Acid
FAD	Flavin Adenine Dinucleotide
KDa	KiloDaltons
mg	milligrams
ml	milliliters
mm	millimeters
NBT	Nitroblue Tetrazolium
nm	nanometer
O/N	OverNight
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
SDW	Sterile Distilled Water
TPF	1, 3, 5 Triphenyl Formazan
TTC	2, 3, 5 Triphenyltetrazolium chloride
µl	microliters
µM	micromoles
XA	Xanthine Agar

XB	Xanthine Broth
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
XOR	Xanthine Oxidoreductase
XVA	Xanthine Vogel’s Agar
XVB	Xanthine Vogel’s Broth

EXECUTIVE SUMMARY

EXECUTIVE SUMMARY

Increasing concern for xanthine oxidase related disorders, like gout, hyperuricemia, aging, myocardial infarction, reperfusion injury and many others has made xanthine oxidase, one of the largest diagnostic enzyme in demand, in clinical industry. Moreover, XO has been used in various other applications, such as, development of miniaturized amperometric biosensors for monitoring substrate utilization in cell culture media and is required to explore answers to a number of questions like what all defects in the gene lead to various XO related disorders and to throw a light on the metabolic pathways of oxidoreductase class of enzymes. Investigators have been searching novel xanthine oxidase sources, with high level of production capacity, so that this high demand of XO 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 xanthine oxidase. The potential of endophytic fungi to degrade/utilize xanthine was assessed by growing fungi on a solid medium containing xanthine as a sole carbon and nitrogen source. Isolates those were capable of growing on such medium showed high filamentous growth indicating utilization of xanthine. Such isolates were then raised in production medium (Xanthine Broth supplemented with 2 mmoles/L xanthine).

#4CMBANEY, #21CMBANEY and #12RSBANEY showed acceptable enzyme activity while #19NOBASVNP demonstrated maximum potential. #19NOBASVNP is an endophytic fungus isolated from *Nerium oleander*, an ornamental 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 Agarwal and Banerjee, 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. INTRODUCTION

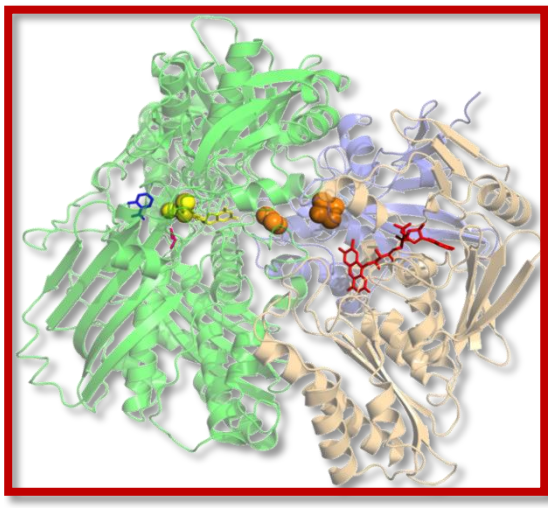


Fig 1: Crystallographic Structure of XO

Purines are heterocyclic aromatic nitrogen compounds which serve as the building blocks of DNA and RNA as well as the energy currency of the cell i.e. ATP. Adenine, Guanine, Hypoxanthine, Caffeine, Theobromine and Isoguanine are examples of purines. The catabolism of purine leads to formation of a poorly soluble compound uric acid. The enzyme responsible for the conversion of purine into uric acid via hypoxanthine is known as Xanthine oxidase (Fig 1). In human blood plasma, the reference range of uric acid is between 3.6 mg/dL and 8.3 mg/dL. Uric acid concentrations in blood plasma above and below the normal range are known, respectively, as hyperuricemia and hypouricemia. A condition wherein the uric acid from plasma precipitates as monosodium urate and gets deposited in joints, tendons or surrounding tissue then it is referred to as Gout or Podagra.

Reactive oxygen species (ROS) either superoxide anion radical or hydrogen peroxide are generated during the oxidation of xanthine to uric acid. Xanthine Oxidase (XO) and Xanthine Dehydrogenase (XDH) are interconvertible forms of the same enzyme, known as xanthine oxidoreductase (XOR) (Hille and Nishino, 1995). The enzymes are molybdopterin-containing flavoproteins that consist of two identical subunits of approximately 145 kDa. The active form of the enzyme is a homodimer of molecular mass 290 kDa, with each of the monomers acting independently in catalysis. XOR is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain as well as the plasma. Physiologically, XO and XDH

participate in a variety of biochemical reactions including the hydroxylation of various purines, pterins, and aromatic heterocycles, as well as aliphatic and aromatic aldehydes, thereby contributing to the detoxification or activation of endogenous compounds and xenobiotics (Borges et al, 2002).

There is overwhelming acceptance that xanthine oxidase serum levels are significantly increased in various pathological states like hepatitis, inflammation, ischemia-reperfusion, carcinogenesis and aging and that ROS generated in the enzymatic process are involved in oxidative damage (Hellsten et. al, 1997). Thus, it may be possible that the inhibition of this enzymatic pathway would be beneficial. Allopurinol is a structural isomer of hypoxanthine, was developed as an inhibitor to reduce the production of uric acid, and thus affects purine synthesis. Allopurinol is metabolized into oxypurinol which is also xanthine oxidase inhibitor. Allopurinol use may also causes severe hypersensitivity reaction in some individuals such as Stevens-Johnson syndrome or toxic epidermal necrolysis and a variety of drug interactions that restrict its use in some patients and limits its widespread use. Febuxostat, a novel non-purine selective inhibitor of xanthine oxidase, is a potential alternative to allopurinol for patients with hyperuricemia and gout (Tokano *et. al*, 2004). However, this is the second molecule which has been developed after Allopurinol in last 40 years.

As Xanthine oxidase is not freely available in blood for screening xanthine oxidase inhibitors; different sources were explored to isolate the enzyme for designing suitable inhibitors. Bovine Milk Xanthine oxidase (XO) also belongs to the family of iron–sulfur–molybdenum flavin hydroxylases that convert xanthine to uric acid. Ball was first to discover that milk XO is present primarily in the cream fraction and described a method of purification from milk and his method is still used for preparation of XO from milk on a commercial scale. Bovine milk XO is commercially available and is widely used for the determination of xanthine, hypoxanthine, and inosine and as an auxiliary enzyme in the determination of guanine and guanosine. It is also used for measurement of guanase and nucleoside– phosphorylase activity. Also, XO is used for development of Miniaturized Amperometric Biosensors for monitoring substrate utilization in Cell Culture Media (Mao *et. al*, 2001). Bovine xanthine oxidase is expensive and hence it is therefore pertinent to explore the microbial resources for the commercial production of xanthine oxidases.

Endophytic microorganisms especially fungi colonize healthy plant tissues and either persist in a dormant phase or comprise more extensive, but symptomless infections (Petrini, 1991). Fungal endophytes have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Bills & Polishook 1991; Strobel & Daisy 2003). A recent comprehensive study has indicated that 51% of bioactive

substances isolated from endophytic fungi were previously unknown (Schultz 2001). Fungal endophytes like *Phomopsis spp*., *Muscudor albus* (Strobel et. al, 2004), *Fusarium spp*, *Pestalotiopsis microspora* (Strobel, 2003), *Cryptosporiopsis quercina* etc are serving as prolific sources of anticancer, antifungal and antimicrobials (Strobel, 2003). Hence, the endophytic fungi are expected to be a potential source for new natural bioactive products. With this background, it can be deduced that endophytic fungi offer themselves to be potential sources of myriad of secondary metabolites and enzymes. There exist only three instances wherein the potential of secondary metabolites of endophytic fungi has been evaluated as XO producers. Hence the present study proposes the screening, isolation and characterization of XO producers from endophytic fungi.

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Xanthine and Xanthine oxidase

Xanthine (3, 7-dihydro-purine-2, 6-Dione) is a purine base which is generally found in all tissues of the body. The physical and chemical properties of this purine base have been studied and mild stimulants like caffeine and theobromine are derived from Xanthine (Konigsberger et. al, 2001). Metabolically Xanthine is produced from three different precursors (a) by action of guanine deaminase on guanine (b) from hypoxanthine by action of xanthine oxidase (c) from xanthosine by action of purine nucleoside phosphorylase (Nuki, 1983). Caffeine is a mild stimulant while Theobromine is a bronchodilator. People with the rare genetic disorder xanthinuria lack sufficient xanthine oxidase and cannot convert xanthine to uric acid (Fig 2).

Xanthine oxidase (**XO**) is a form of **xanthine oxidoreductase** that generates reactive oxygen species (ROS) and catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. This enzyme plays an important role in the catabolism of purines in some species, including humans. It is a highly versatile and ubiquitous complex molybdo-flavoprotein, which controls the rate limiting step of purine catabolism pathway. The protein is large, having a molecular weight of 270 kDa and has 2 flavin molecules (bound as FAD), 2 molybdenum atoms, and 8 iron atoms bound per enzymatic unit. The molybdenum atoms are contained as molybdopterin cofactors and are the active sites of the enzyme. The iron atoms are part of [2Fe-2S] ferredoxin iron-sulfur clusters and participate in electron transfer reactions. The chemical reactions are catalyzed by xanthine oxidase are;

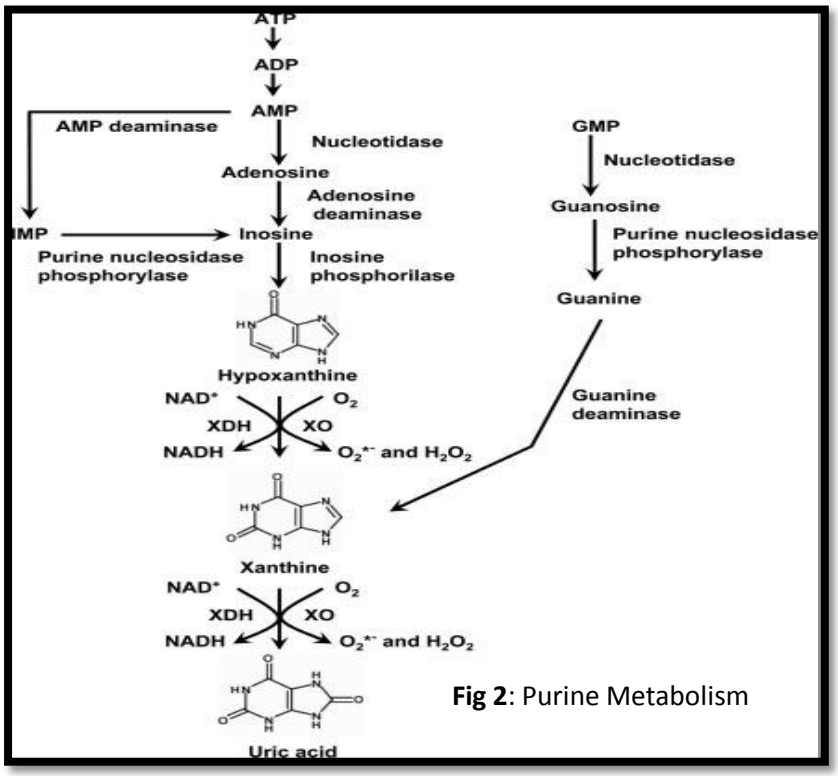


Fig 2: Purine Metabolism

- hypoxanthine + H_2O + O_2 xanthine + H_2O_2
- xanthine + H_2O + O_2 $\rightleftharpoons$ uric acid + H_2O_2

Milk is a rich source of xanthine oxidase (Ball, 1938). The isoelectronic point of milk xanthine oxidase was found to be at pH 6.2 and maximum molecular weight has been calculated to be 74,000 Daltons and kinetic studies have also been done (Escribano *et. al*, 1988). Probable role of the enzyme in milk is its antibacterial activity by virtue of its ability to produce oxygen derived free radicals (Massey *et. al*, 1968). XO also occurs in small intestine, kidney and liver where the enzyme plays the same role, that is, metabolism of purines, catalyzing the conversion of both hypoxanthine and xanthine to uric acid. A number of studies on the mechanism of action of XO enzyme have been done (Choi *et. al*, 2003; Harris *et. al*, 1997).

The mammalian enzymes, which catalyze the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of urate, are synthesized as the dehydrogenase form; Xanthine Dehydrogenase (XDH) and exist mostly as such in the cell but can be readily converted to the oxidase form; xanthine oxidase (XO), by oxidation of sulfhydryl residues or by proteolysis. XDH shows a preference for NAD^+ reduction at the flavin adenine dinucleotide (FAD) reaction site, whereas XO fails to react with NAD^+ and exclusively uses dioxygen as its substrate, leading to the formation of superoxide anion and hydrogen peroxide. The active form of the enzyme is that of a homodimer of molecular mass 290 kDa, with each of the monomers acting independently in catalysis (Enroth *et. al*, 2000).

Xanthine oxidase is commercially available and widely used for the determination of xanthine, hypoxanthine, and inosine and as an auxiliary enzyme in the determination of guanine and guanosine. It is also used for measurement of guanase and nucleoside–phosphorylase activity. Till date, Bovine milk xanthine oxidase is the only commercially available form of enzyme but this form of enzyme is presently not feasible for answering a number of questions about the eukaryotic XO enzyme physical and chemical properties and its mechanism of action.

2.2 Microbial producers of Xanthine oxidase

Microorganisms utilize purines as a nitrogen source. The utilization of these compounds presumably requires enzymes that catalyze the degradation of purines skeleton to simpler compounds. Xanthine Oxidase (XO) enzyme (EC 1.1.3.22) have been isolated from a wide

range of organisms, ranging from bacteria to humans, where the enzyme catalyzes the hydroxylation of a wide variety of substrates (purines, pyrimidines, pterins etc) and hence also plays a significant role in the detoxification or activation of endogenous compounds and xenobiotics. The enzyme has been purified from several sources, including milk. Bovine milk xanthine oxidase (XO) belongs to the family of iron–sulfur–molybdenum flavin hydroxylases that convert xanthine to uric acid and are broadly distributed in different cells and tissues and this is the only commercially available form of enzyme. As bovine milk Xanthine oxidase requires a highly expensive purification procedure and down streaming therefore it is imperative to explore active source of the enzymes requiring less expensive methods for recovering highly purified Xanthine oxidase.

Woolfolk was the first to investigate caffeine degradation by *Pseudomonas putida* (1975). Allam and Elzainy then investigated the degradation of xanthine by *Penicillium chrysogenum* (1969). *P.chrysogenum* showed utilization of the purines hypoxanthine, xanthine, uric acid and adenine as sole nitrogen sources but not the methylated purines Caffeine and Theobromine. Cell-free extracts of *Penicillium chrysogenum* contains the enzymes xanthine dehydrogenase, uricase, allantoinase, allantoicase and urease. Xanthine dehydrogenase, uricase, allantoinase and urease were constitutive whereas allantoicase was inducible by xanthine or allantoin (Allam and Elzainy, 1969). A strain of *P. roqueforti* isolated on caffeine-containing agar from air grew in culture media containing concentrations as high as 0.04 M caffeine as the sole source of nitrogen and found that the metabolism of caffeine involves demethylation at 7-position (Schwimmer *et. al*, 1971).

As the result of a survey of some 47 diverse strains of bacteria containing xanthine-oxidizing enzymes, it was observed that *Arthrobacter* S-2 gave by far the highest specific activities and the properties of the enzyme resemble those of milk xanthine oxidase (EC1.2.3.2), which has been the subject of considerable previous investigation (Woolfolk, 1978). *Alcaligenes* species CF8 isolated from surface water of a lake showed production of a novel serine type metallo-caffeine oxidase and the purified caffeine oxidase had strict substrate specificity towards caffeine and was not able to oxidize xanthine and hypoxanthine (Mohapatra, 2006). Also, the metabolism of Xanthine – 8 – carboxylic acid by *Alcaligenes faecalis* (Dairman *et. al*, 1964) has been studied. Another unique heterotrimeric caffeine dehydrogenase was purified from *Pseudomonas* sp. strain CBB1. This novel enzyme oxidized caffeine to trimethyluric acid stoichiometrically and hydrolytically, without

producing hydrogen peroxide. The enzyme was found to be specific for caffeine and theobromine and showed no activity with xanthine (Subramanian *et. al*, 2008).

Trichophyton mentagrophytes and *Epidermophyton floccosum* showed growth on media containing methylxanthine as the sole carbon and nitrogen source (Hussein *et. al,* 2009). A novel fungal Xanthine Oxidase has been isolated which has a role in sclerotial differentiation (Sclerotia are compact bodies of aggregated hyphae) and a phytopathogenic role during penetration of these fungi in the cell walls of their host plants. XO levels were higher in the sclerotiogenic strains than in the non-differentiating ones and of increasing activity throughout development (Papapostolou *et. al*, 2010).

This clearly exhibits that though the enzyme has been extracted from a number of sources yet the development of a reliable commercial mass production process for Xanthine oxidase remains to be achieved. No specific xanthine metabolizing bacterial or fungal source have been isolated till date, especially only a few caffeine oxidase have been isolated but no specific xanthine oxidase enzyme have been found in any of the known endophytic fungi. The xanthine oxidase/dehydrogenase system has been extensively studied with the bovine milk oxidase and chicken and rat dehydrogenase and has become the standards of comparison for xanthine oxidizing enzymes isolated from other sources. A novel fungal xanthine oxidase can be exploited to address a number of questions presently not possible with the available form of prokaryotic or eukaryotic form of enzymes.

2.3 Need of New XO producer

Identification and purification of a novel, highly specific and active form of xanthine oxidase enzyme from a newer source is a necessity. This search is driven by the fact that the commercially available form of enzyme that is extracted from bovine milk is highly expensive. The identification of a fungal XO can help in providing a less expensive but active and pure form of the enzyme. Also the net cost of the product will decrease as downstream recovery and purification of an enzyme from a fungal fermentation broth would be much simpler than that from bovine milk. Till date, there have been no reports for presence of a specific XO from any of the known endophytic fungi sources. The first report of Xanthine oxidase production by fungi intracellularly has been reported during sclerotial differentiation of phytopathogenic filamentous fungus like *Rhizoctonia, solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, and *Sclerotinia minor* (Papapostolou and Georgiou, 2010).

2.4 Endophytic fungi: Preliminary Screening of XO production

As such methods of screening fungi for production of Xanthine oxidase production have not been devised as this area remains underexplored. However there are methods developed for screening bacteria and fungi degrading caffeine which is a methylated form of Xanthine (Yamaoka and Mazzafera, 1999). It has been reported some genera of Pseudomonas and Serratia among bacteria and fungi belonging to the genera *Aspergillus,* Penicillium, Rhizopus and *Stemphyllium* have the capacity to metabolize caffeine enzymatically (Mazzafera, 2002). The enzymatic catabolism of caffeine in microbes is performed by *N*-demethylation and oxidation with the enzymes demethylases and oxidases, respectively (Gokulakrishnan et al., 2005). Majority of the screening and production of caffeine oxidase have been carried out in medium comprising of caffeine for the induction of enzyme production as a carbon source along with basal salts (Mohapatra et al, 2006). Minimum salt medium containing Xanthine in concentration of 2 mmol/l has been used for screening and isolation of Xanthine oxidase producing bacteria (Aggarwal and Banerjee, 2009). *Aspergillus niger and Penicillium Roqueforti* metabolized caffeine into theophylline as the first degradation product. Hakil *et al.*(1998) tested the ability of twenty strains of filamentous fungi to grown on caffeine as a sole source of nitrogen and only strains of the *Penicillium* and *Aspergillus* genera were able to degrade the alkaloid. Caffeine was degraded to theophylline and then to 3-methylxanthine.

2.5 Fermentation

Fermentation is a process very much similar to anaerobic respiration and is carried out in a mixture of nutrients and metabolites essential for the growth and reproduction of the microbe. It is a metabolic process in many microorganisms and involves oxido-reduction reactions resulting in the breakdown of complex organic molecules into various end products with the release of energy. Fermentation is mostly extracellular and is brought about with the help of enzymes released by the microorganisms. Culture broth extracts produced by fermentation of *Guignardia* species and *Phomopsis* species in Potato Dextrose Broth (PDB) which were isolated from leaves of *Aspidosperma tomentosum* and *Spondias mombin* were evaluated for antimicrobial activity by using agar well diffusion assay (Corrado and Rodrigues, 2004). *Rhizopus delemar* also showed ability to catalyze caffeine and theophylline by solid-state fermentation (SSF) in packed bed column bioreactor and the cell

free extracts showed the presence of significant amounts of caffeine oxidase (Hussein et. al, 2009).

2.6 Secondary screening of XO producing endophytic fungi

2.6.1 Spectrophotometric Assay of Xanthine oxidase

Spectrophotometric techniques are used to measure the concentration of solutes in solution by measuring the amount of light that is absorbed by the solution in a cuvette placed in the spectrophotometer. The experimental approach exploits Beer's Law, which predicts a linear relationship between the absorbance of the solution and the concentration of the analyte. A simplified method was developed using phosphotungstic acid as a reagent, for measurement of uric acid in serum (Carroll *et.al*, 1970). But a number of newer methods have been developed since then and the standard spectrophotometric assay of XO is based on the oxidation of Xanthine / hypoxanthine to uric acid and the activity of xanthine oxidase (XO) is measured as the rate of uric acid production when xanthine is incubated with the fungal culture filtrate (Trivedi *et. al*, 1978). Uric acid is a catabolic end product of purines in the human body and the change in absorbance due to this reaction (in terms of uric acid produced) can be recorded at 292nm. One unit of XO activity is defined as 1 µmol uric acid produced per min at 37°C with excess substrate. This was considered as an accurate method for assay of uric acid, as compared to other calorimetric and enzymatic assays known till date. Xanthine oxidase activity has been assessed based on the oxidation of 2, 2'-azino-di (3-ethylbenzthiazoline-6-sulphonate) (ABTS) by use of uricase and peroxidase. The absorbance increase of the oxidized form of ABTS, measured after 10 min at 410 nm is proportional to xanthine oxidase activity. The method is sensitive, precise and linear up to 20 U/l (Singh *et al.*, 1987). Xanthine oxidase, acting on purine substrates leads to the aerobic oxidation of sulfite. This phenomenon has been used to assay the activity of the enzyme Xanthine oxidase and is helpful in determination of the Michaelis constants for hypoxanthine and Xanthine (Fridovich and Handler, 1958).

2.6.2 Microplate based Xanthine Oxidase Assay

There is an immense requirement of screening a large number of microbial test samples in a shorter, accurate method to isolate the lead microbial systems which could be exploited for enzyme production. This can be achieved by developing high throughput assays using microplate /microtiter plates. This assay would really help in screening for novel compounds

which could be exploited for anti-gout, anti-microbial and anti-tumor activities. Microtiter plate comprises of a flat plate with multiple "wells" used as small test tubes. The microplate has become a standard tool in analytical research and clinical diagnostic testing laboratories. A very common usage is in the enzyme-linked immunosorbent assay (ELISA), the basis of most modern medical diagnostic testing in humans and animals. Microplates are manufactured in a variety of materials; the most common is polystyrene, used for most optical detection microplates. A microbial Xanthine oxidase production assay was developed by Agarwal and Banerjee (2009) for high throughput screening of Xanthine oxidase producing bacteria using Xanthine as the substrate and calorimetric assessment of the formazan formed by reduction of Nitroblue tetrazolium (NBT) as a result of peroxide generated during the conversion of Xanthine into uric acid. The underlying principle was used as a calorimetric assay for screening of XO producers using NBT is (Fig 3).

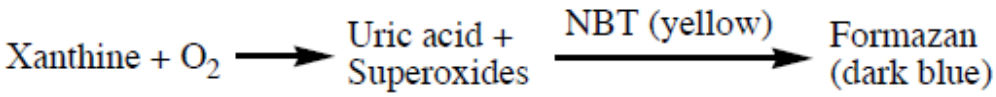


Fig 3: Calorimetric assay for XO

2.6.3 NBT based Xanthine oxidase inhibition assay

Allopurinol is a drug used primarily to treat hyperuricemia (excess uric acid in blood plasma) and its complications, including chronic gout. Also allopurinol has found a role in protection of Type 1 Diabetes (Desco *et. al*, 2002; Klein et. al, 1996). It is a structural isomer of hypoxanthine (that is the naturally occurring purine in the body) and is a competitive enzyme inhibitor of xanthine oxidase. Allopurinol, therefore, not only decreases uric acid formation but also causes feedback inhibition of purine synthesis. Allopurinol is rapidly metabolized by its target, xanthine oxidase, to its active metabolite oxypurinol, which is also

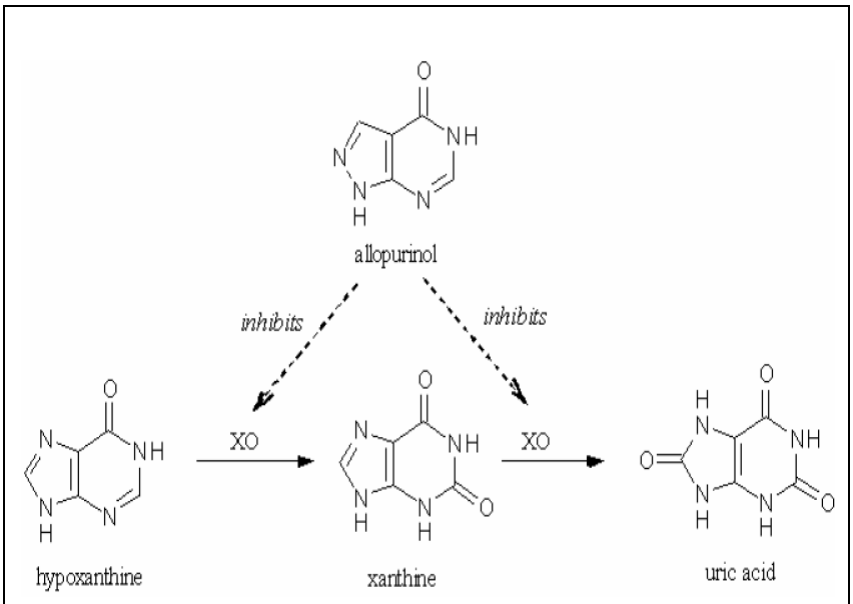


Fig 4: Pharmacological action of Allopurinol

an inhibitor of xanthine oxidase. To further ascertain the production of Xanthine oxidase this inhibitor can be used to find out the affect on NBT reduction (Fried and Fried, 1993; Agarwal and Banerjee, 2009).

2.7 Protein Purification

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps may exploit differences in protein size, physico-chemical properties, binding affinity and biological activity.

Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). Ammonium sulphate precipitation method has been used for isolation and characterization of caffeine oxidase from *Alcaligens* species (Mohapatra *et. al*, 2006). Tannase from *Aspergillus awamorii* has been isolated by acetone precipitation method of the supernatant (Mahapatra *et. al*, 2005). Glycerol oxidase purification from *Penicillium* species TS-622 has been carried out by ammonium sulphate precipitation (Lin *et. al*, 1995).

2.8 XO Activity Assay of Crude Protein Using NBT Based Method

The activity of the xanthine oxidase enzyme was assayed in the protein precipitates obtained by ammonium sulfate precipitation. The NBT based calorimetric assay was used, xanthine was used as substrate and this was incubated with the precipitates obtained at different concentrations of ammonium sulfate, that is; 10%, 20%, 30% and 40%. A microtiter plate assay was performed and the plate was incubated at optimum temperature. The color change was observed due to reduction of NBT to formazan due to superoxides released as a result of activity of XO.

2.9 Protein Estimation by Lowry's Method

The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. It is named for the biochemist Oliver H. Lowry who developed the technique in the 1940s. The Lowry method is best used with protein concentrations of 0.01-1.0 mg/mL and is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin-Ciocalteu reaction). The reaction mechanism is not well understood, but involves reduction of the Folin reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). The concentration of the reduced Folin reagent is measured by absorbance at 750 nm. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Lowry's method has been used for estimation of total proteins and elevated levels of xanthine oxidase in the blood of patients with Myocardial Infarction (Raghuvanshi *et. al*, 2007). Also, the same method has been used for analyzing protein concentration in urinary samples for studying the pivotal role of xanthine oxidase in the initiation of tubulointerstitial renal injury in rats with hyperlipidemia (Gwinner, Scheuer *et. al*, 2006).

2.10 Activity Staining of Xanthine Oxidase

Activity staining on cellulose acetate, agarose and polyacrylamide gels are commonly used for the detection of the activities of several enzymes. Xanthine oxidase is usually used as an auxiliary enzyme in the activity staining of other enzymes such as guanase, ascorbate

peroxidase and in the activity staining of several blotted enzymes. Histochemical staining methods for xanthine oxidase are available (Ozer et. al, 1997). On the other hand, there is no direct staining method for xanthine oxidase on, polyacrylamide gels. Ozer *et. al* (1997) gave a simple and sensitive method for activity staining of XO. This method was obtained by a modification of the method developed for ascorbate peroxidase. Discontinuous PAGE (0.75 mm) is carried out under nondenaturing and nonreducing conditions (Native PAGE), essentially as described by Laemmli and activity staining on polyacrylamide gels is carried out at room temperature with 0.25 mM nitroblue tetrazolium. Staining of the gels is continued till the activity band(s) appear on the gels.

In order to be able to detect the protein bands, corresponding to activity staining, the amount of protein applied per well can be doubled. This direct staining method is very important for the quality control of xanthine oxidase obtained from different sources. This activity staining method has been used for analysis of guanase in serum samples (Nishikawa *et. al*, 1985). Also, the method has been used for the confirmation of the existence of a xanthine oxidase isolated from *Pseudomonas putida* L (Mazzafera *et. al*, 1999).

2.11 Agar Well Diffusion Assay

It is an advanced version of Ditch Plate assay which uses wells prepared in agar to evaluate the xanthine metabolizing qualities of the Endophytic fungi. The technique was initially designed by Heatley in 1944. The principle of method is as follows; a standardized concentration of substrate is provided in the plate and then a well which ranges from 5mm in diameter is punched with the help of a sterile cork borer, at three different positions. A fixed volume of extract is then introduced into the bored well and incubated at optimum temperature and duration depending upon the nature of the test. After incubation the degree of the molecule's movement can be related to the concentration of the molecule and the activity of the extract can be related to the size of the zone of clearance formed and can be compared with the controls. It is a more suitable technique for testing the aqueous solutions of natural products as the suspended particulate matter in the sample being tested is much less likely to interfere with the diffusion of substance under speculation. It is a popular method of screening employed for assessing the antibacterial activity (Das, 2010, Heatley, 1944; Papadopoulou, 2005). This method has been used for studying the

antioxidant and antimicrobial activity of the essential oil and methanolic extracts of *Achillea millefolium sp*. (Candan *et. al*, 2003).

Chapter 3

AIM OF THE STUDY

3.1 Aim of the study

The current study was subjected towards the screening of Endophytic Fungi as a potential source of Xanthine Oxidase.

The objectives of the current study were:

1. To screen and select a potential Xanthine Oxidase producing endophytic fungi.
2. Production and partial purification of the crude enzyme.
3. To assess the activity of fermentation broth and crude enzyme using various *in-vitro* assays.

Chapter 4

MATERIALS AND METHODS

4.1 Maintenance of Pure Cultures of Endophytic Fungi

4.1.1 Preparation of Potato Dextrose Agar (PDA) plates

39.0 g of PDA (Hi Media) was dispensed in liter lukewarm single distilled water and stirred thoroughly. This was then dispensed in 250 ml Erlenmeyer Flasks and autoclaved at 121°C, 15 psi for 15 min. Glass petri plates were sterilized at 121 °C, 15 psi for 20min. Then under sterile conditions 25 ml of autoclaved PDA was dispensed in sterile 90 mm plates and allowed to solidify at room temperature. The plates were stored in incubator at the temperature 26 ± 2°C until further use (Agarwal and Hasija, 1986).

4.1.2 Sub-Culturing

The fungi transferred from the stock culture tube 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 under laminar air flow hood.

4.2 Preliminary Screening for Xanthine Oxidase Producers

Preliminary screening of xanthine oxidase (XO) producers was done on Xanthine Vogel's Agar (XVA) plates. The composition of XVA: 134 ml of Xanthine was dispensed in 35ml of 5x Vogel's salts (Table no. 1 and 2), thereafter 3g of agar was added and the volume was made to 200ml using double distilled water (ddW). The contents were thoroughly mixed using mild heating and the final pH of medium was adjusted to 7.8±0.2. Media was then autoclaved at 121°C/15 lbs for 15 min. and was poured when the temperature was optimum. XVA plates were then inoculated with 5mm mycelial plugs of 7 day old fungal cultures (maintained on PDA plates), under high aseptic conditions and incubated at 26 ± 2 °C for 3 days. After 3 days of incubation, the plates were flooded with NBT and incubated at 37°C for at least three hours.

Table No. 1: Composition of Modified Vogel's Medium (50X): (for 100ml)

Na_3 citrate, 5 1/2 H_20	15g
KH_2PO_4, anhydrous	25g
$MgSO_4$, 7 H_20	1g
$CaCl_2$, 2 H_20	0.2g
Trace Element Solution	5ml

Table No. 2: Composition of Trace Element Solution

Citric acid, 1 H_20	5g
$ZnSO_4$, $7H_2O$	5g
$Fe(NH4)_2(SO4)_2$, 6 H_20	1g
$CuSO_4$, 5 H_20	0.25g
$MnSO_4$, 1 H_20	0.05g
H_3BO_3, anhydrous	0.05g
Na_2MoO_4, 2 H_20	0.05g

4.3 Secondary Screening for Xanthine Oxidase Producers

The fungal isolates which exhibited Xanthine oxidase production in the preliminary screening using XVA plates were retested to ascertain that the production of XO was obligatory in nature or not. Hence Xanthine plates were prepared comprising of 134ml of Xanthine stock , 3 g agar and contents made to a volume of 200ml using ddW. Thus Xanthine served as the sole carbon as well as nitrogen source. These were referred as Xanthine agar (XA) plates. The final pH of medium was adjusted to 7.8±0.2. Media was then autoclaved at 121°C/15 lbs for 15 min. and was poured when the temperature was optimum. XA plates were then inoculated with 5mm mycelial plugs of 7 day old fungal cultures (maintained on PDA plates), under high aseptic conditions and incubated at 26 ± 2 °C for 3 days. After 3 days of incubation, the plates were flooded with NBT and incubated at 37°C for at least three hours.

4.4 Fermentation

Liquid culture fermentation on XVB (Xanthine Vogel's broth) was carried out. The composition of XVB was same as XVA except the solidifying agent i.e. agar. The pH of the broth was also adjusted to 7.8. The media was then autoclaved at 121°C, 15 psi for 15 min. Mycelial plugs of 5 mm diameter of 7-days old fungal cultures maintained on PDA plates were used to inoculate the 200 ml pre- sterilized suitable medium in Erlenmeyer flasks (Schott Duran), under high aseptic conditions The flasks were then incubated at 120 rpm and 26°± 2°C for 10 days for production of secondary metabolites (Rodriguez, 2000; Santamari *et. al*., 2002; Rosa *et al*., 2003). The mycelial growth was then monitored in terms of color, morphology for a period of 10 days. Similarly the translucency of the medium was also taken into consideration to assess Xanthine utilization by the endophytic fungi.

Optimization of fermentation broth

Optimization of the fermentation media was done. Initially, XVB (Xanthine Vogel's Broth) Media was prepared by adding 134ml of 2mM xanthine (prepared in 1N NaOH with constant stirring and boiling on a magnetic hot plate)**,** 35ml of 5X Vogel's Salts and volume make up was done up to 200ml; pH adjusted to 7.8. Later, cultures were put on fermentation in another media containing xanthine as the sole carbon and nitrogen source (without any Vogel's medium). Both the media were then autoclaved at 121°C, 15psi for

15minutes. Mycelial plug of 5mm diameter of 7-days old fungal culture of (maintained on PDA plates) was inoculated in 200ml pre sterilized suitable medium in Erlenmeyer flasks (Schott Duran), under high aseptic conditions (all work done in laminar flow hood). The flasks were then incubated at 120 rpm and 26°± 2°C for 10 days for production of secondary metabolites (Rodriguez, 2000; Santamari *et. al*., 2002; Rosa *et al*., 2003). The mycelial growth was then monitored for a period of 10days to look for the difference in growth in the two media, and hence selection of the optimal media.

The cultures exhibiting the positive results on XA plates were also subjected to liquid culture in a broth containing Xanthine (10mM) as sole carbon and nitrogen source. Briefly XB (Xanthine Broth) Media was prepared by adding 134ml of 10mM xanthine (prepared in 1N NaOH with constant stirring and boiling on a magnetic hot plate) and volume make up was done up to 200ml; pH adjusted to 7.8. Media was then autoclaved at 121°C/15lbs for 15minutes. Mycelial plug of 5 mm diameter of 7-days old fungal cultures (maintained on PDA plates) were inoculated in 200 ml pre sterilized suitable medium in Erlenmeyer flasks, under high aseptic conditions (all work done in laminar flow hood). Bottles were then incubated at 120 rpm and 26°± 2°C for 15 days for production of secondary metabolites. After 15 days, broth was separated from mycelia by filtration. Filtration was carried out aseptically through muslin cloth and subsequently through Whatman Paper 4 (Rodriguez *et. al*, 2000).

4.5 Standard Curve for Xanthine Oxidase activity (Using RANDOX KIT)

Briefly in this test Xanthine stocks from 0.0625 mM to 1 mM were prepared in 1 N NaOH and dispensed in 7 eppendorfs. These were thoroughly vortexed. Further phosphate buffer was prepared and its pH adjusted to 7.8. 2mM stock of NBT was prepared in sterile distilled water (SDW) and stored in amber colored bottle till further use. 134 µl of Xanthine stock solutions were then dispensed in different wells of the 96-well microtiter plate as per the assay template. Thereafter 25 µl of Xanthine oxidase enzyme, 5 µl of 50mM phosphate buffer of pH 7.8 and 36 µl of NBT was added. Control for test stock comprised of 134 µl xanthine stocks, 25 µl of SDW, 5 µl of 50 mM phosphate buffer and 36 µl NBT. The absorbance of the control, test and blank wells were taken at 505 nm using a Biotek throughput reader, Powerwave 340.

Fig 5: Standard Curve for XO Activity using 96- well Micro Titer Plate Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
B	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
C	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
D	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
E	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
F	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
G	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2
H	B1	T1	T2	T3	T4	T5	C1	C2	C3	C4	C5	B2

- T1, T2, T3, T4 and T5 corresponding to 1mM, 0.5mM, 0.25mM, 0.125mM and 0.0625mM respectively.
- C1, C2, C3, C4 and C5 correspond to Controls of test stocks
- B1 and B2 corresponds to blank 1 and blank 2, these wells were kept empty

4.6 Spectrophotometric Analysis of Xanthine Oxidase

The standard spectrophotometric analysis of XO is based on the oxidation of xanthine/hypoxanthine to uric acid and the activity of xanthine oxidase (XO) was measured as the rate of uric acid production when xanthine was incubated with the fungal culture filtrate (Heinz *et. al*, 1979). For this assay, a Standard Curve for Uric Acid was made – Uric Acid stocks were prepared in 1N NaOH (i.e.;t1, t2, t3, t4, t5, t6, t7, t8, t9, t10 corresponding to 5µM, 10µM, 20µM, 30µM, 80µM, 130µM, 180µM, 230µM, 280µM and 330 µM resp.). O.D. was then taken at 292nm against water as a blank (containing an equal amount of NaOH as Uric Acid stocks).

To assess the Xanthine oxidase activity of test culture filtrates 7 test tubes were taken each for each test samples as well as the control samples. The test samples were prepared by adding 0.5 ml of the culture filtrate grown in the XB followed by 3 ml of 50 mM potassium phosphate buffer (pH 7.5) and 0.5 ml substrate solution. Control samples were prepared by

adding 0.5 ml of the un-inoculated XB followed by 3ml of 50mM potassium phosphate buffer (pH 7.5) and 0.5ml of substrate solution. All the test and control samples were then incubated at 37°C. Reaction was stopped in each set after 0min, 10min, 20min, 30min, 40min, 50min and 60min incubation, by adding 250µl of 100%TCA to each sample (Kadam and Iyer, 2007). O.D. was taken at 292 nm to record the uric acid produced in test samples, by the action of XO, keeping control as blank and the quantitative estimation of uric acid produced in test samples was done.

4.7 Micro Titer Plate Assay

Assay for XO activity using NBT - Stocks of xanthine and NBT were freshly prepared. Test samples aliquots were prepared by dispensing 100µl of 2mM xanthine, 100µl of fungal culture filtrate and 50µl of NBT. Control samples were prepared by dispensing 100µl of 2mM xanthine, 100µl of control sample i.e. un-inoculated XB and 50µl of NBT. These assays were carried out in duplicate sets so that they can be incubated under different temperatures and record the change in activity of the XO enzyme activity. After dispensing all the samples, plates were incubated at 37°C and 26°C for 24 hours.

Assay for XDH activity using TTC -- Stocks of xanthine and TTC (Triphenyl Tetrazolium Chloride) were freshly prepared. Test samples aliquots were prepared by dispensing 100µl of 2mM xanthine, 80µl of fungal culture filtrate, 20µl of 50mM Phosphate buffer (pH 7.8) and 40µl of TTC. Control samples were prepared by dispensing 100µl of 2mM xanthine, 80µl of control filtrate, 20µl of 50mM Phosphate buffer (pH 7.8) and 40µl of TTC. After dispensing all the samples, plate was incubated at 37°C and 26°C for O/N.

4.8 XO Inhibition assay by allopurinol

Xanthine Oxidase Inhibition assay was carried out with allopurinol, which is a structural isomer of hypoxanthine and a competitive inhibitor. Microplate assay was carried out using different stock concentrations of Allopurinol within a range of 2 mM to 16 mM. **Test set** comprised of 100 µl of culture filtrate of test fungal strain grown on XB, 50 µl stock concentration of allopurinol (S1 to S5) and 50µl of 2mM Xanthine. The samples were then incubated at 37 °C for 15 min. and then 50 µl of NBT was added. **Control set** comprised of 100 µl of culture filtrate of test fungal strain grown on XB followed by 50 µl of 2 mM Xanthine and then 50 µl of sterile distilled water. The samples were then incubated at 37 °C for 15 min. and then 50 µl of NBT was added. **Negative Control** (Ne) was set to see the interactions between NBT and Xanthine under alkaline conditions since Xanthine stocks were prepared in 1N NaOH. The Negative Control was prepared by adding 100 µl of 2 mM xanthine, followed by 50 µl of 1N NaOH. After 15 min. incubation at 37 °C, 50µl of NBT was added. B corresponds to Blank, that is, these wells were kept empty. **Test sample** (T) comprised of 100µl of test fungal culture filtrate on XB followed by 50 µl of 2mM Xanthine and then 50µl of sterile distilled water. The samples were then incubated at 37 °C for 15 min. and then 50 µl of NBT was added. Finally, **Control over Control** (C/C) was prepared by adding 100µl of test fungal culture filtrate on XB, followed by 50 µl of 2mM Xanthine and then 50 µl of sterile distilled water. After incubation, 50µl of NBT was added. B corresponds to blank, i.e. these wells were kept empty. The change in rate of absorbance was recorded at 575nm (Agarwal and Banerjee, 2009).

Fig 6: A 96-well micro titer plate XO Inhibition assay by Allopurinol

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ne	S1	S2	S3	S5	C1	C2	C3	C5	T	C/C	B
B	Ne	S1	S2	S3	S5	C1	C2	C3	C5	T	C/C	B
C	Ne	S1	S2	S4	S5	C1	C2	C4	C5	T	C/C	B
D	Ne	S1	S2	S4	S5	C1	C2	C4	C5	T	C/C	B
E	Ne	S1	S3	S4	S5	C1	C3	C4	C5	T	C/C	B
F	Ne	S1	S3	S4	S5	C1	C3	C4	C5	T	C/C	B
G	Ne	S2	S3	S4		C2	C3	C4		T	C/C	B
H	Ne	S2	S3	S4		C2	C3	C4		T	C/C	B

- S1, S2, S3, S4 and S5 correspond to 2mM, 4mM, 8mM, 12mM and 16mM allopurinol concentrations
- C1, C2, C3, C4 and C5 correspond to Controls i.e. without allopurinol.
- Ne refers to interaction between NBT and Xanthine stock under alkaline conditions.
- T refers to enzymatic activity of 50% diluted culture filtrate
- C/C refers to uninoculated medium with the assay mixture

4.9 Protein Purification

Ammonium sulfate precipitation was the method used to purify proteins by altering their solubility. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. This method has been used for isolation and characterization of caffeine oxidase from *Alcaligens* species (Mohapatra *et. al*, 2006). Large scale liquid culture fermentation was carried out with the best fungal isolate showing positive results in the *in vitro* assays carried out. The spent medium was filtered through Whatman filter paper 1 and subsequently centrifuged at 10,000 rpm and 4°C for 15min to obtain a cell free culture filtrate for the recovery of protein. Stepwise precipitation was carried out with 10% ammonium sulphate and was kept overnight at 4°C. The filtrate was then centrifuged at 4 °C, 12000 rpm for 15 min.

The pellet was collected in microfuge tube and the supernatant was collected separately in an Erlenmeyer flask. The pellet was re-suspended in minimum amount of 1M Phosphate Buffer (pH 7.0). Subsequently the protein precipitation was done with 20%, 30% and 40% ammonium sulfate with the remaining supernatant. After 40% concentration, no further precipitation was observed. All the pellets were stored at -20°C in 1M Phosphate Buffer.

4.10 XO Activity Assay of Crude Protein Using NBT

The NBT based calorimetric assay was used to assess the Xanthine oxidase activity of the crude precipitated protein. In the assay, xanthine was used as substrate and was incubated with the precipitates obtained at different concentrations of ammonium sulfate, that is; 10%, 20%, 30% and 40%. A microtiter plate assay was performed as discussed previously i.e. section 2.6 to observe the due reduction of NBT to formazan as a result of XO activity.

4.11 Protein Estimation by Lowry's Method

Protein sample was thawed and dilutions of different concentrations of Bovine serum albumin (BSA) viz. were prepared for the standard curve preparation. Protein sample was diluted in double distilled water and thoroughly vortexed. 1ml of the sample/standard dilutions was taken in respective test tubes and 1.4 ml of Lowry's reagent (Sol. A: Sol. B: Sol. C = 100:1:1) (Table No.3) was added, vortexed briefly and incubated in dark for 20 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, 200µl aliquot of each of the standard and sample reaction mixture was withdrawn and dispensed in a 96-well titre plate as per the template given. Thereafter, the absorbance was read at 660nm after the 30min incubation. Amount of protein in the sample was then determined by plotting the values on standard curve, prepared by using absorbance readings of BSA.

	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

- R= Reference
- S= Standards
- E= Empty Wells
- T= Test samples

Fig 7: Protein Estimation by Lowry's method

Fig 8: Folin-Lowry assay on Microplate

Solution A	0.572gm sodium hydroxide + 2.862gm sodium carbonate
Solution B	0.285gm copper sulfate
Solution C	0571gm sodium tartarate

Table No.3: Composition of Lowry's Reagent

4.12 Standard Curve for Xanthine Oxidase activity in crude protein

Briefly in this test, stocks of crude protein were prepared by diluting 200µl of crude protein sample with 1800µl of Phosphate buffer (pH=7.0) [S1]. Serial Dilution was then done to obtain other stocks, i.e., S2, S3, S4 and S5 from S1 resp. These were thoroughly vortexed. Stocks of xanthine (2mM) and NBT (2mM) were freshly prepared. Test samples aliquots were prepared by dispensing 100µl of 2mM xanthine, 50µl of crude protein stock; incubated at 37°C for 6minutes in dark and then 50µl of NBT was added. Control samples were prepared by dispensing 100µl of 2mM xanthine, 50µl of SDW; incubated at 37°C for 6minutes in dark and then 50µl of NBT was added. After dispensing all the samples, plate was incubated at 37°C for 24 hours. The absorbance of the control, test and blank wells were taken at 505 nm using a Biotek throughput reader, Powerwave 340.

4.13 Activity Staining of Xanthine Oxidase

5% native poly-acrylamide gel (that is, resolving gel) was prepared with incorporated 2% xanthine (substrate for XO) for electrophoresis. The 5% resolving gel was prepared using the composition given in the table. Stacking gel was not used. On solidification of the gel the wells were loaded with 80µl of sample. Sample was prepared by taking equal volumes of protein solution and sample buffer containing bromophenol blue (tracking dye) under non-reducing and non-denaturing conditions, that is; a Native PAGE was run without any detergent (SDS) and β mercaptoethanol, also boiling of samples was avoided so that the protein remains active in its native state. Gel was allowed to run for approximately 2 h or till the tracking dye reached the bottom of the gel. After electrophoresis, gel was cut into two halves, one was stained with Coomassie Brilliant Blue G-250 and the other half was incubated in a 2mM solution of NBT for 24hours.

Table No. 4: Composition of components used for 5% Resolving gel

SDW	5.175ml
30%acrylamide/0.8%bis acrylamide	1.485ml
1.5M Tris Buffer (pH 8.8)	2.25ml
10%APS	70µl
TEMED	10µl
Substrate i.e. 2mM Xanthine	166µl

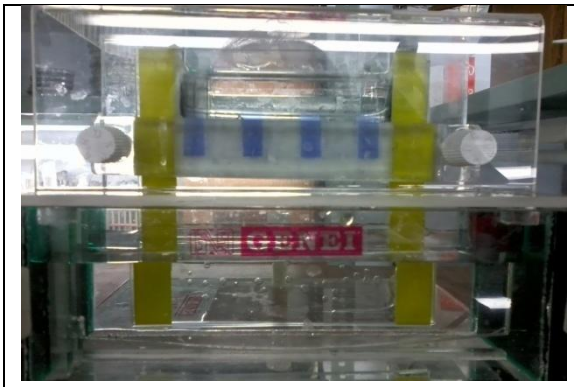


Fig 9: Vertical Polyacrylamide Gel Electrophoresis Unit (Bangalore GENEI)

4.14 Agar Well Diffusion Assay (AWD Assay)

Wells of 5mm diameter were cut with the help of pre sterilized cork borer in 24hours old Xanthine Agar (XA) plates (containing 10mM Xanthine as substrate) to provide a depth of 4 to 5mm. Another XA plate was prepared in which 2mM Nitroblue Tetrazolium (NBT) was also added. 20µl of test extract (that is, the crude protein extracted) was dispensed in one well, 40µl in another well and 40µl of Sterile Distilled Water (SDW) was added in third well, which was used as a control. All the samples were allowed to diffuse for one hour and the plates were incubated at 37°C. After one hour, a zone of clearance was observed near the test extracts, and the size of zone of clearance was found to be related to the concentration of the test extract.

Chapter 5
RESULTS AND DISCUSSIONS

5.1 Preliminary Screening for Xanthine Oxidase Producers

Cultures of endophytic fungi, maintained on Potato Dextrose Agar (PDA), were screened for xanthine degradation/utilization on XVA (Xanthine Vogel's Agar) plates. 85 cultures were screened on XVA. Out of the 85 culture screened, 38 fungal isolates were from *Aegle marmelos (AM)*, 3 were from *Tabarnaemontana divaricata (TMD)*, 9 were from *Rauwolfia serpentine (RS)*, 18 isolates were from *Cinnamomum mulbaricum (CM)*, 5 isolates were from *Cinnamomum zeylanicum (CZ)*, 4 were from *Jatropha curcas (JT)*, 6 were from *Nerium oleander (NO)*, 3 were from *Bamboo (BB)*, 2 were from *Cimmelia sinensis (CS)* and 3 were from *Wild ginger (WG)*.

S No.	Culture Codes	Part Isolated from	XVA media	Other Metabolism
1	## 10AMSTITYel	Stem	-	
2	## 22AMSTITYel	Stem	-	
3	# 4AMSTYel	Stem	+	
4	# 7AMSTYel	Stem	+	
5	## 11AMSTITYel	Stem, Internal tissue		☑
6	# 21AMSTYel	Stem		☑
7	# 9(b)AMSTYel	Stem	±	
8	# 20AMSTYel	Stem	±	
9	# 1077AMSTITYel	Stem, Internal tissue	±	
10	# 1003AMSTITYel	Stem, Internal tissue		☑
11	# 1070AMSTITYel	Stem, Internal tissue		☑
12	# 1048AMSTITYel	Stem, Internal tissue		☑
13	# 18AMSTYel	Stem		☑
14	# 1022AMSTITYel	Stem, Internal tissue	+	
15	# 23AMSTYel	Stem	+	
16	# 23(b) AMSTYel *	Stem	+ +	
17	#32AMSTYel	Stem	-	
18	# 2TMDSTYel	Stem	=	
19	# 4TMDSTYel	Stem		☑
20	# 2(a) TMDSTYel **	Stem	+ + +	
21	## 8AMLBRT *	Leaves	+ +	
22	# 9AMLBRT	Leaves	+	
23	# 17AMLBRT	Leaves	-	
24	# 1016AMLBRT	Leaves	±	
25	# 12AMLBRT	Leaves		☑
26	# 1RSLBRT	Leaves	-	
27	# 15RSLBRT	Leaves		☑
28	# 16RSLBRT **	Leaves	+ + +	
29	## 4CMBABRT	Bark	+	
30	## 7CMBABRT	Bark	+	
31	# 12CMBABRT *	Bark	+ +	
32	# 21CMBABRT	Bark	±	
33	# 16CMBABRT	Bark	±	
34	# 9 CMLBRT **	Leaves	+ + +	
35	# 5111CMSTITBRT	Stem, Internal tissue	-	
36	#5109CMSTITBRT	Stem, Internal tissue	±	
37	# 6610 CMSTITBRT	Stem, Internal tissue	-	
38	# 7 CZSTITBRT	Stem, Internal tissue	±	
39	# 16AMLWLS	Leaves	±	
40	# 6AMLWLS *	Leaves	+ +	
41	# 26AMSTWLS	Stem		☑
42	# 17AMSTWLS	Stem	-	
43	# 1118AMSTITWLS	Stem, Internal Tissue	±	
44	# 1088AMSTITWLS	Stem, Internal Tissue		☑

45	# 12 AMBAWLS **	Bark	+ + +	
46	# 5 CZBAWLS **	Bark	+ + +	
47	# 20 AMSTWLS	Stem	-	
48	# 29/23CZBAWLS	Bark		☑
49	# 19CZBAWLS	Bark		☑
50	#6CZBAWLS	Bark	-	
51	# 1099AMSTITWLS	Stem, Internal Tissue		☑
52	# 1082AMSTITWAY	Stem, Internal Tissue	±	
53	# 2(b)JTLSVNP	Leaves	+	
54	# 31JTLSVNP **	Leaves	+ + +	
55	# 33(b)JTLSVNP *	Leaves	+ +	
56	# 23 JTLSVNP *	Leaves	+ +	
57	# 5 NOBASVNP	Bark	+	
58	# 19 NOBASVNP **	Bark	+ + +	
59	# 20 NOBASVNP *	Bark	+ +	
60	# 21 NOLSVNP	Leaves		☑
61	# 3 NOBASVNP	Bark		☑
62	#16 NOBASVNP **	Bark	+++	
63	# 10BBSMf	Stem	±	
64	# 1BBSMf	Stem	-	
65	# 6BBSMf	Stem	+	
66	# 4CSSTOT	Stem	±	
67	# 2CSSTOT	Stem	-	
68	# 15WGSTNEY	Stem	+	
69	# 15(a)WGSTNEY	Stem	+	
70	# 13(a)WGSTNEY *	Stem	+ +	
71	# 3RSSTNEY	Stem	-	
72	# 23(a)RSSTNEY	Stem	±	
73	# 14RSBANEY	Bark	-	
74	# 1RSBANEY	Bark		☑
75	# 7 RSBANEY *	Bark	+ +	
76	# 12 RSBANEY **	Bark	+++	
77	# 20CMBANEY	Bark	+	
78	# 4CMBANEY **	Bark	+ + +	
79	# 9CMBANEY	Bark		☑
80	# 26CMBANEY *	Bark	+ +	
81	# 21CMBANEY *	Bark	+ +	
82	# 18CMLNEY	Leaves	-	
83	# 16CMLNEY	Leaves	-	
84	# 1562 CMSTITNEY	Stem, Internal Tissue	-	
85	# 1622 CMSTITNEY	Stem, Internal Tissue		☑

(-) no growth (±) poor activity; (+) average ;(++) good; (+++) very good; ☑ only growth no activity

Table No. 5: Screening for XO producers on XVA plates

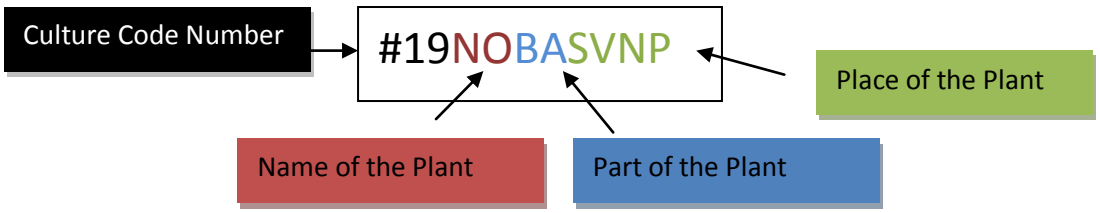
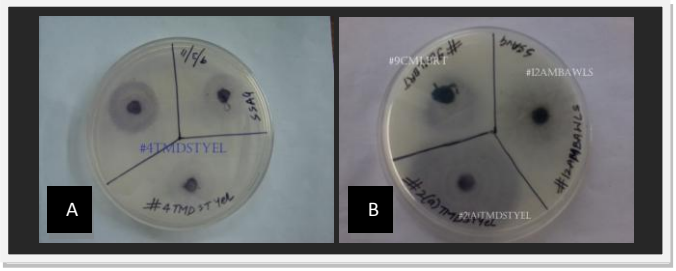


Fig 10: Culture Code

Out of these 85 cultures screened, 10 cultures, i.e. endophytic fungal strains isolated from different parts of different plants (Table 6); showed very good filamentous growth on XVA plates which were then again screened on the media containing only xanthine as the sole carbon and nitrogen source. Also, another set of 11 cultures, i.e. # 23(b) AMSTYel, ## 8AMLBRT, # 12CMBABRT, #6AMLWLS, #33(b) JTLSVNP, #23 JTLSVNP, #13(a) WGSTNEY, #20NOBASVNP, #26CMBANEY and #21CMBANEY showed acceptable filamentous growth. All the above cultures were selected for liquid culture fermentation. Fungal strains showing positive growth on XVA plates; reacted with NBT and gave dark blue color and appearance of this blue color can be attributed to the production of XO by the fungal strains. As assumed, no color change was observed in control. A Bacterial strain *C. acidovorans* was cultured in mineral salts medium with hypoxanthine as the sole carbon and nitrogen source, the culture reached late-log phase in 24hours (Xiang *et. al,* 1996), but no such screening for XO producers have been done for fungi on XVA plates. The cultures marked as ☑ [Table No.5] showed good filamentous growth on XVA plates but were unable to reduce NBT to formazan, so no color change was observed. It was concluded that these endophytic fungi were utilizing xanthine as a carbon and nitrogen source but probably they have shifted their metabolism to some other biochemical pathway, since formazan is formed only as a result of interaction of NBT and superoxides produced during the course of the oxidation of xanthine to uric acid by xanthine oxidase (Agarwal and Banerjee, 2009).

Culture Codes	Plant Obtained from
#16NOBASVNP, #19NOBASVNP	*Nerium oleander*
#12RSBANEY, #16RSLBRT	*Rauwolfia serpentine*
#4CMBANEY, #9CMLBRT	*Cinnamomum mulbaricum*
#2(a)TMDSTYEL	*Tabarnaemontana divaricata*
#12AMBAWLS	*Aegle marmelos*
#5CZBAWLS	*Cinnamomum zeylanicum*
#31JTLSVNP	*Jatropha curcas*

Table No.6: Cultures showing positive growth on XVA plates



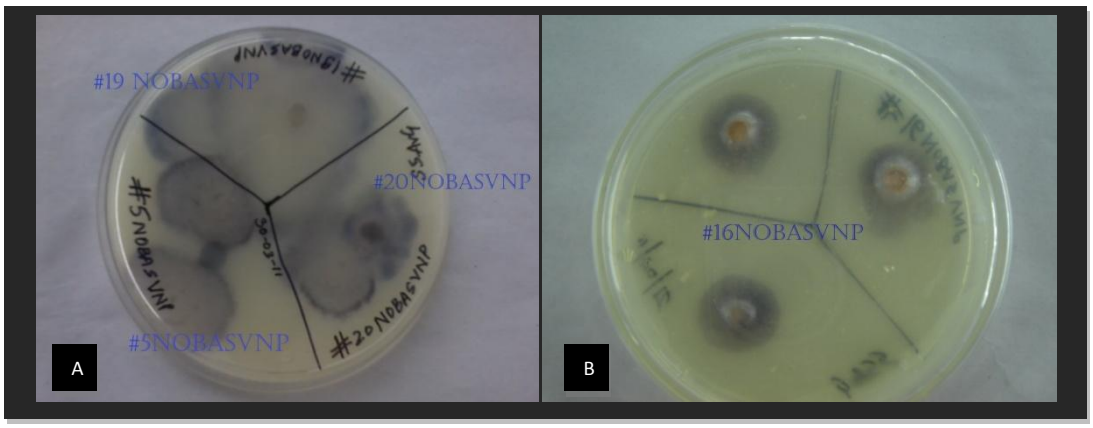
Photograph No.1: Oxidation of NBT by Endophytic Fungi screened on XVA plates; (A) #4TMDSTYEL; (B) #2(a) TMDSTYEL, #12AMBAWLS, #9CMLBRT

5.2 Secondary Screening for Xanthine Oxidase Producers

The fungal isolates which exhibited Xanthine oxidase production in the preliminary screening using XVA plates were retested to ascertain that the production of XO was obligatory in nature or not. All the above cultures (Table 7) were again screened on agar plates containing xanthine (XA) as the sole carbon and nitrogen source. All of them showed good filamentous growth even on media containing only xanthine and also showed color change of NBT from yellow to dark blue, confirming the reduction of NBT to formazan as a result of superoxides released over the course of reaction. On the basis of maximum growth on XA plates after an incubation period of 3 days, 21 cultures were selected for *in vitro* enzyme production in XVB broth.

Culture Codes	Plant Obtained from
#16NOBASVNP, #19NOBASVNP, #20NOBASVNP	*Nerium oleander*
#12RSBANEY, #16RSLBRT	*Rauwolfia serpentina*
#4CMBANEY, #21CMBANEY, #26CMBANEY, #12CMBABRT, #9CMLBRT	*Cinnamomum mulbaricum*
#2(a)TMDSTYEL	*Tabarnaemontana divaricata*
#12AMBABRT, ##8AMLBRT, #23(b)AMSTYEL, #6AMLWLS, #12AMBAWLS	*Aegle marmelos*
#5CZBAWLS	*Cinnamomum zeylanicum*
#31JTLSVNP, #33(b)JTLSVNP, #23JTLSVNP	*Jatropha curcas*
#13(a)WGSTNEY	*Wild ginger*

Table No.7: Cultures showing positive growth on XA plates



Photograph No.2: Oxidation of NBT by Endophytic Fungi screened on XA plates; (A) #5NOBASVNP, #19NOBASVNP, #20NOBASVNP; (B) #16NOBASVNP

5.3 Fermentation

XVB (Xanthine Vogel's Broth) was used as the fermentation media for the production of secondary metabolites in the 21 fungal strains selected for liquid culture fermentation (Table No. 8). Out of 21 strains tested for their ability to grow on XVB (Xanthine Vogel's Broth), five strains showed the fastest ability to degrade xanthine and utilized it for their mycelia growth. So, these five strains (Table No. 9) were again fermented on XB i.e. media containing xanthine as the sole carbon and nitrogen source, without any mineral salts. After 10 days incubation (Rodriguez, 2000; Santamari *et. al*., 2002; Rosa *et al*., 2003), turbidity of medium was decreased suggesting consumption of xanthine by fungus. The mycelial growth was also monitored in terms of color, morphology for a period of 10 days. But in XB, turbidity was decreased after 15days incubation. The decrease in turbidity of the fermentation broth was primarily used for monitoring growth of fungus. As assumed, no such decrease in turbidity was observed in the control. A study on degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi; *Aspergillus tamarii* and *Penicillium commune*, has been done, here caffeine was provided as the sole carbon and nitrogen source (Hakil *et. al*, 1998) and most of the other studies have been done on these fungal strains only.

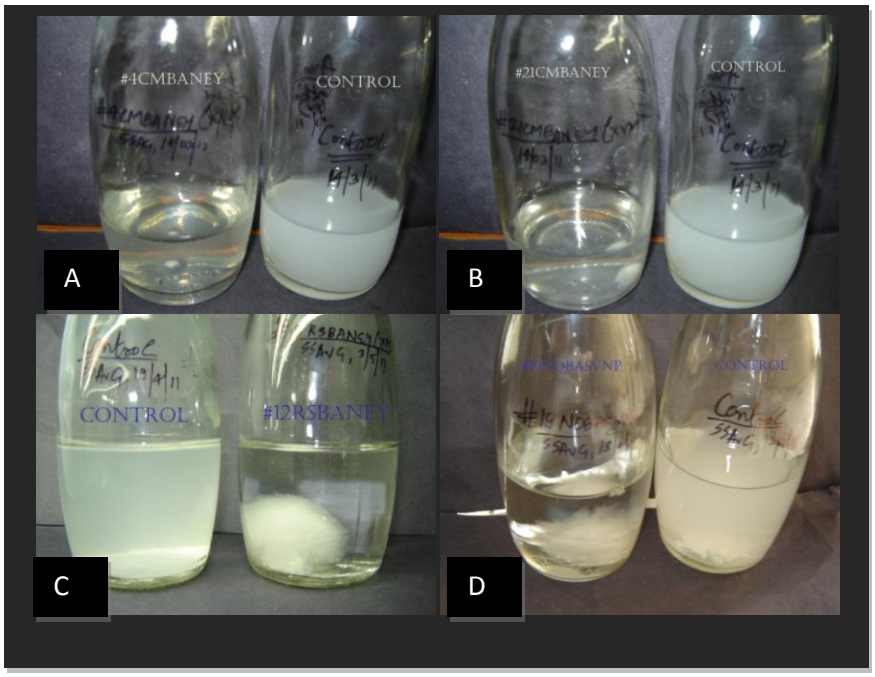
Table No.8:List of cultures fermented on XVB media

S.No.	Culture Code
1	#16RSLBRT
2	#23JTLSVNP
3	#5CZBAWLS
4	##8AMLBRT
5	#33(b)JTLSVNP
6	#6AMLWLS
7	#7RSBANEY
8	#19CZBAWLS
9	#21CMBANEY
10	#20NOBASVNP
11	#13(a)WGSTNEY
12	#23(b)AMSTYEL
13	#1022AMSTITYEL
14	#2(a)TMDSTYEL
15	#2TMDSTYEL
16	#19NOBASVNP
17	#23(a)RSSTNEY
18	#31JTLSVNP
19	#12RSBANEY
20	#4CMBANEY

Table No.9: List of cultures fermented on XB media

S.No.	Culture Code
1	#4CMBANEY
2	#31JTLSVNP
3	#19NOBASVNP
4	#12RSBANEY
5	#21CMBANEY

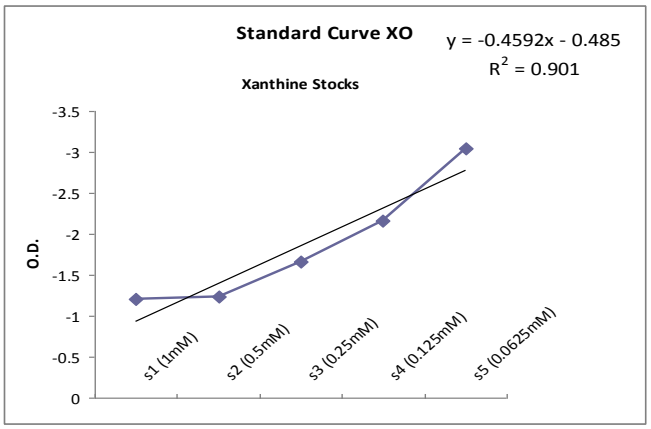
All the five cultures fermented on XB showed consumption of xanthine within 15 days of incubation. This suggests that these fungal strains can utilize xanthine as their sole carbon and nitrogen source, without any mineral salts in the fermentation media. Since the endophytic fungi showed better mycelial growth on XB media, so this was only preferred for further large scale liquid culture fermentation. It has been noticed that the ability of fungi to utilize a purine (xanthine, hypoxanthine, caffeine etc.) source is characteristic of a strain but not of a genus, suggesting enzymatic differences between different strains (Hakil *et. al*, 1998).



Photograph No.3: Liquid Fermentation broths of; (A) #4CMBANEY; (B) #21CMBANEY; (C) #12RSBANEY and (D) #19NOBASVNP, along with their controls.

5.4 Standard Curve for Xanthine Oxidase activity (Using RANDOX KIT)

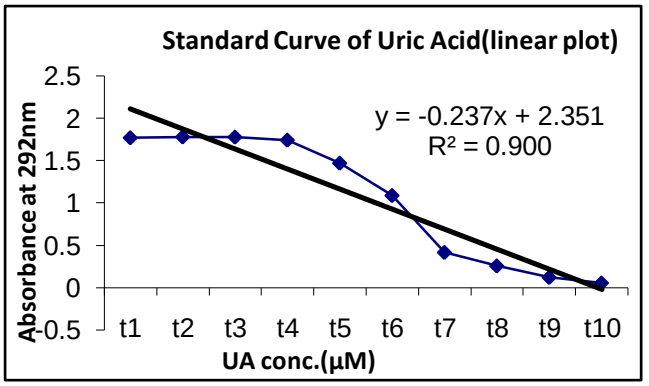
Randox (RANSOD) Kit provides a simple and accurate method for quantifying xanthine oxidase activity. A standard curve was prepared for activity of pure XO enzyme and the standard curve equation was y= -0.4529x-0.485 with R^2= 0.901 exhibiting a high accuracy of the estimation method.



Graph No.1: Standard Curve for XO

5.5 Spectrophotometric Analysis of Xanthine Oxidase

The activity of xanthine oxidase (XO) was measured as the rate of uric acid production when xanthine was incubated with the fungal culture filtrate (Heinz et. al, 1979). For this assay, a Standard Curve for Uric Acid was made first. The standard curve equation was y= -0.237x + 2.351 with R^2= 0.900 exhibiting a high accuracy of the assay. Measurement of XO activity, as a function of uric acid production, has been done on plasma, liver and intestinal tissue (Hashimoto et.al, 1974), but no such studies have been done on cell free culture filtrates of endophytic fungi.



Graph No.2: Linear Plot for standard stocks of Uric Acid

Uric Acid produced in test sample was calculated using the following equation:-

$$Y = -0.237X + 2.3519$$

$$X = \frac{Y - 2.3519}{-0.2372}$$

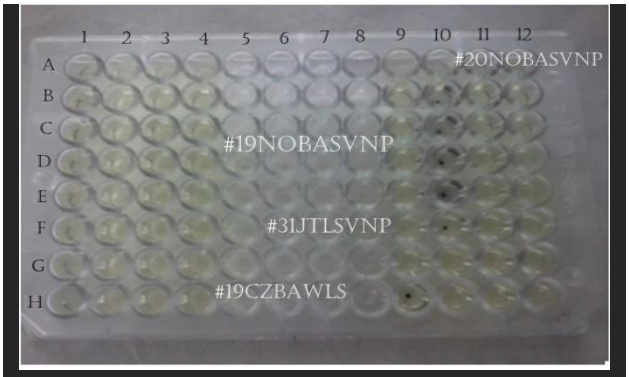
Y= Absorbance at 292nm

X= Uric Acid Conc. (µM)

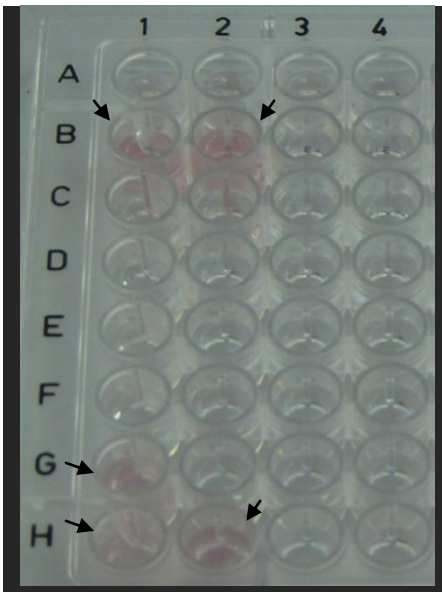
The test culture filtrate (fermented on XB media) was tested for XO activity by recording the final product obtained by the activity of this enzyme, that is, Uric Acid. From the above equation, uric acid produced in test sample was found to be 7.24µM. This confirms the production of XO by culture filtrate of #19NOBASVNP. Thus, a quantitative estimation of uric acid produced as a result of XO activity was accurately done by this method.

5.6 Micro Titer Plate Assay

The test culture filtrates were incubated with 2mM NBT and incubated at 37°C for O/N (Agarwal and Banerjee, 2009). As a result of XO activity, the color of NBT was changed from yellow to dark blue, but no such color change was observed in control wells, as assumed (Photograph 4). This color change confirms the activity of XO in the test fermentation broths. Another assay was performed with TTC (Triphenyl Tetrazolium Chloride). Since, XO is present as Dehydrogenase form when inactive, so a color change of TTC from white to red was also observed (Photograph 5). This showed that the test culture filtrates also have a Xanthine Dehydrogenase activity. Fridovich I. has also studied the quantitative aspects of the production of superoxides anion radical by milk xanthine oxidase (1970).

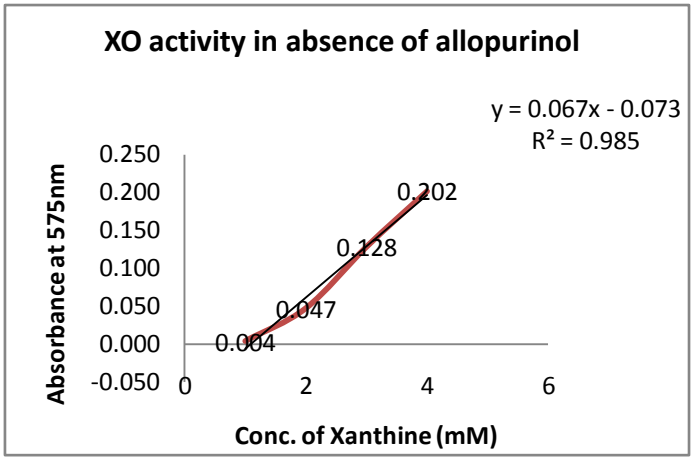


Photograph No.4: Micro Titer plate assay using NBT;A1:H4;Control;H9:#19CZBAWLS; B10;C10:#20NOBASVNP D10;E10:#19NOBASVNP;F10:#31JTLSVNP.



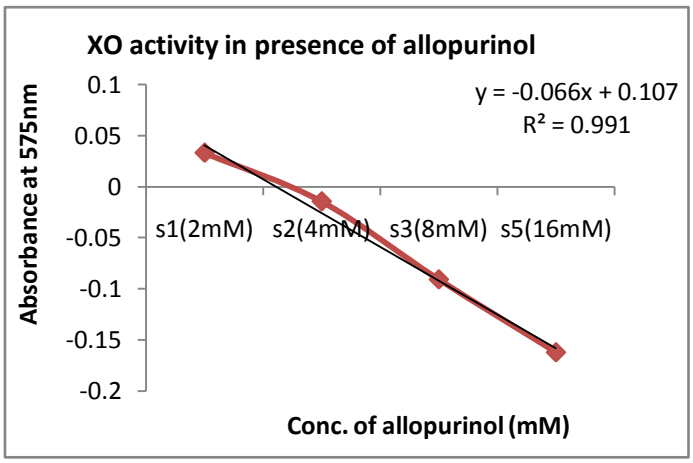
Photograph No.5: Micro Titer plate assay using TTC; arrows depict the wells having different test culture filtrates.
B1: #23JTLSVNP; G1: #7RSBANEY H1:#19CZBAWLS;B2:#20NOBASVNP H2: #19NOBASVNP; A3: H4; Control

5.7 XO Inhibition assay by allopurinol



Graph No.3: XO activity in absence of Allopurinol

Further confirmation of specific XO enzyme activity in culture filtrate of #19NOBASVNP was done by Xanthine Oxidase Inhibition assay, with allopurinol which is a competitive inhibitor of xanthine oxidase. Allopurinol and other 6-unsubstituted pyrazolo [3, 4-d] pyrimidines have been shown to reduce all the oxidation-reductive groups of bovine milk XO (Massey *et. al*, 1970). Since the culture filtrate of #19NOBASVNP gave the best results with other *in-vitro* assays, so further studies were done with it only.

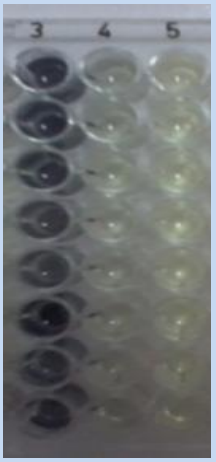


Graph No.4: XO activity in presence of Allopurinol

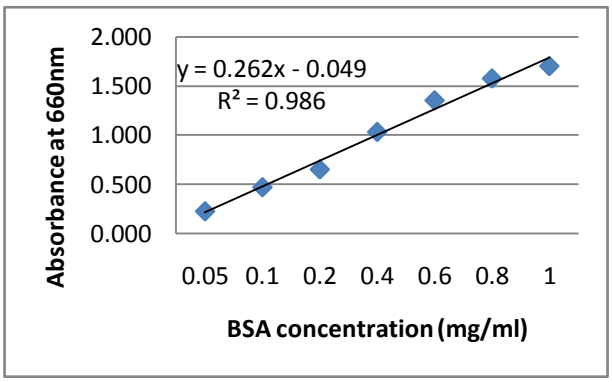
Graph 3 and Graph 4 clearly depicts the activity of XO in absence and presence of allopurinol, respectively. It can be concluded from the graphs that in presence of allopurinol, the activity of XO goes on decreasing. But no such inhibition was observed when the culture filtrates were incubated with the substrate (xanthine) without any allopurinol. This assay confirms that the formation of formazan from NBT was only due to superoxides released during the course of the re conversion of xanthine to uric acid, due to XO activity and is not due to any other superoxides present in the fungal culture filtrate. The antioxidant potential of *Cassia fistula* seeds extract has been evaluated by 2, 2 –diphenyl -1-picrylhydrazyl (DPPH) radical scavenging assay and xanthine oxidase inhibition assay, using allopurinol (Jothy *et. al,* 2011). Also, a new animal-model Bioassay procedure has been developed for the evaluation of Xanthine Oxidase inhibitors (Mohammad et. al, 2010).

5.8 Production, purification and estimation of crude protein from #19NOBASVNP

Further production of enzyme by #19NOBASVNP was done in a total volume of 2 L of XB broth. Crude protein was sufficiently recovered from the cell free filtrate by ammonium sulfate precipitation. This method has been used for isolation and characterization of caffeine oxidase from *Alcaligens* species (Mohapatra et al, 2006). The total protein in the culture filtrate of #19NOBASVNP was found to be 0.416mg of protein/ml of filtrate and in the pellet; protein amount (w/w) was calculated as 0.074 mg of protein/mg of dry pellet as calculated by the Bovine Serum Albumin standard curve by Folin-Lowry's method (Graph No.5). The crude protein obtained was assayed for XO activity, using NBT based calorimetric assay (Agarwal and Banerjee, 2009). Based upon the observations, maximal activity was found in the 10% protein precipitate.

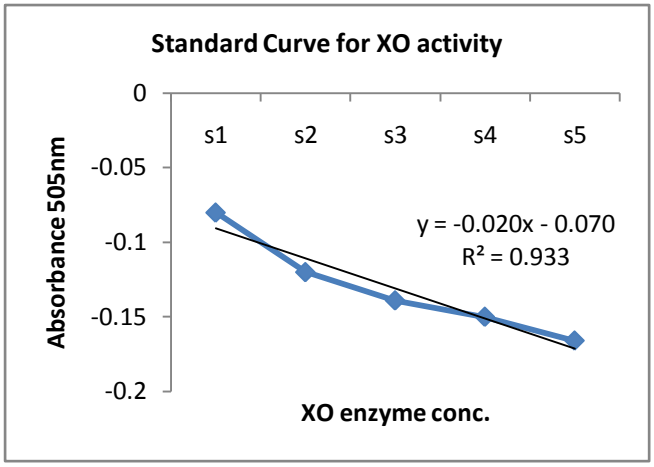


Photograph No.6: XO Activity of 10% (Row 3), 20% (Row 4) and 30% Protein Precipitate of #19NOBASVNP, using NBT Based Calorimetric Method



Graph No.5: BSA Standard Curve

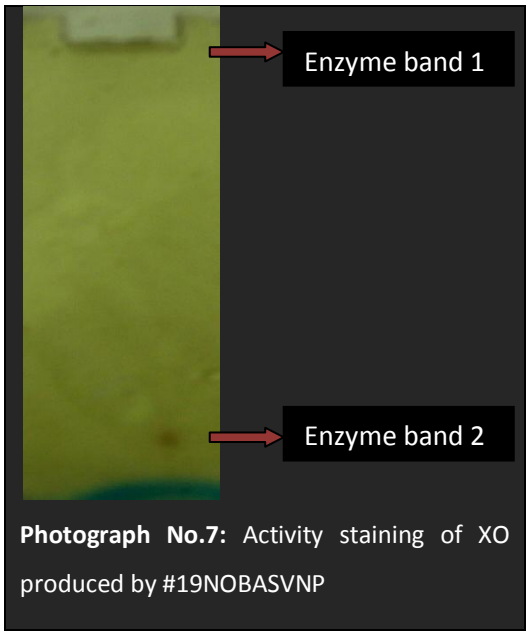
The crude protein obtained was also assayed for XO enzyme activity using different stocks of the protein pellet obtained by ammonium sulfate precipitation. A linear plot was obtained, confirming that as the conc. of XO in test samples decreases from S1 to S5, the specific enzyme activity also decreases linearly. The standard curve equation was y= -0.020x – 0.070 and the R^2 = 0.933, exhibiting a high accuracy of the estimation method.



Graph No.6: XO enzyme Standard Curve

5.10 Activity Staining of Xanthine Oxidase

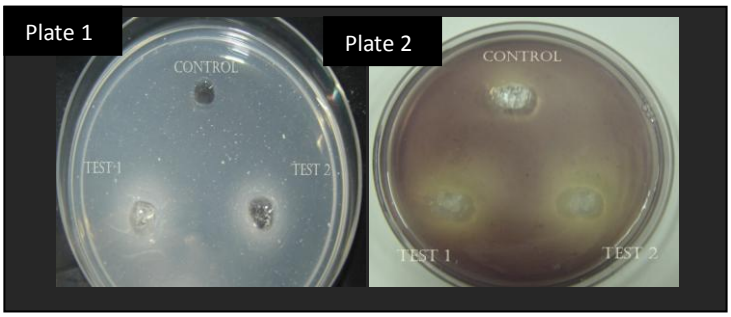
Presence of xanthine oxidase (XO) enzyme in the crude protein was assessed by the activity staining technique. On native polyacrylamide gel containing xanthine as a substrate for enzyme, dark blue colored bands were observed due to the accumulation of formazan at the site of enzyme action, indicating specific activity of XO for its substrate. As per the mechanism, oxidation of xanthine by XO 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 dark blue 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 and no color change was observed in any other part of the gel, thus it could not be regarded as an artifact. Since XO is a large protein, with molecular weight ranging from 80KDa to 290KDa (Ball, 1938), in different prokaryotic and eukaryotic sources, it is possible that during extraction and purification of the enzyme, the protein might get fragmented. The two bands obtained on the Gel with different nature and molecular weights suggest that the enzyme has lost its native state and is thus observed as two different bands on a PAGE Gel.



Photograph No.7: Activity staining of XO produced by #19NOBASVNP

5.11 Agar Well Diffusion Assay (AWD Assay)

This method was used for assessing the XO activity of the test protein (#19NOBASVNP). The test protein was introduced on two agar plates, one containing only 10mM xanthine, i.e., the substrate (Plate 1) for XO activity and another plate containing 2mM NBT along with 10mM xanthine (Plate 2). On agar plates containing xanthine as a substrate for enzyme activity, a zone of clearance was observed and this was compared with the control well, in which no change was observed, as per the assumption. A change in color of NBT from yellow to dark blue (Plate No.2) was also observed as a result of formation of formazan, due to XO activity. The size of clearance zone was found to be related to the concentration of the enzyme. As this result was obtained and no color change was observed in control wells, this confirms the specific activity of the enzyme (XO) in the test protein obtained from #19NOBASVNP.



Photograph No.8: Plate 1; Zone of clearance observed in Test 1 and Test 2 but no such zone in the control well; Plate 2; Color change of NBT from yellow to Dark blue, due to XO activity and a Zone of clearance observed in Test 1 and Test 2 but no such zone in the control well.

Chapter 6

CONCLUSION

CONCLUSION

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

#4CMBANEY, #21CMBANEY (from *Cinnamomum mulbaricum)* and #12RSBANEY (from *Rauwolfia serpentine)* showed satisfactory enzyme activity while #19NOBASVNP (from *Nerium oleander)* demonstrated maximum potential of degrading/transforming xanthine to uric acid at an optimal temperature of 37^{o}C.

Presence of #19 NOBASVNP xanthine oxidase was demonstrated partially by the enzyme assay and rest by enzyme activity staining method. But further work is required to study the enzyme kinetics and its mode of action. Also decoding of the protein sequence would help in the comparison of this protein with all the other already known, sequenced and submitted xanthine oxidases in the protein database. Such comparisons often tell about the novelty of the proteins.

Chapter 7

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