Optimization of media for mass production of Xanthine Oxidase from endophytic fungus.

A

Thesis submitted

In partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted By:

Pallavpreet kaur

Under the supervision of

Dr. Sanjai Saxena

Department Of Biotechnology And Environmental Sciences

Thapar University, Patiala

July, 2012

Certificate

This is to certify that the thesis entitled ***"Optimization for production and partial purification of xanthine oxidase from endophytic fungus"*** being submitted by **Ms. Pallavpreet kaur (Roll No. 301001017)** 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.

14/7/12

Dr. Sanjai Saxena,
Associate Professor / Supervisor
DBTES, Thapar University

Dr. M.S. Reddy
Professor & Head
DBTES, Thapar University

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

Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled ***"Optimization for production and partial purification of xanthine oxidase from endophytic fungus"*** 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 2012 to June 2012, 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.

Pallavpreet Kaur.

Date: July 16th, 2012
Patiala

Pallavpreet kaur
Roll No. 301001017

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

Sanjai Saxena 14/7/12

Dr. Sanjai Saxena
Associate Professor / Supervisor
DBTES, Thapar University

Dr. M.S. Reddy
Professor & Head
DBTES, Thapar University

Department of Biotechnology & Environmental Sciences
Thapar University, Patiala-147004

Contents

List of Tables

List of figures

List of Graphs

Abbreviations

BSA	Bovine Serum Albumin
FAD	Flavin Adenine Dinucleotide
KDa	Kilodalton
mg	milligrams
PDA	Potato Dextrose Agar
ROS	Reactive oxygen pecies
rpm	Revolutions per minute
SDW	Sterile distilled water
PAGE	Polyacrylamide gel electrophoresis
PDB	Potato Dextrose Broth
NBT	Nitroblue Tetrazolium
µl	Microlitre
µM	Micromoles
XA	Xanthine Agar
XB	Xanthine Broth
XO	Xanthine Oxidase
XDH	Xanthine Dehydrogenase
XOR	Xanthine Oxidoreductase
nm	Nanometer
RSM	Response surface Methodology
PBD	Placket Burman Design
ABTS	2, 2 –azino –di (3- ethylbenthiazoline-6- sulphonate)

Executive summary

Xanthine Oxidase has been in lot of demand in clinical industry due to its crucial role in purine metabolism pathway. Various ailments like gout, hyperuricemia, myocardial infarction and reperfusion injury are related to the levels of expression of Xanthine Oxidase .A plethora of questions that need to be answered regarding the implication of genes in XO related disorders and mechanism of action and metabolic pathway of oxidoreductase class of enzymes. Xanthine Oxidase can also find application in the development of biosensors for monitoring substrate utilization in cell culture media.

#19 NOBASVNP, an Endophytic fungus isolated from *Nerium oleander* , demonstrated the maximum potential to produce Xanthine Oxidase. The pure strain of this Endophytic fungus was grown on synthetic media to assess the yield and activity of Xanthine Oxidase. The natural media demonstrated higher ability to produce XO than synthetic media. The natural media was optimized by conventional methods optimizing one parameter at a time and by use of design expert version 8. The optimized media gave higher XO activity than unoptimized media. The enzyme activity of crude enzyme precipitate obtained from culture filtrate was assessed by Nitro blue tetrazolium assay. The crude enzyme was purified by gel filtration chromatography to achieve 34% fold purity. The various kinetic parameters of the partially purified enzyme were also deduced.Further studies on full- purification and characterization of enzyme would allow the commercial exploitation of enzyme from Endophytic fungus.

Chapter 1

Introduction

1. Introduction

Purines are heterocyclic aromatic organic compounds, consisting up of pyrimidine ring fused to an imidazole ring. There are many naturally occurring purines like Adenine, Guanine, uracil hypoxanthine and isoguanine. Aside from the pivotal role of purines in DNA and RNA , purines also act as significant components in a number of other important biomolecules such as ATP, GTP, cyclic AMP, NADH and coenzyme A. The purine metabolism is essential to the body, it provides components of nucleic acids, DNA and RNA, and produces ubiquitous metabolites like ATP. Purine catabolism leads to the formation of a poorly soluble compound Uric acid. The reference range of uric acid in human blood plasma is 3.6mg/dL to 8.3mg/dL. Elevated levels of uric acid leads to hyperuricemia which is linked to Gout. Xanthine Oxidase, the major enzyme of purine metabolic pathway that control the rate limiting step of the pathway i.e. the conversion of hypoxanthine to uric acid via Xanthine with the concomitant production of reactive oxygen species (ROS). Reactive oxygen species are chemically reactive species that are formed as a natural byproduct of normal metabolism oxygen and have important role in cell signaling and homeostasis.

Xanthine Oxidase is highly versatile and ubiquitous complex molybdoflavoprotein that oxidizes Xanthine to uric acid and transfer electrons to NAD^+ (Xanthine dehydrogenase) or oxygen (Xanthine Oxidase). Xanthine Oxidase (XO) and Xanthine Dehydrogenase (XOD) are interconvertible forms of the same enzyme Xanthine Oxidoreductase (XOR) (Hille and Nishano,1995). These different forms of enzyme are conserved in living organisms including archea, bacteria, fungi, plants and metazoans (Amit Aggarwal *et al.*.2009). XOR is widely distributed throughout various organs including liver, intestine, kidney, lungs, myocardium, brain, plasma. The enzyme consist up of 2 monomers each independently capable of catalysis. XO and XDH are capable of detoxification of endogenous compounds apart from oxidation of purines (Borges *et al.*, 2002). The Oxidase form of Xanthine utilizing enzyme uses oxygen as electron acceptor whereas dehydrogenase uses NAD^+ (although it is capable of utilizing O_2.).The presence of NAD^+ binding site constitutes the primary difference between the dehydrogenase and Oxidase. Xanthine Oxidase family, commonly referred to as molybdenum hydroxylases,

which catalyze the hydroxylation of large variety of substrates ranging from aldehydes to aromatic heterocyclic. Members of this enzyme family have the same basic constitution of redox active centers: a molybdenum centre, two 2 Fe/2S centre's of the ferrodoxin variety and one flavin adenine dinucleotide.

The elevated levels of Xanthine Oxidase can lead to various pathological conditions like inflammation, carcinogenesis, ischemia-reperfusion and hepatitis. The level of Xanthine Oxidase is elevated in injured human skeletal muscle in association with inflammatory events (Ylva Hellesten *et al*.,1997). Thus there is utmost need to explore various inhibitors of Xanthine Oxidase. Allopurinol, structural isomer of hypoxanthine is being used to reduce the uric acid level and for the treatment of Gout. Due to the unavailability of free XO in the blood, diverse sources were exploited for the isolation of enzyme so as to design suitable xanthine oxidase inhibitors. On the other hand , Type I Xanthinuria, a condition due to loss of Xanthine Oxidase activity is due to mutations in genes encoding Xanthine oxidoreductase. Type II Xanthinuria results from loss of both Xanthine Oxidase and aldehyde Oxidase activities. Patients with type I and type II xanthinuria are asymptotic. The condition is due to inability to synthesize unique pterin co factor cheated to molybdenum and result in loss of activity of all mononuclear molybdenum enzymes.

Xanthine Oxidase have been isolated and purified from bovine milk. Bovine milk Xanthine Oxidase is commercially available and is used for determination of Xanthine, hypoxanthine and as an auxiliary enzyme for determination of guanine. It has also been suggested that Xanthine Oxidase, due to wide range of substrate specificity may play a role in detoxification. Xanthine Oxidase may detoxify potentially oncogenic and dangerous compounds such as Purine N Oxidase by oxidizing them to less toxic form. It has also been suggested that uric acid may function as anti-oxidant and radical compounds by scavenging singlet oxygen and radicals.

Milk is a rich source of Xanthine Oxidase (Ball, 1938). The probable role of enzyme in milk is its antibacterial activity by virtue of its ability to produce oxygen derived free radicals (Massey *et*

al., 1968). The Xanthine Oxidase from cow's milk crude or purified, appears as Oxidase (type O) and can be converted almost completely into a NAD+ dependant dehydrogenase (type D) by treatment with dihydrolipoic acid, but only to some extent by other thiols. The D form of enzyme is inhibited by NADH which competes with NAD+. The kinetic constants of the two forms of enzyme are similar to those of the corresponding form of rat liver Oxidase. The enzyme on electrophoresis shows a major faster band and a minor slower band on gel electrophoresis. Xanthine Oxidase has also been isolated and purified from Bovine Small intestine (G.G.Roussos *et al*., 1956). Purification procedures have been reported for the chicken liver and kidney dehydrogenases. The calf and pig liver and bovine intestinal and spleen oxidases have also been purified. The purification of milk Oxidase has also been fully investigated and procedures have been reported which are more convenient to carry out and are capable of homogeneous material in high yield (G, G, Rousse*, et al*.,1956).The various purification procedures used till date to purify Xanthine Oxidase from various prokaryotic and eukaryotic sources include acetone precipitation, ammonium sulphate precipitation, dialysis, sephrose 4B column gel filtration, ultrafilteration, DEAE gel filtration etc.

Bovine milk Xanthine oxidase is quite expensive and it is therefore important to explore the microbial communities for the production of xanthine oxidase. Also, endophytic fungi are great source of Xanthine Oxidase. Xanthine Oxidase has been isolated from diverse sources. The Xanthine Oxidase isolated from bacterial sources like *Arthrobacter sp. Arthrobacter sp.* produced high levels of Xanthine Oxidase activity requiring as little as 20 fold purification to approach homogeneity.

Endophytic fungi are a special and important group of microorganisms which reside within the healthy tissue of the plant without providing any obvious clue of their existence and are hypothesized to be providing protection to host plant from the attack of herbivorous insects or vertebrate grazers by production of bioactive compounds (Tan and Zou, 2011). Endophytic fungi are a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity. Fungal endophytes like *Phomopsis sp.,Muscudar albus (* Strobel *etal .,* 2004 *), Fusarium spp,Aspergillus sp,Pestalotiopsis microspora* (Strobel, 2003) are serving as a

source of antifungal , antimicrobial and anticancerous products (Strobel, 2003).Hence, it can be inferred that they are potential sources of plethora of secondary metabolites and enzymes. Endophytes are the resources of diverse enzymes. Tannase has been isolated from *Penicillium chrysogenum*.It has also been purified to 24 fold with a recovery of 18.5 % after ammonium sulphate precipitation, DEAE cellulose chromatography and Sephadex -200 gel filtration. *Penicillium chrysogenum* has also been shown to utilize hypoxanthine and xanthine as sole nitrogen source. This suggests the presence of various enzymes like Xanthine dehydrogenase, uricase and urease.

The first report of Xanthine Oxidase production from Endophytic fungus came from the study of superoxide involved in the sclerotial differentiation of filamentous phytopathogenic fungi (Papapostolou,Georgiou,2010). The present study proposes the optimization of media for production of maximum Xanthine Oxidase, its purification and characterization.

Chapter 2

Review of literature

2. Review of literature

2.1 Xanthine Oxidase

Purines are heterocyclic aromatic compounds which serve as building blocks of genetic repository and energy currency of cell. Xanthine is a purine base normally found in almost all tissues of the body. 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).Mild stimulants like caffeine are derived from Xanthine (Konisberger *et al.*, 2001).

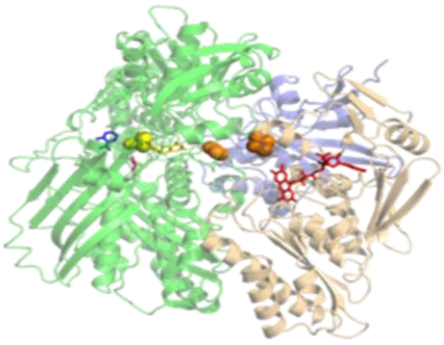


Figure 1: Crystallographic structure of Xanthine Oxidase

Xanthine Oxidase plays a crucial role in catabolism of purines in various species including humans. It is highly versatile and ubiquitous complex molybdo-flavoprotein which control rate limiting step of purine catabolism. Xanthine Oxidase is a large molecule having a molecular weight of 270 kDa and has 2 flavin molecules bound as FAD, 2 molybdenum atoms, and 8 iron atoms per enzymatic unit. It is not only confined to milk, but also having been detected in all species examined to date, including bacteria (Krenitsky, Tuttle, Cattau, & Wang; 1974;Parks and Granger., 1986). In mammalian tissues, the enzyme is widely distributed, particularly high levels being found in liver and intestine (Parks and Granger, 1986). It is conventionally seen as late enzyme of purine catabolism, catalyzing the oxidation of hypoxanthine to Xanthine and of Xanthine to uric acid. However as will be seen, XOR is capable of much more than this and over the last two decades, attention has focused on physiological roles (Harrison, 1997). Apart from hypoxanthine and Xanthine, XOR has wide range of reducing substrates , including many nitrogen heterocyclic and also relatively simple aldehydes. Once reduced, XOR passes electrons to NAD^+, yielding NADH or to molecular oxygen. Reduction of oxygen yields the reactive oxygen species (ROS), hydrogen peroxide and superoxide anion. The enzymes from sources, ranging from bacteria to avian liver, react only poorly with oxygen,

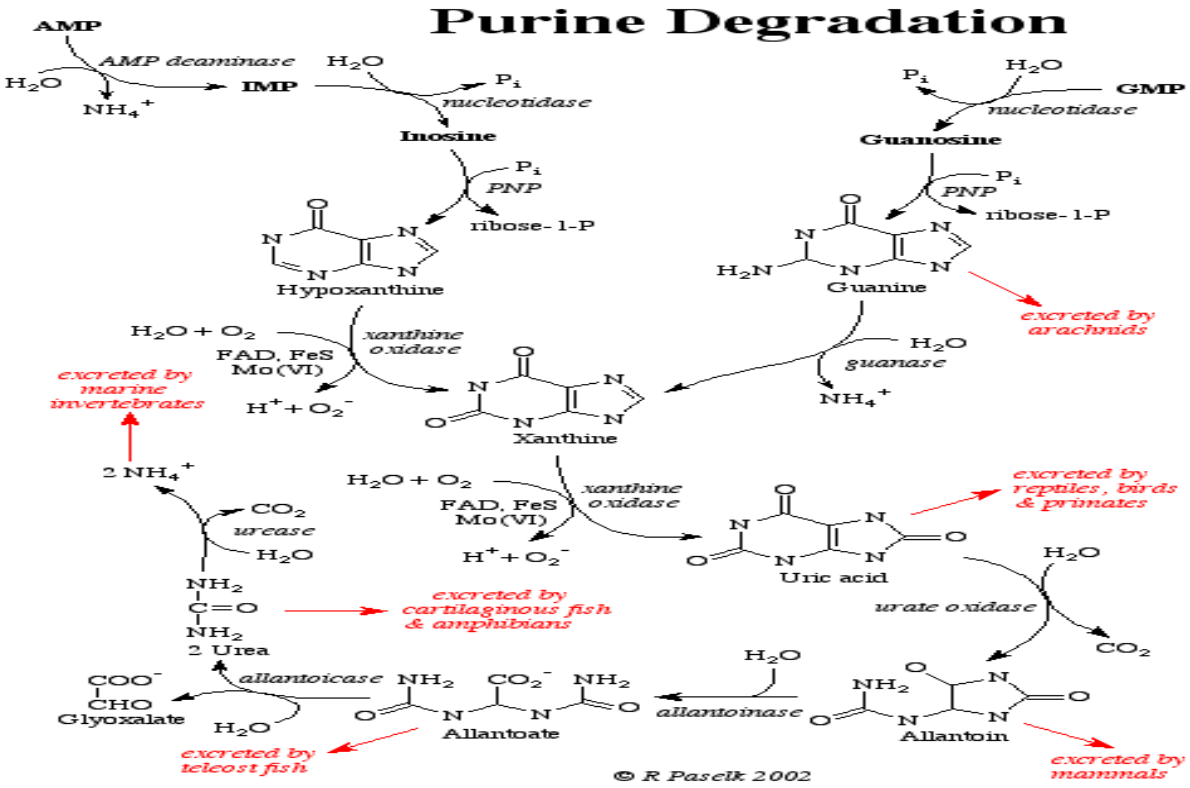
especially when NAD is also present as an alternative acceptor and appears to exist as single stable form. On the other hand, the enzymes from mammalian sources can exist can exist in two reversible forms, in one of which oxygen is preferred acceptor and in the other NAD+ is the preferred acceptor. The enzyme from these sources can be converted irreversibly from the dehydrogenase to Oxidase form by limited proteolysis (Russ Hille, Takeshi nishino, 1995).

2.1.1 Various sources of Xanthine Oxidase.

Xanthine Oxidoreductase has been long known to be present in cow's milk, from which it is readily available even in gem scale. Indeed, XOR was purified from this source over 60 years ago and is consequently one of the best studied of all enzymes (Massey & Harris, 1997). In cow's milk, XOR occurs in milk fat globule membrane (MFGM). Fat droplets originate in the endoplasmic reticulum of mammary secreatory cell. In the secretion process, they migrate to luminal surface and bud off from the cell, enveloped by the apical cell membrane. This becomes MFGM (Keenan, 2001, Patton and Keenan , 1975) .The MFGM contains eight major proteins (Mather , 2000). XOR is prominent among these and procedures for purification are established. The enzyme is accordingly well characterized (Bray 1975, Harisson, 1996). Xanthine Oxidase and Xanthine Dehydrogenase are the enzymes catalyzing the oxidation of Xanthine, are widespread in nature and found to be remarkably similar in overall size and composition of redox centre, as well as their ability to oxidize variety of purine, pyramiding, pterin and aldehyde substrates. In terms of electron acceptor, they fall into two broad groups by using preferentially molecular oxygen (the Oxidase) or NAD^+ (the dehydrogenases).

The enzymes from sources, ranging from bacteria to avian liver, react only poorly with oxygen, especially when NAD is also present as an alternative acceptor and appears to exist as single stable form. On the other hand, the enzymes from mammalian sources can exist can exist in two reversible forms, in one of which oxygen is preferred acceptor and in the other NAD^+ is the preferred acceptor. The enzyme from these sources can be converted irreversibly from the dehydrogenase to Oxidase form by limited proteolysis (Russ Hille, Takeshi nishino, 1995).

Xanthine Oxidase is commercially available and is widely used for the determination of Xanthine, hypoxanthine and inosine and an auxiliary enzyme in the determination of guanine and guanosine. It is also used for the measurement for the measurement of guanase and nucleoside phosphorylase activity. Till date, Bovine milk Xanthine Oxidase is the only commercially available enzyme. As Bovine milk Xanthine Oxidase requires highly expensive purification procedures and down streaming, therefore it is imperative to explore active sources of the enzymes that require less expensive methods for recovering highly purified Xanthine Oxidase.



Source: R Paselk *et al* .,2002

Fig 2: Purine metabolism pathway.

2.2 Microbes as a source of Xanthine Oxidase

A vast diversity of microbes act as a source of Xanthine Oxidase. A plethora of microbes have shown to produce high levels of Xanthine Oxidase, requiring very low fold purity too approach homogeneity with some preparations. The Xanthine Oxidase isolated from bacterial source has been shown to posses high K_m for Xanthine and is relatively specific. The bacterial enzyme was relatively small and may be dimeric with approximate native and subunit molecular weights of 146,000 and 79,000 Da respectively. The molecular oxygen is relatively efficiently utilized in case of bacterial Xanthine Oxidase.

Wool folk was the first to scrutinize caffeine degradation by *Pseudomonas putida* (1975). Tahany A. Elzainy investigated degradation of Xanthine by *Pencillium chrysogenum* (1969). *Penicillium chrysogenum* utilized the purines hypoxanthine, Xanthine, uric acid and adenine as sole nitrogen source but not as methylated purines, caffeine and theobromine. Cell free extracts of this organism contained the enzyme Xanthine dehydrogenase, uricase, allantoises and uricase.

Arthrobacter S-2, originally isolated by enrichment on Xanthine, produced high levels of Xanthine Oxidase activity requiring as little as a 20 –fold purification to approach homogeneity with some preparations. The enzymes was relatively specific when compared with previously studied Xanthine oxidizing enzymes . As a result of the survey of 47 diverse bacteria containing Xanthine Oxidase, we observed two strains of *Arthrobacter* gave the highest specific activities when ferricyanide was used as an electron acceptor (C.A .Woof lock *et al.*, 1978). 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 a sole source of nitrogen and found that metabolism of caffeine involves demethylation at seventh position (Schimmmer *et al* ., 1971).

A novel caffeine Oxidase has also been isolated , purified and characterized from Alcaligenes species (B.R.Mohapatra *et al.*, 2006). The methyluric acid production in *P.putida* has also been

witnessed. Also, the oxidative stress superoxide radical is involved in sclerotial differentiation of phytopathogenic filamentous fungi *Rhizoctonia solani, Sclerotinia sclerotium, sclerotinia* minor. Also, the metabolism of Xanthine -8 – carboxylic acid by *Alcaligens faecalis* (Dairman *et al.*, 1964) has been studied. The caffeine dehydrogenase has also been purified from *Pseudomonas species* strain CBB1. It oxidized caffeine to trimethyuric acid hydrolytically without producing hydrogen peroxide. The caffeine Oxidase was shown to be specific for caffeine and showed no activity for Xanthine (Subramanian *et al*., 2008).

2.3 Endophytic Fungi –Source of novel Xanthine Oxidase

Endophytic fungi colonize healthy plant tissues and either persist in a dormant phase or compromise more extensive , but symptomless infections (Petrini, 1991). They are ubiquitous and have been found in all the species of plants studied to date. They may improve the ability of the plant to tolerate abiotic stress such as drought as well as improve their resistance to insects. The endophytes benefit host by preventing pathogenic organism from colonizing them by secreting chemicals that inhibit the growth of competitors. The vast range of compounds produced by endophtyes have been shown to combat pathogens and even cancers in animals including humans (Strobel and Daisy , 2003). Fungal endophytes like *Phomopsis spp., Fusarium spp., Muscudor albus* and *Pestalotiopsis microspora* serve as potential sources of antimicrobial and anticancerous compounds. The various compounds and enzymes produced by endophytes include anticancerous compounds like Taxol , Campthothecin , Ergoflavin ,Phenypropanoids and Podophyllotoxins , Antimicrobial agents like Pestalachlorides A and Emodin and antioxidants like Pestacin , Isopestacin and Exopolysaccharides. Cell free Extracts of *Penicillium chrysogenum* contains enzyme Xanthine dehydrogenase , uricase , allantoinase, allantoicase and urease . There has also been instances eliciting the role of Endophtic fungi as Xanthine Oxidase producers. Xanthine Oxidase has been implicated a role especially in the transition developmental stage between the undifferentiated and the differentiated stage suggesting phytopathogenic role of Xanthine Oxidase in plant infection (Ioannis *et al.*,2010)

Xanthine Oxidase (XO) was detected for the first time in the fungi in general was localized in the cytoplasm membrane. The contribution of XO in overall oxygen production was very significant, reaching 30–70% among the strains. The additional finding that these fungi is excreted extracellular can be related to their protection from the response of plants to produce O_2 at infection sites.

There is utmost need for new Xanthine Oxidase producer. This is due to the fact that the enzyme extracted from bovine milk is highly expensive. Although xanthine Oxidase has been extracted from a number of sources yet the development of reliable commercial mass production process for xanthine Oxidase remains to be achieved. A novel fungal Xanthine Oxidase can be exploited to address a number of questions presently not possible with the available form of prokaryotic and eukaryotic enzymes. The net cost of purified enzyme will decrease as downstream recovery and purification of enzyme from fungal broth would be much simpler than bovine milk. The first report of Xanthine Oxidase production from Endophytic fungus came from the study of superoxide involved in the sclerotial differentiation of filamentous phytopathogenic fungi (Papapostolou, Georgiou, 2010). There are developed methods for screening bacteria and fungi degrading caffeine (Yamaoka and Mazzafera , 1999).

The enzymatic degradation of caffeine in microbes is performed by N-demethylation and oxidation with demethylases and oxidases respectively (Gokulakrishnan *et al.*, 2005). The medium comprising up of basal salts and caffeine has been used for screening of microorganisms producing caffeine Oxidase (Mohaptara et al., 2006). Minimal salt media consisting up of 2mm/L Xanthine concentration has been used for screening of Xanthine Oxidase producing bacteria (Aggarwal and Banerjee, 2009).

Fermentation involves breakdown of complex organic molecules into various simple products and release of energy. Fermentation is carried out by the enzymes released extracellularly. *Rhizopus delemar* showed ability to catalyze caffeine and theophylline by solid state fermentation (SSF) in packed bed column reactor and cell free extracts showed considerable amount of caffeine Oxidase (Hussein et al., 2009).

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 and a phytopathogenic role during penetration of these fungi in cell wall of their host palnts.the first report of Xanthine Oxidase production by fungi intracellularly has been reported during sclerotial differentiation of phtyopathogenic filamentous fungi like *Rhizoctonia,solani* and sclerotinia minor (Papapostou , *et al .,* 2010). Also, it has been reported that some genre of *Aspergillus , Penicillium, Rhizopus and Stemphyllium* have the capacity to metabolize caffeine enzymatically (Mazzaafera, 2002). *Aspergillus niger* and *Penicillium Roqueforti* metabolized caffeine into theophyllline as the first degradation product (Hakil *et al*.,1998) tested the ability of vast strains of filamentous fungi to to grow on caffeine as a sole source of nitrogen .

Culture broth extracts produced by fermentation of Guignardia species and Phomopsis species in Potato dextrose Broth were evaluated for antimicrobial activity by using agar well diffusion assay (Corrado and Rodrigues, 2004)

2.4 Spectrophotometer Assay of Xanthine Oxidase

This experimental approach exploits the beer's law which predicts a linear relationship between absorbance of a solution and concentration of the analyte. The simplified alkaline phosphotungstate assay for uric acid in serum was developed (Carrol *et al*; 1970). In the present study, a high-throughput microtitre plate-based colorimetric assay for Xanthine Oxidase producing microorganism was developed. Superoxide produced by microbial cultures grown on Xanthine rich medium interacts with nitroblue tetrazolium (NBT) solution and produces dark blue color, which facilitates the rapid screening of Xanthine Oxidase producing microorganisms and could be adapted for quick quantitative assessment and distribution of Xanthine Oxidase in many heterogeneous microbial communities. The method developed may be utilized for the rapid screening of a variety of Xanthine Oxidase producing microorganisms from the nature (Aggarwal and Banergee, 2009).

The standard spectrophotometer assay of Xanthine Oxidase is based on the oxidation of Xanthine / hypoxanthine to uric acid and activity of Xanthine Oxidase is measured as the rate of uric acid production when Xanthine is incubated with the culture broth (Trivedi *et al.*, 1978). The change in absorbance due to uric acid production can be recorded at 292 nm. One unit of XO activity is defined as 1 µM of uric acid produced per min at 37^{O}C with excess substrate. Xanthine Oxidase activity has also been assessed based on the oxidation of 2, 2-azino-di (3-ethylbenthiazoline-6-sulphonate) (ABTS) by use of uricase. The absorbance increase of oxidized form of ABTS, measured after 10 minutes at 410 nm is proportional to Xanthine Oxidase activity (Singh *et al.*, 1987).

2.5 Microtiter based Xanthine Oxidase assay

Microtitre plate consists up of a flat plate with multiple wells. It has become a standard tool in analytical and clinical research. The screening of Xanthine Oxidase producing microorganisms was done using nitroblue tetrazolium salt based calorimetric assay. Microbial culture, grown on Xanthine as a sole source of carbon and nitrogen was centrifuged and the pellet was suspended in Tris-HCl buffer (50 mmol/L, pH 7.6). Each reaction contained 150µl microbial cell (concentration varied from 25 to 150 mg/ml), lysed with NaOH(1 mol l-1); 150 µl NBT solution containing 50 mmol/L Tris-HCl, pH 7.6, 2mmol/L Xanthine and 0.50mmol/L nitroblue tetrazolium The reaction mixture was incubated for 5 minutes at ambient temperature, centrifuged and absorbance of supernatant was measured by microplate readers (Emax, Molecular Devices, California) at 575 nm. Dark blue color appears due to the formation of formazan, formed as a result of interaction of the NBT and superoxide produced during the course of the oxidation of Xanthine to uric acid by Xanthine Oxidase (Agarwaal and Banergee ; 2009).

2.6 Optimization of media for maximum yield of Xanthine Oxidase possessing maximum activity

Although various synthetic media are suitable for the growth of Endophytic fungi producing Xanthine Oxidase such as Czapek dox broth, Richard's broth and Xanthine broth, but they are expensive. Hence it is requisite to look for alternative media. The natural media was defined

and optimized by varying concentration of various constituents. Various parameters were optimized for getting the maximum yield of the enzyme with maximum activity. The parameters optimized include both the physical as well as the nutritional parameters such as pH, temperature, revolutions per minute (rpm), sucrose concentration, potato extract concentration, Xanthine concentration, inoculum size.

Various compounds can serve as substrate for Xanthine Oxidase. Formamide is a substrate of xanthine oxidase. At pH 8.2 and 1.14mM-02, V_{max} (app.) is 3.1 s- I and K_m (app.) is 0.7M. Mo (V) e.p.r. signals obtained by treating the enzyme with form amide were studied, and these provide new information about the ligation of molybdenum in the enzyme and about the enzymatic mechanism.

The substrate is the first compound that is not a nitrogen-containing heterocyclic to give a Very Rapid signal. This supports the hypothesis that the very Rapid signal, though it is not detectable with all substrates, represents an essential intermediate in turnover. Form amide also gives the inhibited signal and is the first non-aldehyde substrate to do so. The single oxygen atom detectable in the signal is shown to be strongly and anisotropically coupled. This indicates that this atom remains as an oxoligand of molybdenum in this signal-giving species.

The optimization of media has been achieved by using Design expert version 8. Design Expert is a piece of software designed to help with the design and interpretation of multi-factor experiments. The software offer a wide range of designs , including factorials, fractional factorial and composite designs. In analyzing an experiment, we fit models relating to a response to a set of controllable variables. For continuous control variables, we often use a linear, factorial or quadratic model.Half-normal and normal plots are used for highlighting active factors while pareto chart gives a picture of relative sizes of the different effects.

Design expert version 8 has been used to carry out statistical media optimization and alkaline protease production from *Bacillus mojavenesis* in a bioreactor (Qasim et al ; 2003). Alkaline Protease production was in *Bacillus mojavenesis* was improved up to 4.2 fold in a 14 litre bioreactor during validation of a predicted stastical model .

The final enzyme concentration was 2389 U/ml obtained within 10-12 hours in comparison to 558 U/ml after 24 hours in shake flask cultures.

Analysis of variance (ANOVA) of face centered central composite design showed a high coefficient of determination value of 0.9473, thus ensuring a satisfactory adjustment of the quadratic model with the experimental data. The co-ordinates of three factors were positive whereas negative coordinates were obtained for other two factors. The response surface curves predicted curves for optimal enzyme production. Response surface methodology was also used to study the cumulative effect of the nutritional parameters to enhance the production of extracellular amylase in solid state fermentation by *Thermomyces lanuginosus* .For obtaining mutual interaction between the variables and optimizing these variables ,a central composite design using response surface methodology was employed.

Thus, this study provides useful information about the regulation of protease synthesis through manipulation of various physiochemical factors.

2.7 In –vitro Assays of Xanthine Oxidase

2.7 .1 Xanthine Oxidase based inhibition Assay

Allopurinol is the structural isomer of hypoxanthine and is a competitive enzyme inhibitor of Xanthine Oxidase. It reduces the uric acid formation as well as cause feedback inhibition of purine synthesis. Allopurinol is oxidized to oxypurinol, an inhibitor of Xanthine Oxidase. Allopurinol is a drug primarily used to treat hyperuricemia and chronic gout. Allopurinol is also useful in prevention of diabetes type 1 (Desco *et al.*, 2002). The microbial cultures which were treated with Allopurinol is a standard inhibitor of Xanthine Oxidase, showed the formation of light blue color and the corresponding decrease in absorbance (Aggarwal and Banerjee, 2009).

2.7.2 Determination of enzymatic activity of crude protein by NBT Assay method.

The Xanthine Oxidase activity was assayed by using NBT based method .The crude protein obtained by acetone precipitation was incubated with Xanthine as a substrate and Nitro blue tetrazolium as a dye .The reaction is initiated by the attack of the hydroxyl group attatched to the molybdenum centre of the enzyme on the substrate. It is followed by deprotonation of the

Xanthine and the subsequent rearrangement yield uric acid and superoxides. The blue colour was obtained due to farmazan formation by interaction of superoxide radicals released by Xanthine Oxidase and the NBT. The absorbance obtained hence was used to deduced the enzymatic activity by comparision with the standard curve. Hence U/ ml of Xanthine Oxidase in crude protein was obtained.

2.7.3 Determination of protein concentration by Folin –Lowry method.

This is based on reaction of Cu^{+}, produced by oxidation of peptide bonds with folin-ciocalteu reagent. The reaction involve reduction of folin reagent and oxidation of aromatic residues. The concentration of reduced folin reagent is measured by absorbance at 660 nm. The amount of aromatic amino acids present in the protein will decide the intensity of the color developed. The total protein content in the crude protein obtained by the acetone precipitation was obtained by lowry method. The lowry method has been used for determining protein concentration in urinary samples for studying the crucial role of Xanthine Oxidase in initiation of renal injury in rats with hyperlipidemia (Gwinner,Scheuer *et al*., 2006).

2.7.4 Determination of uric acid by Phosphotungstate Assay.

Phosphotungstic acid is a heteropoly acid and is normally present as a hydrate. In alkaline medium, uric acid reduces phosphotungstate reagent producing tungsten blue color, whose absorbance is proportional to uric acid concentration.

Uric acid is commonly determined in biological fluids by measuring the blue colour produced by its reaction with phosphotungstic acid under conditions which minimize interference by other reducing substances. The determination of uric acid in biological materials was done by using the phosphotungstate assay (A.E steel, 1956). It was shown that it is feasible to make calorimetric uric acid determinations by means of blue colour which uric acid produces with a reagent which was supposed to be phosphotungstic acid. The reaction mixture consisted up of uricacud , sodium bicarbonate and phosphitungstate and absorbancewas taken at 292 nm .The intensity of blue colour is directly proportional to the amount of uricacid produced by the xanthine oxidase (Kalpana Luthra, 2010).

2.7.5 Activity staining of crude and purified Xanthine Oxidase.

Ozer *et. al* (1997) gave a method for activity staining of Xanthine Oxidase. A method of activity staining after Native Polyacrylamide gel electrophoresis was done analyze several glycolytic enzymes (Jean Rivoal *et al.,* 2001).The method has also been used to confirm the presence of Xanthine Oxidase isolated from *Pseudomonas putida* L (Mazzaferra et .al ,1999). Laemmli described the technique of activity staining that is essentially carried out non-denaturing and nonreducing conditions. The staining of gels is done in 2mM Nitroblue tetrazolium chloride till the appearance of dark blue bands due to the formation of formazan .The activity staining has also been done in exploration of Xanthine Oxidase produing microorganisms from hypoxanthine supplemented soil (Mahajan *et al*., 2012).

2.8 Protein Purification

The various steps in protein purification may free the protein from the matrix that confines it , separates 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. The separation employs difference in protein size , biological and binding affinity.

Various purification procedures have been employed to purify Xanthine Oxidase from diverse sources. The ultra fractionated extract has been treated with streptomycin, ammonium sulphate and precipitate obtained as such was applied to Sepharose 4 B column and then ultra filtration and dialysis was carried out to purify Xanthine Oxidase from *Arthrobacter S-2* (C.A.Woolfolk *et al* ., 1978).

A typical purification procedure of caffeine Oxidase isolated from *Alcaligens sp* CF8 involves treating crude extract with 60 % ammonium sulphate , DEAE cellulose chromatography followed by Sephadex G-100 chromatography.

Protein samples commonly contain substances that interfere with downstream applications. Several strategies exist for eliminating these substances from samples. Small soluble substances may be removed and the samples exchanged into appropriate buffers by dialysis or gel filtration (desalting columns). A variety of Thermo Scientific dialysis and desalting products are available for performing such buffer exchanges with small or large sample volumes .Another strategy for removing undesirable substances is to add a compound that causes protein to precipitate. After centrifugation to pellet the precipitated protein, the supernatant containing the interfering substance is removed and the Protein pellet is re-dissolved in a buffer that is compatible with the downstream application.

Several methods for protein precipitation are described in the literature. Tannase from *Asperigillus awamorii* has been isolated by acetone precipitation method of the supernatant (Mahaptara *et al*., 2005).

The ammonium sulphate precipitation is method used to purify proteins by altering their solubility. It is a specific case of more general technique known as salting out. Ammonium sulphate is used as its solubility is exceptionally high. The solubility of protein varies according to the salt concentration. At low concentration of ammonium sulphate, the solubility of protein increases with increasing salt concentration, an effect called salting in. At very high salt concentration, protein will be precipitated out from the solution. An effect called salting out .The purification and characterization of caffeine Oxidase from *Alcaligens sp* has been carried out by using ammonium sulphate precipitation. Glycerol Oxidase purification from *Penicillium* species TS-622 has been carried out by ammonium sulphate precipitation (Lin *et al.*, 1995).

2.9 Protein Purification via dialysis and Gel filtration chromatography

Chromatography is separation method that relies on differences in partition behavior between a flowing mobile phase and a stationary phase to separate components of mixture. A column holds the stationary phase and the mobile phase carries the sample.

Gel fitration chromatography, also called as Size exclusion or Gel permeation chromatography, separates molecules based on differences in size. The sample is applied to the top of column containing porous beads of cross linked agarose. Large molecules cannot enter the pores and

elute as the first peak in the chromatogram. They elute fast and this is called total exclusion. Intermediate molecules may enter the pores and may have an average residence time in the particles. Different molecules therefore have different total transit times through the column. The portion of chromatogram is called the selective permeation region. Small molecules enter the pores and have longest residence time on the column and elute together as the last peak in chromatogram. The last peak in the chromatogram is the total permeation limt. Sepharose 6B has a pore size ranging from 45-165µm and a fractionation range of 10KDa-1,000 KDa. It is stable in chaotropic salts and has potential applications in separation of viruses, polysaccharides and proteins. The crude caffeine Oxidase was purified by treating it with 60% ammonium sulphate , dialysis with 10KDa molecular weight cut-off and passage of dialyzates through DEAE cellulose column pre-equiliberated with 0.2 M phosphate buffer.

Xanthine Oxidase has been purified from *Micrococcus lactilyticus* by protamine sulfate, 50-65% ammonium sulphate fractionation, acetone precipitation followed by pooling of samples through DEAE sephadex and sephadex G-200(Susan T.smith *et al*.,1967). Purification of *Arthrobacter S-2* Xanthine Oxidase has been done by precipitation with ammonium sulfate followed by elution from a column of Sephrose4 B. The enzyme obtained hence was concentrated fivefold by ultrafiltration through Amion XM50 filters (C.A.woolfolk *et al* .,1978). Adenosine deaminase has been purified from *Asperigillus oryzae* by Sephadex G-100 Gel filtration chromatography. (Hye-Seon choi , 1963).NADH Oxidase has been purified from Serpulina hyodysenteriae by size –exclusion columns (Thad B. Stanton , 1993).

Chapter 3

Aims and Objectives

3. Aim of Study

The current study was aimed at purification and characterization of Xanthine Oxidase from an Endophytic fungal strain #19 NOBASVNP.

The Objectives of current study were:

1. To optimize various nutritional and parameters to attain maximum activity of Xanthine Oxidase from Endophytic Fungal strain #19 NOBASVNP.

2. To Assess activity of fermentation broth, crude and purified enzyme by in-vitro assays. Purification of enzyme via various methods .

3. To study enzyme kinetics and assess the effect of substrate concentration, temperature, pH and inhibitors on enzyme activity.

Chapter 4

Materials and methods

4.1 Maintenance of Pure cultures of Endophytic fungi

4.1.1 Preparation of Potato Dextrose agar Plates

39g of PDA (Hi Media) was dissolved in 1 liter of double distilled water and stirred thoroughly. It was dispensed in 250 ml Erlenmeyer flask and autoclaved for 15 minutes at 121°C. Aseptically 22.5 ml of PDA was dispensed in sterile 90mm petriplates and allowed to solidify at room temperature.

4.1.2 Sub -culturing of Endophytic fungus on PDA plates

#19 NOBASVNP Endophytic fungal strain, isolated from *Nerioum oleander*, was found to produce xanthine oxidase, the major enzyme involved in the purine degradation pathway (Avom Gupta , M.Sc thesis 2011). The loopful of culture was transferred from stock cuture tube slant on to the fresh PDA plates. The plates were incubated at 26^{o}C for maximum of 5 days till profuse fungal growth was observed. All the requisite procedures are carried out aseptically in Laminar air flow hood.

4.1.3 Pure culture maintainence on PDA slants:

The pure culture was transferred from the 5-6 day old PDA plate on to the PDA slants for long term storage. The stock of the culture was also maintained on to the PDA slants containing 10 % glycerol for long term storage at 26°C.

4.2 Fermentation

Liquid culture fermentation was carried out initially in synthetic media. The synthetic media used were- CzapekDox broth, Richards broth, Xanthine broth. In addition to synthetic media, the fermentation was also carried out in natural media Potato dextrose Broth. Xanthine Broth was prepared by dispensing 134 ml of filter sterilized 10mM Xanthine into an Erlenmeyer flask and making up volume to 200 ml by autoclaved double distilled water (pH-7.8). Xanthine is not autoclaved as it is heat-sensitive. The pH of rest of the broths were adjusted to 7.3. The broths

were then autoclaved at 121^{o} C, 15 psi for 15 minutes. The spore suspension of 10^{8} cells of fungal culture was used to inoculate 100 ml pre-sterilized broth in Erlenmeyer flasks under aseptic conditions. The flasks were incubated at 120 rpm, 26 ^{0}C for 10 days for production of secondary metabolites (Rosa *et al*., 2003) The color and morphology of mycelia growth was monitored during 10 days.

4.3 Optimization of Natural Fermentation broth

Optimization of nutritional components

4.3.1 Optimization of Xanthine (substrate for Xanthine Oxidase):

The media components were optimized so as to obtain the maximum yield of xanthine oxidase. The natural media was used for carrying out the fermentation as the synthetic media is too costly to carry out mass fermentation and isolate protein in bulk in order to carry out plethora of purification procedures. Initially, natural media is prepared by adding 80 ml of potato extract, 2g of sucrose and making up volume to 90 ml by single distilled water. The potato extract was prepared by adding 250 g of peeled and finely chopped potatoes to 650 ml of water. The mixture was heated for 45 minutes until the volume of mixture was reduced to half. The potato extract was then dispensed in the flasks and 2 g of sucrose was added to each flask. The pH was adjusted to 7. The media was then autoclaved at 121^{0}C ,15 psi for 15 minutes .The different concentration of Xanthine ranging from 0.2mM-0.8mM was prepared from a stock of 10 mM Xanthine. The Xanthine was filter sterilized by using nitrocellulose membrane aseptically in a Laminar air flow hood. 10 ml of filter sterilized Xanthine solution of varying concentration was then dispensed in flask aseptically.

The spore count was determined by preparing spore suspension of Endophytic fungal strain and then preparing various dilutions and counting the cells in a haemocytometer using a compound microscope. The spore count of 10^{6} was then used to inoculate 100 ml of media in each flask with varying Xanthine concentration. Uninoculated flask containing media was used as a control. The flasks were incubated at 26^{0} C at 120 rpm for 10 days (Rodriguez, 2000; Santamari

et al., 2002; Rosa *et al*.,2003). The mycelia growth was monitored for a period of 10 days to look for the difference in growth in various media and hence the selection of optimal media. After 10 days, broth was separated from mycelia by filtration. Filtration was carried out aseptically through Whatmann filter paper no.4 (Rodriguez *et al*., 2000). The culture filtrate was then centrifuged at 12000 rpm for 15 minutes to get a filtrate devoid of any fungal biomass. The optimal concentration of Xanthine was then deduced by preliminary test like Nitroblue tetrazolium assay (Aggarwal and Banerjee, 2009).

4.3.2 Optimization of Sucrose

Once the Xanthine concentration has been optimized, the sucrose was optimized by carrying out fermentation in media containing varying percentage of sucrose ranging from 0.5%, 1%, 2%. 3%, 4% and 5%. The pH was adjusted to 7. The media was autoclaved at 121 ^{0}C, 15 psi for 15 minutes. The optimized concentration of Xanthine was then filter sterilized .The spore count of 10^6 spores /ml of endophytic fungal strain #19 NOBASVNP was then used to inoculate 100ml of media in each flask. The flasks were kept at 26^{0}C , 120 rpm for 10 days. After 10 days ,broth was separated from mycelia by filtration. Filtration was carried out aseptically through Whatmann filter paper 4 (Rodriguez et.al; 2000). The culture filtrate was then centrifuged at 12000 rpm for 15 minutes to get cell-free filtrate. The culture filtrates was then screened for maximum xanthine Oxidase activity by Nitro blue tetrazolium assay so as to predict the optimal concentration of sucrose to be used for the production the maximum amount of xanthine oxidase.

4.3.3 Optimization of Potato extract

Having optimized the sucrose and xanthine concentration, the next step was to optimize the volume of potato extract. This is done by carrying out fermentation in varying volume of potato extract. The media containing optimized concentration of sucrose and varying volume of potato extract ranging from 60 ml, 70 ml , 80 ml and 100 ml was prepared (pH-7.0) by autoclaving at 121^0 C, 15 psi for 15 minutes. Then the optimized concentration of filter sterilized Xanthine was

was dispensed into the media. Each 100ml flask was inoculated with spore suspension having 10^6 cells/ml. The flasks were kept at 26^{o}C, 120 rpm for 10 days.After the incubation was over, ,broth was separated from mycelia by filtration whatmann filter paper 4. (Rodriguez et.al; 2000).The filtered broth was then centrifuged to make it cell free as previously and tested for Xanthine Oxidase activity by taking absorbance of reaction mixture at 575 nm . The reaction mixture consisted of 1.5 ml of culture filtrate, 1 ml of 2mM Xanthine, 0.9 ml of 0.5 mm NBT and 0.1 ml of 50 mM Tris-HCL buffer (pH 7.8).

4.3.2 Optimization of Physical parameters:

Optimization of temperature, pH and rpm

For optimization of physical parameters, The media is prepared by adding optimized volume of potato extract, optimized concentration of sucrose, optimized concentration of filter sterilized Xanthine. To optimize the temperature, pH and rpm, the flasks containing rest of the optimized components of varying pH ranging from 5 to 9, inoculated with 10^6 cells/ml were incubated at 26°C, 29°C, 31°C and 37^0 C, at 120 rpm and 150 rpm for 10 days. After 10 days, broth was separated from mycelia by filtration through Whatmann filter paper 4. (Rodriguez et.al; 2000). It was followed by centrifugation at 12000 rpm for 15 minutes. The culture fitrate was then screened for maximum Xanthine Oxidase activity in various flasks by Nitroblue tetrazolim assay.

Optimization of inoculum size

For the optimization of inoculum size, the optimized media was prepared and inoculated with 10^2, 10^4, 10^6 cells/ml in different flasks. The varying concentration of spores is made by scraping spores from the master plate of endophytic fungus #19 NOBASVNP , diluting the spore suspension and counting the number of spores in 4 big squares of haemocytometer to determine the optimum count. The flasks were incubated at optimized temperature and rpm for 10 days. After 10 days, broth was separated from mycelia by filtration through Whatmann filter paper 4. (Rodriguez *et al.*, 2000). The filtered broth was then centrifuged at 12000 rpm for

15 minutes. The cell free broth hence obtained was then tested for Xanthine Oxidase activity by nitoblue tetrazolium salt assay so as to deduce the optimum inoculum size.

4.4 Optimization via Design expert version 8

4.4.1 Placket Burman design

In order to optimize culture conditions for Xanthine Oxidase production by # 19 NOBASVNP, a two step approach is employed. At first, the positive and negative effects of various parameters on the Xanthine Oxidase activity are scrutinized. In second step, Composite design response surface methodology was used to determine the optimum values. The conventional method for medium optimization involves changing one parameter at a time while keeping all others constant. This method may be very expensive and time consuming. It fails to determine the combined effect of different factors. This conventional method was performed and optimum values of various parameters were revealed. In order to check the authenticity of conventional method, the media optimization via Design Expert version 8 was performed. A number of statistical experimental designs have been used to address these problems. Among theses, full factorial designs provide more complete information, but they require lots of experiments (Lk, where k is the number of variables and L is the number of levels for each variable) which makes them impractical when a large number of variables are to be studied. The Plackett-Burman design (Noshin Tasharoffi *et.al*.,2011), as a two level fractional factorial design, is especially useful in screening studies by estimating the main effects of k variables in just k+1 experiments according to a linear model. However, this design does not consider the interaction between variables. The variables screened by Plackett-Burman design may be optimized by using statistical and mathematical optimization tools such as Response Surface Methodology (RSM) (Adinarayana Kunamneni, Suren Singh ;2005). This empirical technique enables to evaluate the relationship between independent variables and to predict the response in an effective experimental design. The aim of this study was to screen and optimize the most important factors affecting the production of Xanthine Oxidase by *Endophytic fungus #19NOBASVNP* using Plackett-Burman design and RSM (Adinaryana Kunameni *et al.*, 2005)

Experimental design and statistical analysis for optimization.

Optimization of medium components for Xanthine Oxidase production by Endophytic fungus #19NOBASVNP was performed in two stages. At the first stage, the components that have significant effect on enzyme production were identified. At the second stage, the optimum values of these components for Xanthine Oxidase production were determined.

Screening design

Initial screening of the most important components affecting Xanthine Oxidase production by Endophytic fungus #19NOBASVNP was performed by Plackett-Burman design. A total number of seven components were selected for this study, with each being represented at two levels, high (+1) and low (-1) . In this design, it was assumed that the main factors have no interactions and a first-order multiple regression models is appropriate.

$$Y = \beta\circ + iix\Sigma\beta(i = 1, \ldots, k)$$

Where Y is the response function (Xanthine Oxidase production) and βi is the regression coefficient. In Table 2, the design matrix built by the statistical software package Design-Expert 7.0.0 (Stat-Ease,Inc., Minneapolis, MN, USA) for the evaluation of eight variables in nine experiments is presented. Variables *X1* through *X7* represent the medium components and *D* represents a dummy variable. In The main effect of each variable was determined according to Equation :

$$E(xi) = 2(\sum Pi + \sum Pi -)/N$$

Where E(xi) is the effect of tested variable, *Pi +* and *Pi –* represent Xanthine Oxidase production from the trials where the tested variable was present at high and low levels, respectively, and *N* is the number of trials (Noshin Tasharoffi *et.al*.,2011). Data were analyzed through analysis of variance (ANOVA) Statistical significance was at p-value < 0.05.

Optimization design

After selecting the most important components influencing production Xanthine Oxidase by Endophytic fungus #19 NOBASVNP by Box-Behnken response surface methodology (Noshin tashroffi *et al* ., 2011) was used to determine the optimum levels of these variables. Selected variables (Sucrose concentration , pH) were studied at three different concentrations coded as -1, 0, and 1. According to the Box-Behnken design matrix generated by Design-Expert software, a total number of 13 experiments including factorial points and replicates at the center point were performed .

Predicted Xanthine Oxidase activity was calculated using the following second order polynomial equation:

$$XO\ activity(Y) = +1.02 + 8.321\ E\text{-}003 * A + 0.032 * B - 0.058 * A * B - 0.13 * A^2 - 0.18 * B^2$$

Where Y is predicted response, where A and B are independent variables. The independent variables used here are pH and sucrose. By using this equation, it is possible to evaluate the linear, quadratic and interactive effects of the independent variables on the response appropriately. Statistical analysis and graph plotting was performed using Design-Expert software. ANOVA through Fisher's test was used to evaluate the effect of independent variables on the response and significant results were identified by a p-value of < 0.05. Multiple correlation coefficient (R^2) and adjusted R^2 were used as quality indicators to evaluate the fitness of the second order polynomial equation. Contour and three-dimensional surface plots were employed to demonstrate the relationship and interaction between the coded variables and the response. The optimal points were determined by solving the equation derived from the final quadratic model and grid search in RSM plots.

4.5 Standard curve of Xanthine Oxidase activity

Xanthine stocks of varying concentrations ranging from 0.25 mM to 3mM were prepared in 1N NaOH and dispensed in 7 test tubes. These were vortexed thoroughly. Further 50mMTris-HCl buffer was prepared and its pH was adjusted

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
B	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
C	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
D	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
E	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
F	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
G	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2
H	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	B1	B2

Fig.3: showing template of microtitre plate used for standard curve of Xanthine Oxidase.
S1, S2, S3,S4 and S5 corresponding to 1mM, 1.5 mM, 2 mM , 2.5 mM and 3 mM.
C1, C2, C3 , C4 and C5 correspond to controls of test stocks. B1 and B2 are Blank

to 7.8. As per assay template, requisite amount of Xanthine solution was then dispensed into a 96-well microtitre plate. The Standard Xanthine oxidase enzyme (Source- Ex butter milk), buffer and NBT was added to allow formation of a blue color thereby showing the presence of formazan. The control consisted up of same reaction mixture except for the enzyme, sterile distilled water was added. The absorbance was taken at 575 nm using Biotek throughput reader, Power wave 340.

Stock solution	**Test sample**	**Control sample**
2m M Xanthine	134 µl	134 µl
Xanthine Oxidase (pure enzyme)	25 µl	-
50mM Tris – HCl buffer (pH -7.8)	5µl	5µl
Nitroblue tetrazolium salt	36 µl	36µl
Sterile distilled water	-	25µl
Total reaction mixture	200µl	200µl

Table No. 1: Reaction mixture for Standard curve of Xanthine Oxidase

4.6 Assay of Xanthine Oxidase activity using NBT

Stocks of Xanthine and NBT were freshly prepared. Test sample was prepared by dispensing 1 ml of Xanthine (2mM), 0.9 ml of NBT (0.5mM) and 0.1 ml of 50 mM Tris HCL buffer of pH 7.8 and 1.5 ml of culture filtrate. The blank was prepared by adding 1 ml of Xanthine (2mM), 0.9 ml of NBT (0.5mM), 0.1 ml of Tris –HCL buffer of pH 7.8 and 1.5 ml of sterile distilled water. After dispensing, the test tubes were incubated at 37°C for 15 minutes until there is development of blue color.

4.7 XO inhibition assay by Allopurinol.

Allopurinol, a structural isomer of hypoxanthine, is a competitive inhibitor of Xanthine Oxidase. Microtitre plate assay was carried out with different concentrations of Allopurinol with a range of 2mM to 16mM. The test sample consists up of 100 µl of fermentation broth, 50 µl of 2mM Xanthine, 50 µl of stock concentration of Allopurinol. The samples were incubated at 37^{o}C for 15 minutes and then 50 µl of NBT was added. The control set comprised of 100 µl of fermentation broth, 50 µl of 2mM Xanthine, 50 µl of sterile distilled water. The sample was incubated for 15 minutes and 50 µl of NBT was added. To see the interactions between NBT and Xanthine under alkaline conditions, a negative control (Nc) was set. The negative control was prepared by adding 100 µl of 2mM Xanthine, 50µl of NaOH and then incubated for 15 minutes. Then 50 µl of NBT solution is added. B refers to the wells that are kept empty. The absorbance was then recorded at 575 nm (Aggarwal and Banerjee,2009).

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

Fig no. 4: Showing template for Xanthine oxidase inhibition S1 , S2 , S3 . S4 and S5 refers to 2mM, 4 mM , 1mm , 12 mM and 16 mM allopurinol concentrations. C1, C2 , C3 , C4 and C5 refers to the controls , without allopurinol.NC refers to interaction between NBT and Xanthine under alkaline conditions. B refers to empty wells .

4.8 Protein purification

4.8.1 Protein precipitation

Acetone precipitation was performed to precipitate Xanthine Oxidase isolated from Endophytic fungus fermented on optimized media. This strategy was used to remove undesirable substances from protein solution. Acetone causes protein to precipitate .After centrifugation to pellet the precipitated protein, the supernatant containing the interfering substance is removed and the protein pellet containing desired enzyme along with various other protein fractions is redissolved in buffer that is compatible with downstream application.

Large scale liquid fermentation was carried out with optimized nutitional and physical parameters .The spent broth was filtered through Whatman filter paper 4 and subsequently centrifuged at 12000 rpm for 15 minutes to obtain cell free filtrate for recovery of protein .The cell free filtrate was then placed in a falcon and cold acetone (-20 °C) with four times to that of sample volume was added. Then the falcon was vortexed and incubated for 60 minutes at -20°C. After incubation, the sample was centrifuged for 10 minutes at 13,000g. The supernatant was decanted carefully so as not to dislodge the protein pellet. Acetone was allowed to evaporate from the uncapped tube at room temperature for 30 minutes. The pellet was dissolved in 50mM Tris-HCL buffer of pH7.8 and it was stored at at -20°C (Dirce Mithico yamaoka- yano, 1998).

Xanthine Oxidase activity assay of crude protein

The NBT based assay was used to assess the Xanthine Oxidase activity of crude protein precipitated with acetone . The reaction mixture consisted of 500µl of crude protein, 0.5ml of Xanthine, 0.5ml of NBT and 0.1 ml of 50mM Tris-HCL buffer. The NBT will be reduced to formazan as a result of XO activity.

4.9 Protein estimation by Lowry method

This method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay .The Phenol group of tyrosine and tryptophan residues in a protein will produce a blue purple colour complex with Folin-ciocalteau reagent. The amount of aromatic acids will decide the intensity of colour. The different concentrations of BSA ranging from 0.05mg/ml to 1mg/ml were prepared from the stock concentration of BSA (1mg/ml).

Solution	Volume added
Solution A	50 ml of 2% sodium carbonate+50 ml of 0.1N NaOH
Solution B	10 ml of 1.56% copper sulphate + 2.3 % sodium potassium tartarate.
Alkaline Copper sulphate solution	100 ml of solution A+ 2 ml of solution B
Folin - Ciocalteau reagent solution (1N)	Dilute commercial reagent (2N) with an equal volume of water

Table no 2: Composition of Lowry's reagent

0.2ml of the protein solution was pipette out from different test tubes and 2 ml of alkaline copper sulphate reagent was added. The solution was incubated at room temperature for 10 minutes. Then 0.2 ml of Folin-ciocalteau reagent was added and incubated for 30 minutes. The absorbance was measured at 660 nm to get a standard curve. Then absorbance was measured at 660 nm and the concentration of protein sample was deduced from the standard curve.

4.8 Activity Staining of Xanthine Oxidase (crude protein)

The Native PAGE electrophoresis was done with 10% resolving poly-acrylamide gel. The stock solution of 2mM Xanthine was prepared and 166 µl of it was incorporated inside the gel. On the solidification of gel, the comb was removed carefully and load the 20 µl of sample into the wells. The sample to be loaded was prepared by mixing 10 µl of protein sample and 10 µl of the 1X loading dye. The SDS and ß-mercaptoethanol was not used. In order to allow the protein to remain in the active state, no boiling of the samples was done. A layer of agarose of 0.07 % was allowed to form at the bottom of gel in order to prevent leakage. No stacking gel was used. The gel was allowed to run for 2 hours until the dye entered into the agarose layer. After electrophoresis, the gel was dipped inside 2mM NBT solution and observed after 24 hours for the appearance of dark blue bands due to formazan formation by Xanthine Oxidase.

Protein purification via Gel filtration Chromatography:

The Gel filtration kit KT39 (Bangalore Genei) was used for the purification of Xanthine Oxidase .The kit is packed with Sepharose 6B designed to separate molecules on the basis of their size. Sepharose 6B has cross linked Agarose type of matrix. The particle size of hydrated particle beads is 45-165µm. The fractionation range is from 376Da to 2000 KDa. The enzyme was reconstituted with 50mM Tris –HCL buffer (pH 7.8) to get a homogenous solution. The column was stored at 4°C. The column was fixed to a vertical stand and was equilibrated with 4 ml of gel filtration buffer. Then 0.2 ml of crude enzyme was loaded onto the column and it was allowed to sink completely and then 0.2 ml of buffer was added. Then, the buffer was allowed to flow completely. The eluted fractions were then collected in different microcentrifuge test tubes and then each fraction was tested for XO activity and the fraction showing maximum XO activity was then screened for XO inhibition by Allopurinol in the same way as done with crude protein.

Chapter 5

Results and Discussions

5.1 Pure culturing and long term storage:

The given Endophytic fungal strain was maintained on PDA plates for sub culturing and on to PDA slants for long term storage at 28° C.

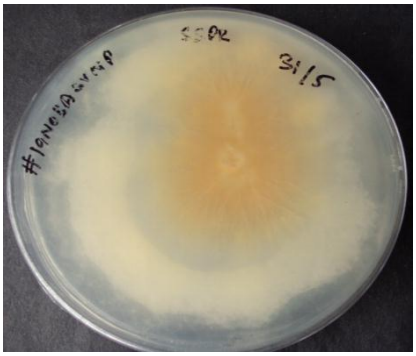


Figure no.5: Pure culture of #19NOBASVNP grown on PDA plate

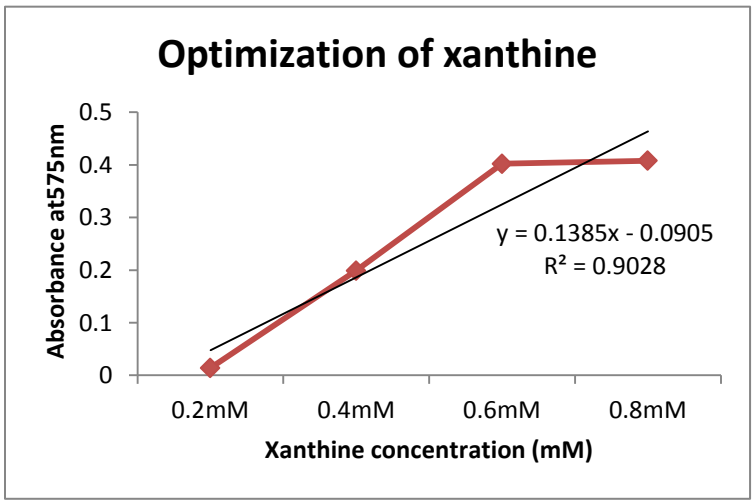
5.2 Fermentation in synthetic media

The culture filtrate obtained from all the three synthetic media i.e. Czepak dox broth, Richard's broth, Xanthine broth and natural media PDB, on screening for Xanthine Oxidase enzyme activity by NBT assay it was observed that culture filterate of Czepak Dox broth, Richards broth and PDB showed XO activity of about 0.256 U/ml, 0.275 U/ml and 0.294 U/ml respectively but on the other hand the culture filtrate of Xanthine broth showed up least activity of about 0.193 U/ml .Hence, there was a need to explore other media to get high enzymatic activity of Xanthine Oxidase .

5.3 Optimization of nutritional parameters

5.3.1. Optimization of Xanthine

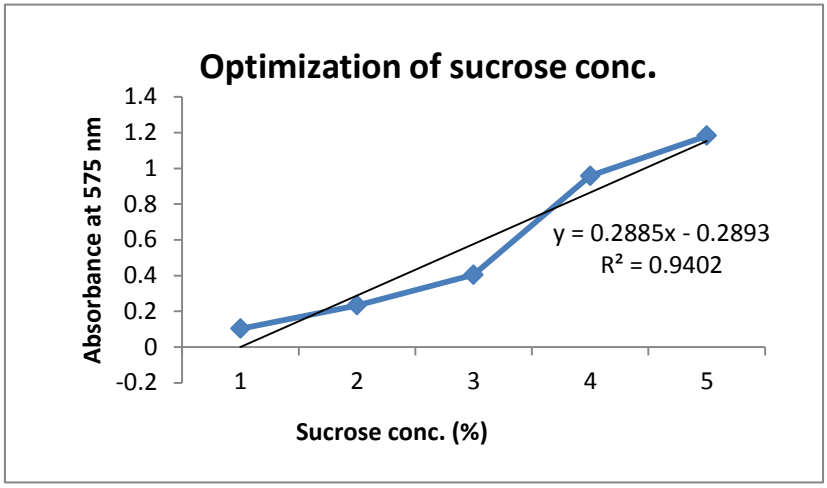
The concentration of Xanthine was optimized by varying the concentration of Xanthine from 0.2mM-0.8mM, keeping all the other nutritional components and physical parameters constant. The culture filtrate of media containing respective Xanthine concentrations showed increase in XO activity with increasing xanthine concentration, maximum XO activity was obtained in the culture filtrate of 0.6mM xanthine, decrease in XO activity was obtained on increasing the Xanthine concentration beyond 0.6mM. (Graph no.1). The optimum concentration of Xanthine which showed maximum XO activity was found to be 0.6mM.



Graph 1: Optimization of Xanthine by Nitro blue tetrazolium assay.

5.3.2 Optimization of sucrose percentage

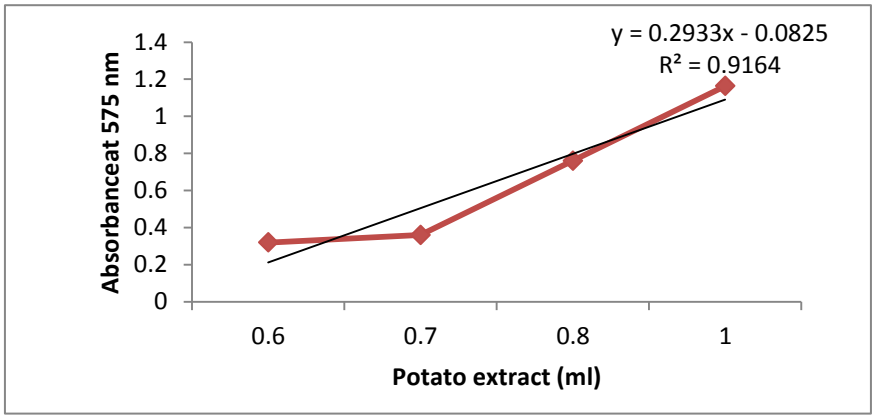
Once the Xanthine concentration was optimized, the sucrose concentration was optimized by varying sucrose concentration from 0.5% to 5%. The culture filtrate of flask containing 5% sucrose showed maximum XO activity by NBT assay. (Graph 2).



Graph 2: Optimization of sucrose concentration

5.3.3 Optimization of Potato extract

The volume of potato extract in the media containing optimized concentration of xanthine (0.6mM) and sucrose (5%) was varied from 60:40, 70:30, 80:20 and 100 ml of potato extract. The XO activity was increasing with the increasing volume of potato extract used. So The culture filtrate of 100 ml potato extract was found to show maximum XO activity. (Graph 3)



Graph 3: The optimization of Potato extract .

5.3.4 Optimization of temperature

On screening the culture filtrate from the different incubation temperatures for XO activity, the culture filtrate from the fermented broth incubated at 29°C was showing higher Xanthine Oxidase activity (Graph no.4)

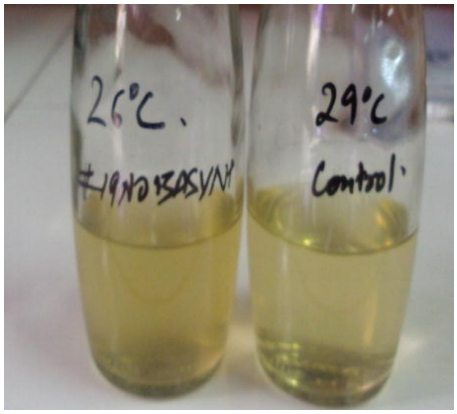
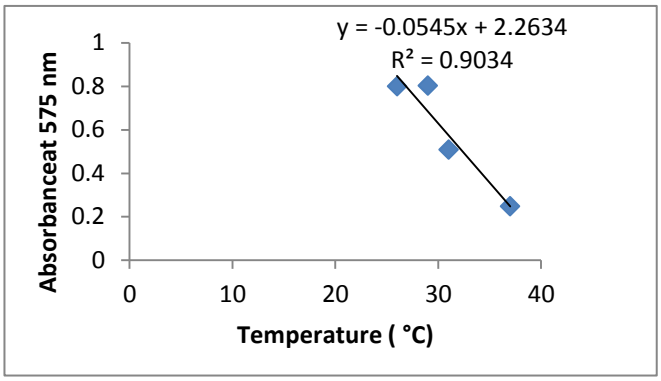


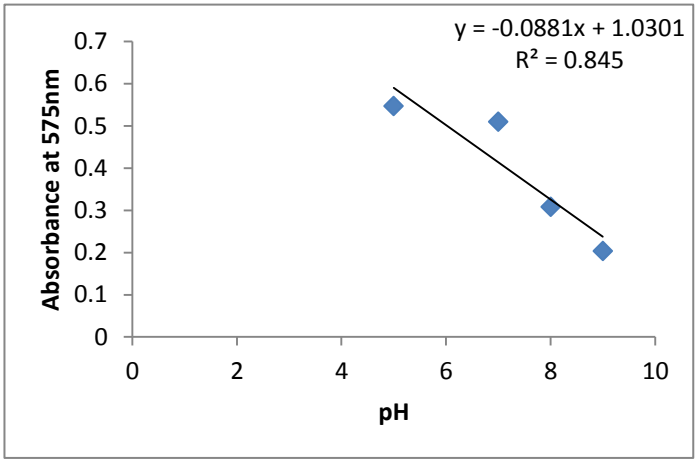
Fig 6: Culture filtrates fermented at 29°C



Graph no. 4 – Optimization of temperature by NBT assay.

5.3.5 Optimization of pH and rpm

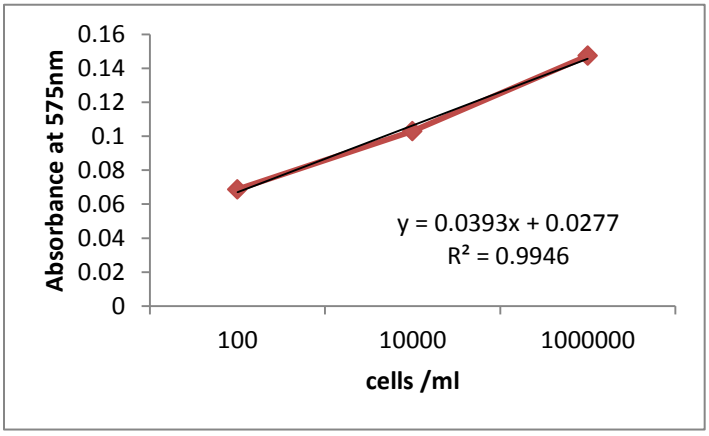
The culture filtrate of the optimized media incubated at 29°C and of pH 5 was giving maximum XO activity as illustrated by Graph no.5. The enzyme activity decreases on moving from culture filtrate of higher pH as shown in the graph that the filtrate of pH 6,7,8shows decline in enzyme activity.



Graph 5: Optimization of pH

5.3.6 Optimization of inoculums size

The inoculums size was varied from 10^2 cells/ml of Endophytic fungus to 10^6 cells/ml. The inoculums size of 10^6 cells/ml spore suspension showed maximum XO activity. The enzyme activity was found to be increasing with increasing inoculum size. The optimum inoculum size was 10^6 cells/ml



Graph 6 : optimization of inoculum size

5.4 Standard curve for Xanthine Oxidase activity

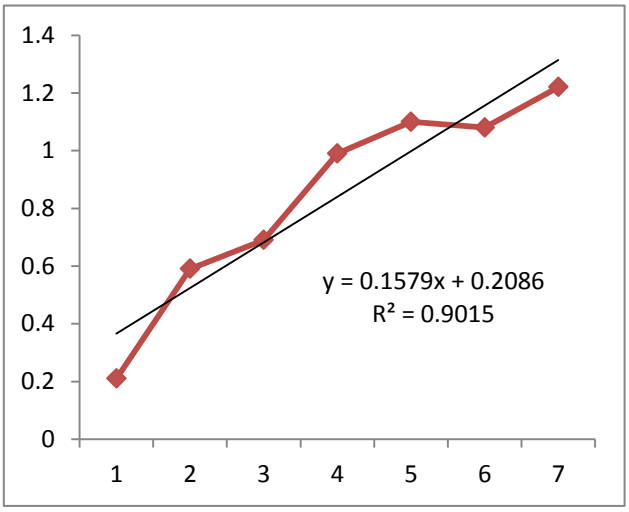
A standard curve was prepared for activity of pure Xanthine Oxidase enzyme. (Graph no.7)

The standard curve equation was

$$y = 0.157x + 0.208$$

$$R^2 = 0.901$$

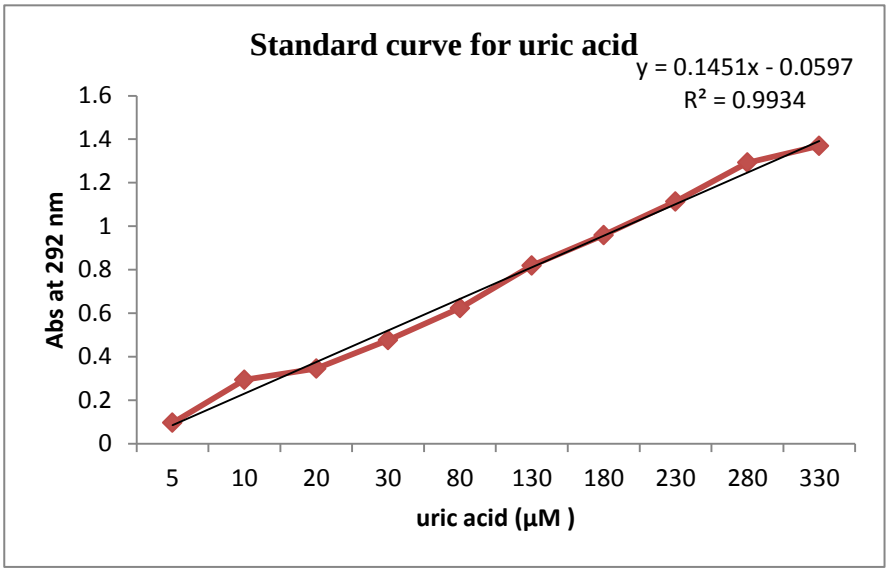
The high value of R^2 exhibit high precision of the method used for estimation of XO activity



Graph -7: Standard curve of Pure Xanthine Oxidase

5.5 Standard curve of uric acid.

The rate of uric acid formation is related to Xanthine Oxidase activity when Xanthine is incubated with fungal culture filtrate. The standard curve for uric acid estimation was plotted.



Graph 8: Standard Curve of uric Acid

The standard curve equation was

$$Y=0.145X - 0.059$$

The concentration of uric acid produced in a test sample was calculated from the standard curve. The concentration of uric acid (product formed) was then divided by reaction time to obtain the Xanthine Oxidase activity. The uric acid was estimated in 12 flasks given different concentrations of nutritional components and kept at different physical parameters. The absorbanse readings of the flasks were put in standard curve equation thereby giving the concentration" x " i.e. the substrate converted into product - Uric acid.

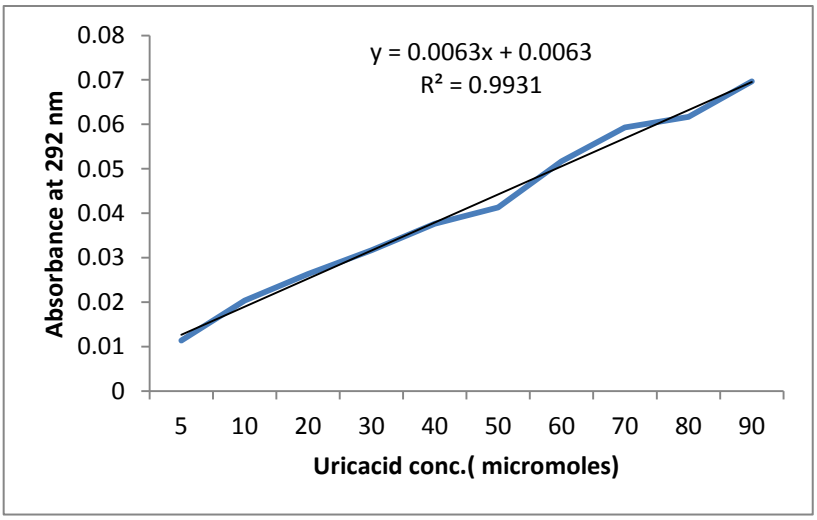
S.No	Average of triplicate readings (absorbance at 292 nm)	Concentration of uric acid formed(µM)
1	.204	1.81
2	.2190	1.91
3	0.1896	1.714
4	0.20.	1.78
5	0.184	1.675
6	.204667	1.81
7	0.26066	2.20
8	0.2476670	2.11
9	.231	2.00
10	.227	1.97
11	0.198	1.774
12	.210330	1.85

Table no. 3: uric acid estimation at 292 nm

These readings are in concordance with the readings obtained for absorbance at 575 after the NBT assay of culture filtrate of 12 flasks thereby suggesting that more the activity of Xanthine Oxidase, more is the yield of product i.e. uric acid.

5.6 Phosphotungstate assay for uric acid estimation.

Uric acid is commonly determined in biological fluids by measuring the blue color produced by its reaction with reagents containing phosphotungstic acids under conditions which minimize interference by other reducing substances (Folin & Dennis,1912; Benedict,1912;Bidmead, 1951). The standard curve of uric acid was obtained on reaction of uric acid with sodium bicarbonate and phospotungstic acid (Kalpana luthra, 2001).



Graph 9: Standard curve of uric acid .

The uric acid was estimated by phosphtungstate method in 12 flasks having different concentration of various nutritional components and different physical parameters.

Flask no	Average of triplicate readings	Uric acid concentration
1	0.019	2.1667
2	0.022	2.667
3	0.011	0.833
4	.0.014	1.333
5	0.01	0.66
6	0.019	2.1667
7	0.027	3.5
8	0.029	3.83
9	0.026	3.33
10	0.026	3.33
11	0.0130	1.16
12	0.021	2.5

Table 4: Uric acid estimation by phosphotungstate assay .

These readings obtained after the reaction of culture filtrate with phophotungstate and sodium bicarbonate are in concordance with the readings obtained for absorbance at 575 nm after the NBT assay of culture filterate of 12 flasks thereby suggesting that more the activity of Xanthine Oxidase, more is the yield of product i.e. uric acid.

Parameters	Optimized value
Xanthine	0.6mM
Sucrose	5%
Temperature	29
pH	5
Rpm	150
Inoculums size	10^6 cells /ml
Volume of Potato extract	100 ml (undiluted)

Table No. 5: optimized nutritional and physical parameters.

5.7. Optimization of media via design expert version 8

5.7.1 Placket Burman design

Among the various components of media under investigation, sucrose and pH showed positive effect on enzyme production while potato extract, temperature had negative effect. However, the linear model proved to be insufficient for determining the optimum values for these components due to highly significant curvature effect. It was noted that quadratic polynomial equation fitted the experimental data appropriately. The optimum pH was 5.7 and optimum concentration of sucrose was 5 % with a predicted Xanthine Oxidase production of 1 U/ml. These values were in concordance with the parameters optimized conventionally taking one parameter at a time.

When more than five independent variables are to be investigated, the Placket-Burman design may be used to find the most important variables in a system, which are then optimized in further studies (Placket and Burman, 1946). These authors give a series of designs for up to one hundred experiments using an experimental rationale known as balanced incomplete blocks. This technique allows for the evaluation of *X-I* variables by *X* experiments. *X* must be a multiple of 4, e.g. 8, 12, 16, 20, 24, etc. Normally one determines how many experimental variables need to be included in an investigation and then selects the Placket-Burman design which

meets that requirement most closely in multiples of 4. Any factors not assigned to a variable can be designated as a dummy variable. The incorporation of dummy variables into an experiment makes it possible to estimate the variance of an effect(experimental error).

Table no. 6 shows a Placket-Burman design for seven variables *(A to G)* at high and low levels in which two factors, *E* and G, are designated as 'dummy' variables. These can then be used in the design to obtain an estimate of error.

Select	Run	Factor 1 A:xanthine mM	Factor 2 B:sucrose %	Factor 3 C:rpm rpm	Factor 4 D:temperature deg C	Factor 5 E:pH numeric	Factor 6 F:inoculum sie cells/ml	Factor 7 G:Potato extr.. ml	Factor 8 H:dummy 1	Factor 9 J:dummy2	Factor 10 K:dummy3	Factor 11 L:dummy4	Response 1 XO activity U/ml
	1	0.80	6.00	120.00	37.00	9.00	1000000.00	0.60	-1.00	-1.00	1.00	-1.00	1.4872
	2	0.80	6.00	150.00	26.00	4.00	100.00	1.00	-1.00	1.00	1.00	-1.00	1.5477
	3	0.20	0.50	120.00	26.00	4.00	100.00	0.60	-1.00	-1.00	-1.00	-1.00	0.7098
	4	0.20	0.50	120.00	37.00	4.00	1000000.00	1.00	-1.00	1.00	1.00	1.00	1.2728
	5	0.80	0.50	150.00	37.00	4.00	1000000.00	1.00	1.00	-1.00	-1.00	-1.00	0.5958
	6	0.80	6.00	120.00	26.00	4.00	1000000.00	0.60	1.00	1.00	-1.00	1.00	1.396
	7	0.20	6.00	150.00	37.00	4.00	100.00	0.60	1.00	-1.00	1.00	1.00	1.5813
	8	0.20	6.00	120.00	37.00	9.00	100.00	1.00	1.00	1.00	-1.00	-1.00	1.5858
	9	0.80	0.50	120.00	26.00	9.00	100.00	1.00	1.00	-1.00	1.00	1.00	1.5805
	10	0.20	6	150.00	26.00	9.00	1000000.00	1.00	-1.00	-1.00	-1.00	1.00	1.5768
	11	0.20	0.50	150.00	26.00	9.00	1000000.00	0.60	1.00	1.00	1.00	-1.00	1.2683
	12	0.80	0.50	150.00	37.00	9.00	100.00	0.60	-1.00	1.00	-1.00	1.00	1.4887

Table no 6: showing Placket burman design

Normally three dummy variables will provide an adequate estimate of the error. However, more can be used if fewer real variables need to be studied in an investigation (Stowe and Mayer, 1966). Each horizontal row represents a trial and each vertical column represents the H (high) and L (low) values of one variable in all the trials. For those trials in which *A* is low, *B* will be high two times and low two times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of *A.* The same logic then applies to each variable. However, no changes are made to the high and low values for the *E* and G columns.

Greasham and Inamine (1986) stated that although the difference between the levels of each variable must be large enough to ensure that the optimum response will be included, caution must be taken when setting the level differential for sensitive variables, since a differential that is too large could mask the other variables. The trials are carried out in a randomized sequence. The effects of the dummy variables are calculated in the same way as the effects of the experimental variables. If there are no interactions and no errors in measuring the response, the effect shown by a dummy variable should be 0. If the effect is not equal to 0, it is assumed to be a measure of the lack of experimental precision plus any analytical error in measuring the response (Stowe and Mayer, 1966).This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum values to use.

The reaction mixture was prepared by adding culture filtrate of the 12 flasks fermented under different conditions to Xanthine (2mM), NBT (0.5mM) and Tris HCL buffer (50 mM). The absorbance was taken at 575 nm. It was seen that the absorbance readings of 12 flasks obtained by NBT assay were concordant with the readings obtained from the uric acid estimation at 292 nm and phosphotungstate assay .The absorbance readings were then put in standard curve of Xanthine Oxidase to yield the substrate concentration. The substrate concentration hence obtained was then divided by the reaction time to yield Xanthine Oxidase activity. These Xanthine Oxidase activity values were lodged in Placket burman to analyze the effect of various nutritional and physical parameters on Xanthine Oxidase activity. Their results obtained from placket burman can be analyzed from Table -3.

5.7 .2 The Pareto chart exhibiting the positive and negative factors.

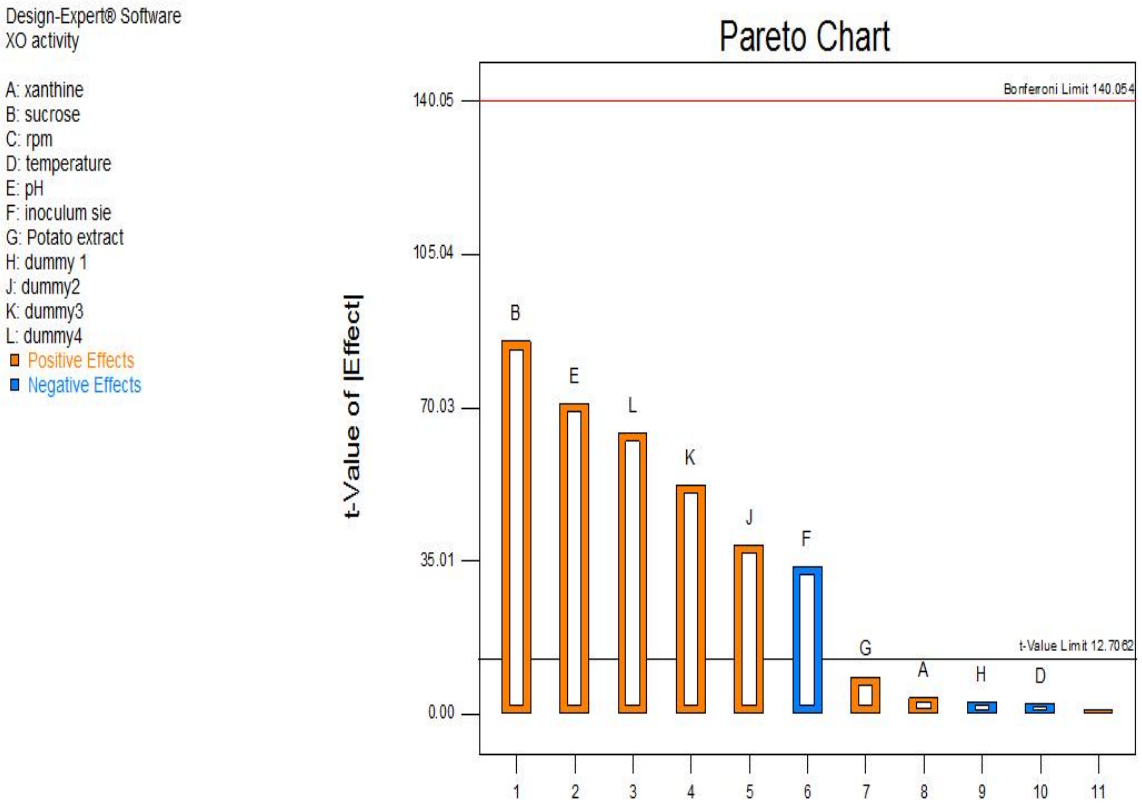


Fig -7 : Pareto chart illustrating the effect of various parameters on XO activity

Various factors were analyzed for their positive and negative effect on Xanthine Oxidase activity. The positive factors include sucrose percentage, pH, volume of potato extract and concentration of Xanthine. The factors exhibiting negative role on the Xanthine Oxidase activity were found to be inoculum size and temperature.

5.7.3 ANOVA for seleted factorial model

The model F- value of 2192.7 implies that model is significant.There is only 1.6 % chance that model can occur due to noise. Moreover values of P> F indicates that model is significant.

The "Pred R-Squared" of 0.9934 is in reasonable agreement with the "Adj R-Squared" of 0.9995.

Source	Sum of squares	df	Mean square	F value	P value P>F value
Model significant	1.28	10	0.13	2192.7	0.0166
Xanthine		1		14.55	
sucrose	0.43	1	0.43	7266.11	
Temperature		1		6.49	
pH	0.30	1	0.30	5053.87	
Inoculum size	0.067	1	0.067	1145.5	
Potato extract		1		74.09	
Dummy 1		1		8.07	
Dummy2	0.088	1	0.088	15.04	
Dummy3	0.16	1	0.16	27.31	
Dummy4	0.24	1	0,24	41.22	

Table -7 : ANOVA for Placket burman design.

5.7.4 The normal plot of residues vs normal % probability.

The range of xanthine oxidase activity as analyzed in 12 flasks is illustrated in the Graph no..The xanthine oxidase activity varies from 0.5958 to 1.5856 depending on various nutritional and physical parameters provided to the media used for fermentation of Endophytic fungal strain # 19 NOBASVNP .

Final equation in terms of Actual factors:

XO activity = +0.72714* xanthine +0.028083 * sucrose +0.068452 -1.02273E-0* temperature

+0.062797 * pH -1.49498E-00 * inoculum size + 0.095042 * Potato extract

-6.27500E-0 * dummy 1 + 0.085658 * dummy2 + 0.11541* dummy 3

+0.14179*dummy

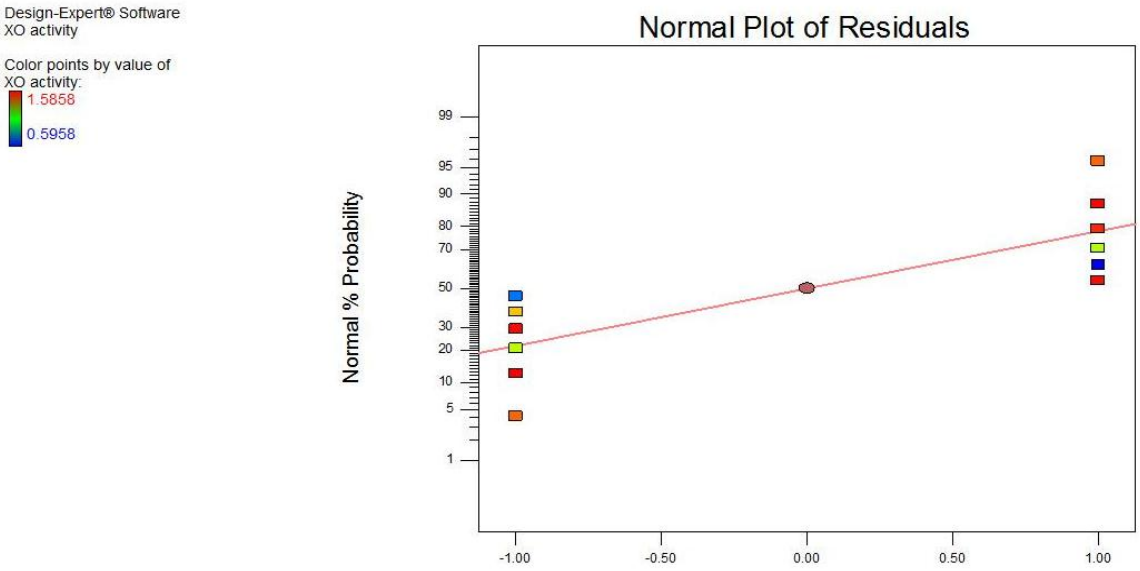


Fig no.8 : Range of xanthine oxidase activity

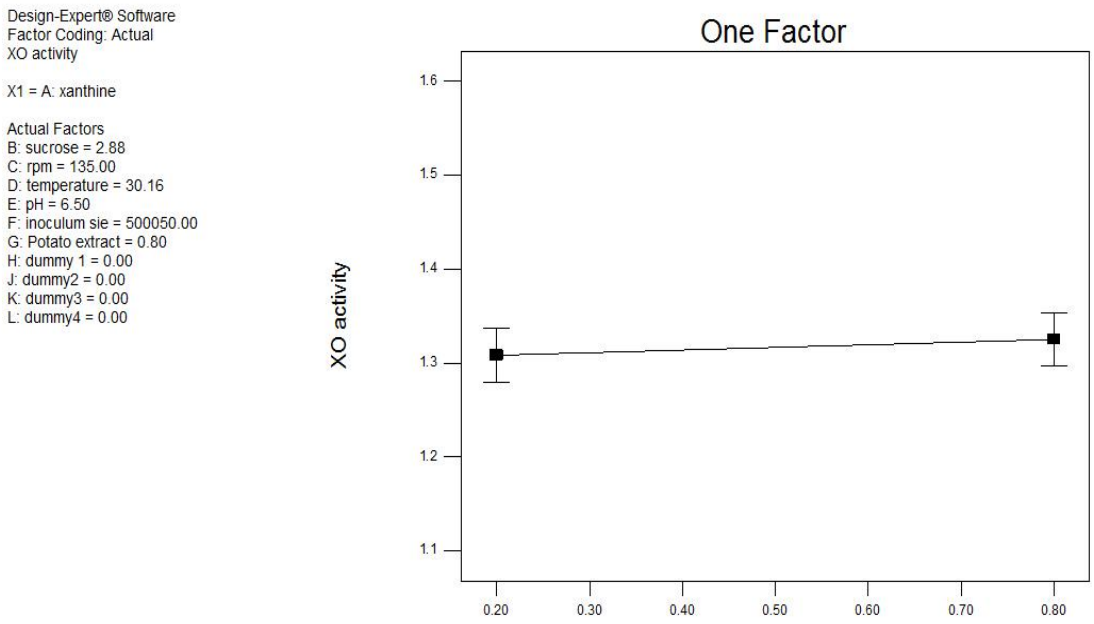


Figure no 9: Effect of Xanthine concentration on XO activity

5.7.5 Response surface methodology

The next stage in medium optimization was to determine the optimum level of each key variable which has been identified by the Placket-Burman design. This may be done using response optimization techniques which were introduced by Wilson (1951). Hendrix (1980) has given a readable account of this technique and the way which it may be applied. Response surfaces are similar to contour plots or topographical maps. Whilst graphical maps show lines of constant elevation, tour plots show lines of constant value. Thus, the contours of a response surface optimization plot show lines of identical response. In this context, response means the result of an experiment carried out at particular values of the variables being investigated. The axes of the contour plot are the experimental variables and the area within the axes is termed the response surface.

Run	Sucrose percentage	pH	XO activity
1	1	9	0.8
2	3	6.5	0.99
3	1	4	0.62
4	5	4	0.74
5	5	9	0.69
6	3	6.5	1.03
7	3	6.5	1.02
8	3	6.5	1.038
9	3	10.04	0.72
10	0.17	6.5	0.76
11	3	6.5	1.04
12	5.83	6.5	0.8
13	3	2.96	0.63

Table 8 : XO activity obtained in 13 flasks fermented in media with varying sucrose concentration and pH.

5.7.6 Anova for Response surface methodology.

The "Pred R-Squared" of 0.9758 is in reasonable agreement with the "Adj R-Squared" of 0.9872. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 32.049 indicates an adequate signal. This model can be used to navigate the design space.

Standard deviation	0.018
Mean	0.84
R- squared	0.9925
Adjusted R suared	0.9872
Pred R squared	0.9758
Adequate precision	32.049

Table 9 : ANOVA of response surface methodology

Factor	Coefficient estimate	df	Standard error	95% CI low	95 % CI high	VIF
Intercept	1.02	1	8.248E-003	1.00	1.04	1.00
A-sucrose	8.321E-003	1	6.521E-003	7.098E-003	0.024	1.00
B-pH	0.032	1	6.521E-003	0.017	0.048	
AB	1	9.222E-003	-0.079	-0.036	1.00	
A-square	1	6.993E-003	-0.14	-0.11	1.02	

Table 10 : coefficient estimate and standard error in rsm.

Final equation in terms of actual factors:

XO activity = -0.78285 + 0.26724 * sucrose + 0.41771 * pH 0.011500 * sucrose * pH -

$0.031388*\text{sucrose}^2-0.028488*\text{pH}^2$

5.7.7 Contour

Whilst graphical maps show lines of constant elevation, contour plots show lines of constant value. Thus, the contours of a response surface optimization plot show lines of identical response. In this context, response means the result of an experiment carried out at particular values of the variables being investigated. The axes of the contour plot are the experimental variables and the area within the axes is termed the response surface. To construct a contour plot, the results (responses) of a series of experiments employing different combinations of the variables are inserted on the surface of the plot at the points delineated by the experimental

conditions. Points giving the same results (equal responses) are then joined together to make a contour line.

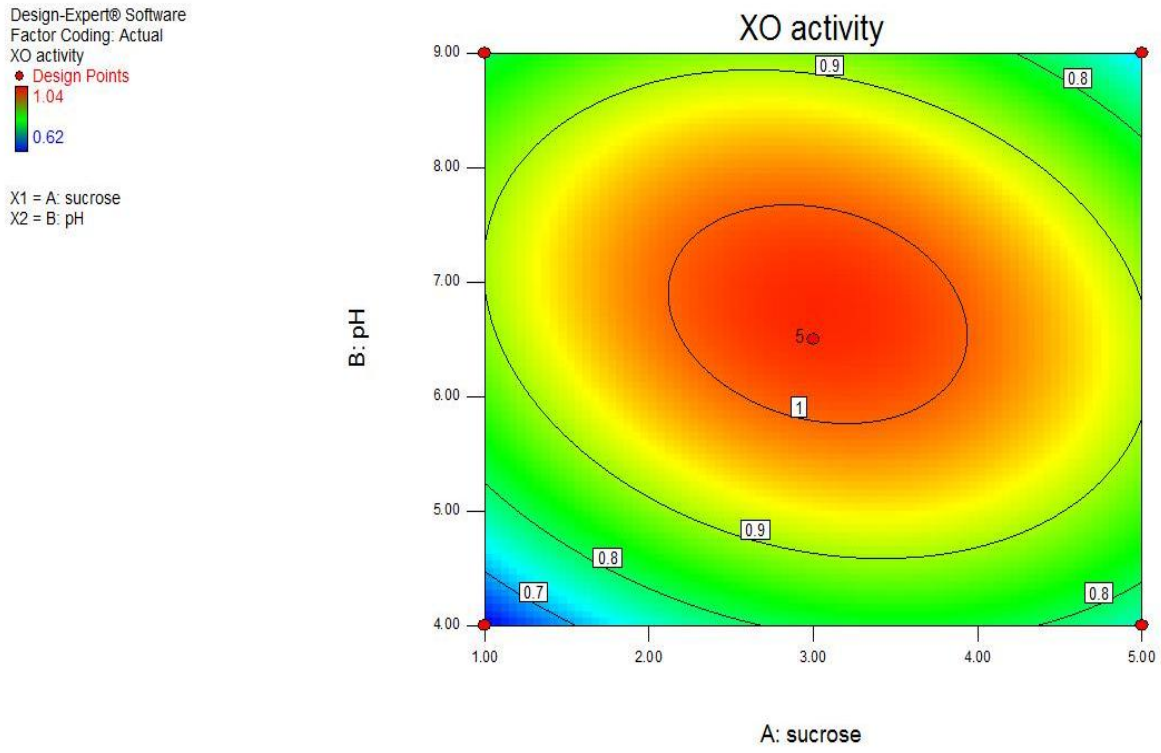


Fig 10 : Contour Plot showing effect of pH and sucrose percentage on XO activity.

5.7.8 3-D plots showing dependence of XO activity on pH and sucrose

In its simplest form two variables are examined and the plot is two dimensional. It is important to appreciate that both variables are changed in the experimental series , rather than one being maintained constant, to ensure that data are disturbed.

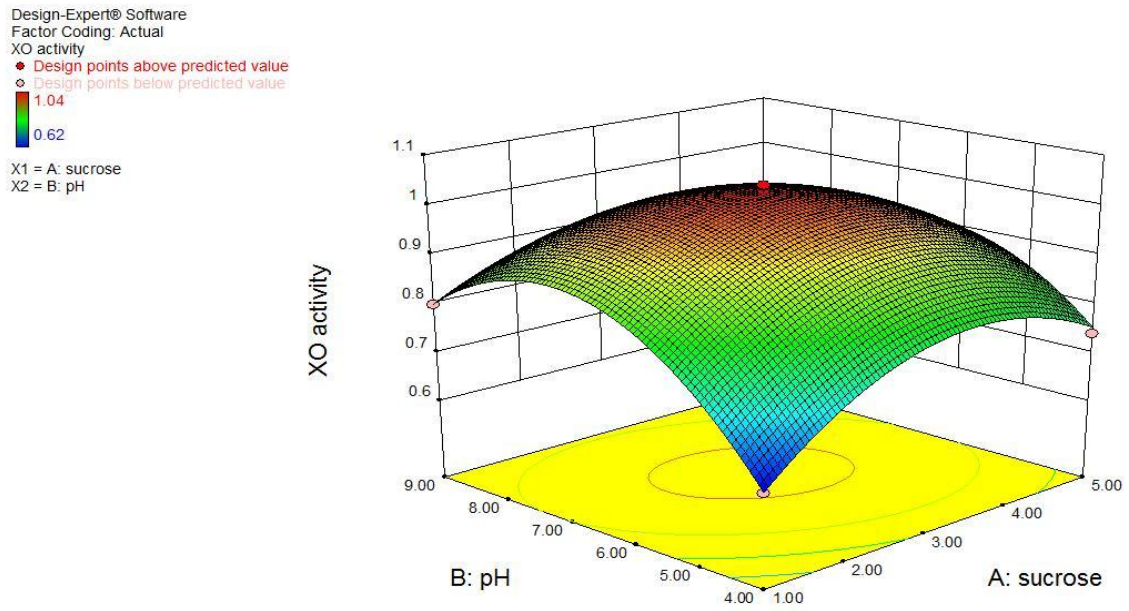


Figure 11 : 3D plot illustating dependance of XO activity on pH and sucrose.

Media	Enzyme activity U/ml
Unoptimized media	0.46 U/ml
Optimised media (predicted)	1.02U/ml
Optimized media (actual)	0.9889 U/ml

Table 11 : The XO activity obtained in unotimized and optimized media

Parameter	Optimized value as per RSM
pH	5.36
Sucrose	5 %

Table 12: The values of optimized parameters by RSM.

5.7.9 The Prediction of enzyme activity by Design expert software

Design expert software predicted the enzymatic activity (U/ml) of the xanthine oxidase produced by the endophytic fungus # 19 NOBASVNP fermented on optimized media.The predicted enzyme activity was 1 U/ml if the optimized media contained 5 % sucrose and if the pH of the media was set at 5.3 .The enzyme activity of the culture filtrate containing xanthine Oxidase was deduced by NBT assay after 10 days of fermentation of pure endophytic atrain on the optimized media. It was found to be 0.9889 U/ml .Hence the validation of the prediction was

done and predicted value lied close to the actual one thereby validating the authenticity of the design expert software.

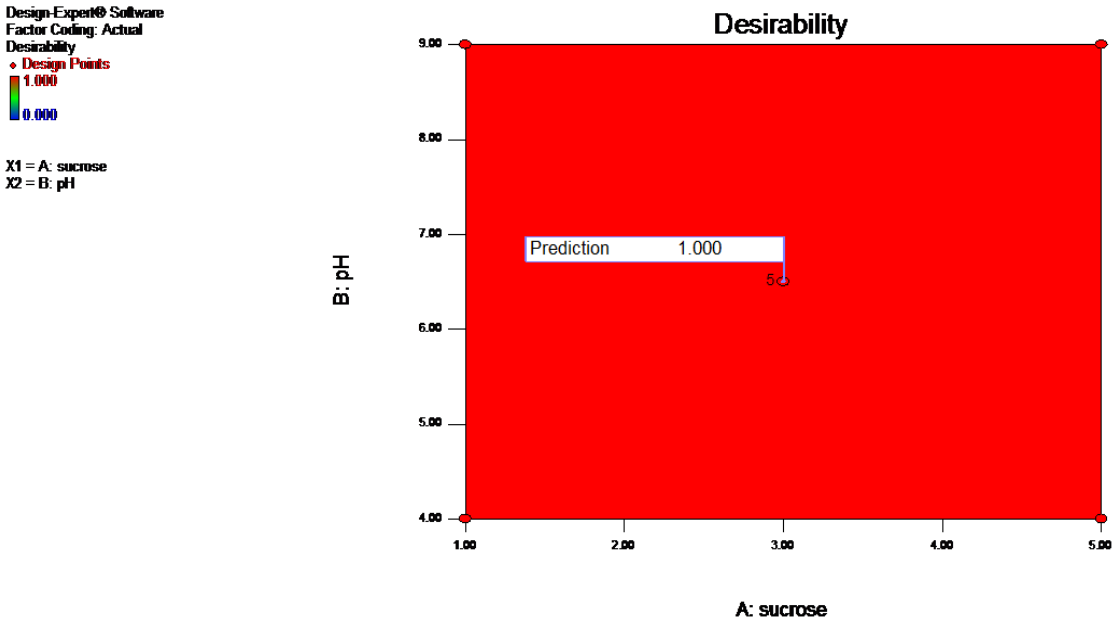


Figure 12: Pediction of XO activity by RSM.

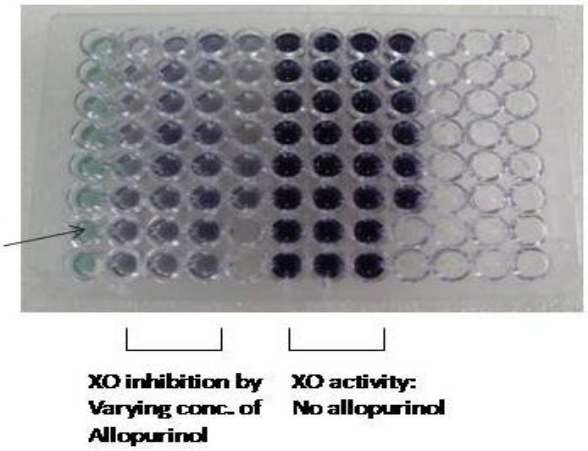
5.9 *In vitro* NBT assay of the crude enzyme:

From the culture filtrate of optimized media, the protein was precipitated by cold acetone. This crude enzyme on investigating for its potential to utilize xanthine by NBT assay shows 1.21 U/ml of XO activity.

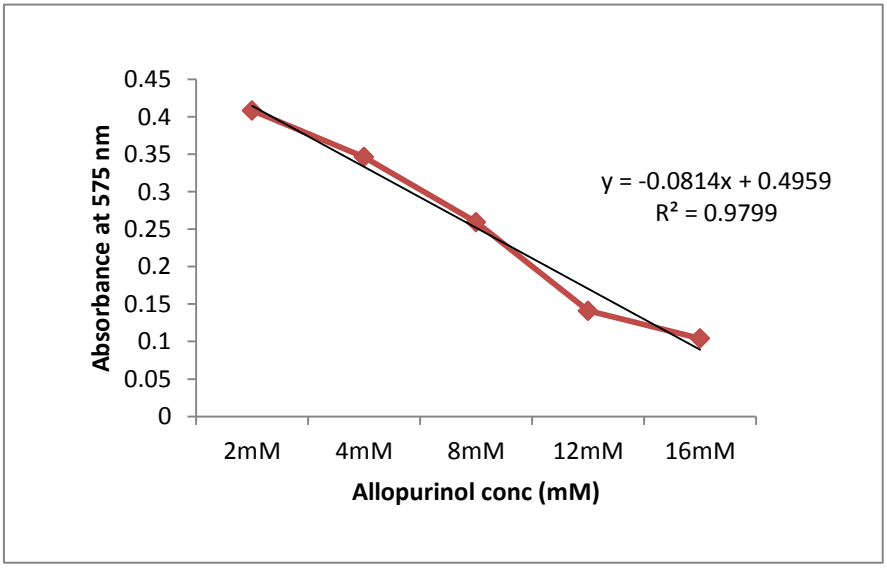
5.10 Xanthine oxidase inhibition assay

The confirmation of xanthine oxidase activity in culture filtrate of #19 NOBASVNP was done by xanthine Oxidase Inhibition assay, with allopurinol which is a competitive inhibitor of xanthine oxidase.

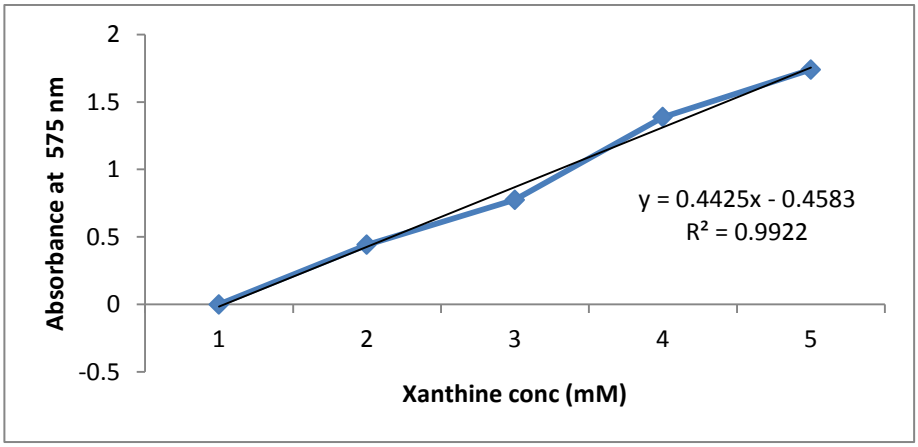
Fig 12: showing the microtiter plate showing XO inhibition by allopurinol in crude enzyme.



From the graph no. 9 and 10 it was clearly understood that xanthine oxidase activity goes on decreasing with increasing concentration of Allopurinol but on the other hand in the absence of Allopurinol the XO activity was increasing. This inhibition test confirms the presnce of xanthine oxidase enzyme in crude enzyme due to the formation of formazan due to the interaction of superoxide radicals ,produced by XO, with NBT.



Graph No.10: xo activity in presence of Allopurinol



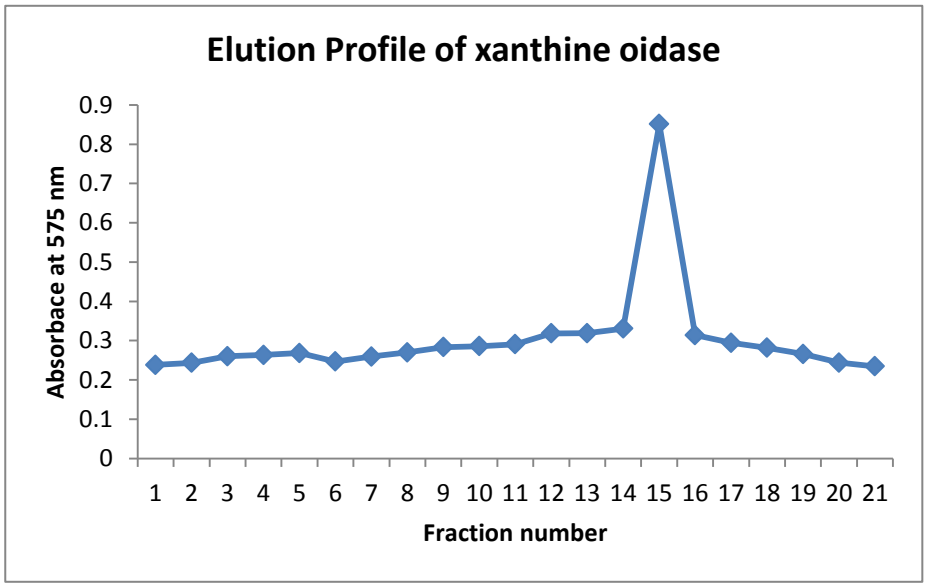
Graph No.11: XO activity in absence of Allopurinol

5.11 Purification of xanthine oxidase

The crude enzyme was purified by preliminary procedure involving acetone precipitation. The acetone precipitation has an advantage over dialysis and desalting method in that it enables concentration of protein samples as well as purification from undesirable substances. A single acetone precipitation may not be sufficient to remove all types and concentrations of interfering contaminants. In such case , repeated precipitaion may be performed. However because sample loss will accompany each cycle of precipitation .

Elution profile of protein from Gel filteration column

The crude enzyme was applied to the Sepharose 6B column pre-equiliberated with Tris HCl buffer (pH- 7.8). The eluted various fractions were collected and screened for xanthine oxidase activity by NBT assay. There was increase in XO activity observed from fraction 1 to fraction 15 and after the 15th fraction enzyme activity declined sharply. There by suggesting that the 15th fraction was found to have maximum enzymatic activity.



Graph 12: XO enzymatic activity of purified eluted fractions

Step	Fraction	Vol (ml)	Total units	Protein(mg)	Sp act	Yield /recovery (%)	Purification fold
1	Unfractionated extract/culture filtrate.	1.5	0.9889	5.04mg	0.1962	100	1
2	Acetone precipitation	0.5	1.212418	1.11742	1.090	81.7	5.55
3	Eluate from Sepharose 6 B	0.1	2.1056	0.310606	6.779	46.96	34.55

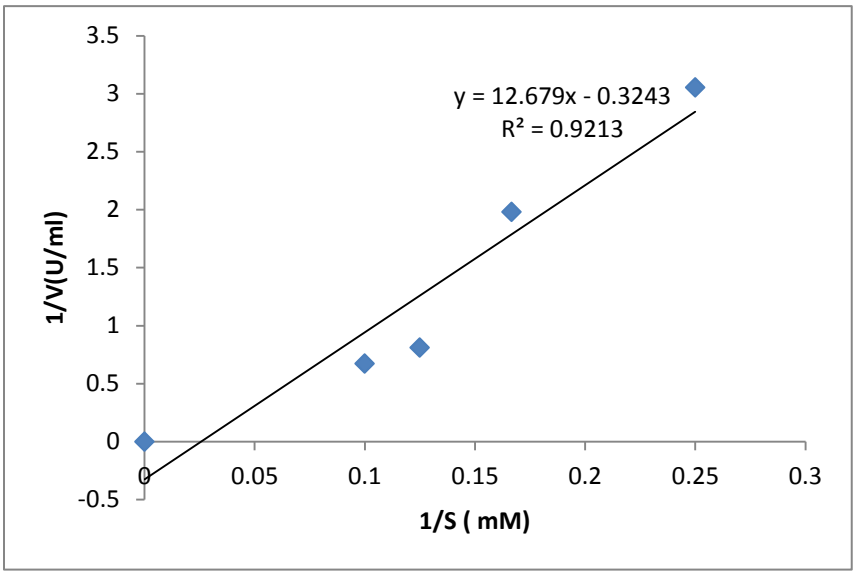
Table 13: The purification of XO from Endophytic fungus #19 NOBASVNP

5.12 Line weaver Burk plot.

Optimum substrate concentration for maximum enzyme activity was determined in terms of V_{max} and K_m using Xanthine. V_{max} and K_m values were interpreted from Line-Weaver Burk plot. Line weaver plot gives an indication of the affinity of enzyme towards substrate. The intercept of the graph is represented by $1/V_{max.}$ Also, the slope of the graph represents Km/Vmax, hence we deduced Km that give an indication of the affinity of enzyme towards substrate. The results from the Graph no. 13 revealed that alkaline Xanthine Oxidase from #19NOBASVNP had a V_{max} of 3.08 U/mg of protein and K_m value of $3.8 * 10^{-4}$ M. Xanthine Oxidase isolated fron *Arthrobacter sp.* had a relatively high K_m for xanthine (1.3×10^{-4} M), and substrate inhibition was not observed with this compound, in contrast to the standard enzyme. In fact, an opposite effect was observed, and double-reciprocal plots with Xanthine as the variable substrate showed a concave downward deviation at high concentrations. The values of K_m and V_{max} hence deduced are represented in Table no. 14 .

Kinetic parameters	Value
Km	3.8×10^{-4} M
V max	3.08 U/mg

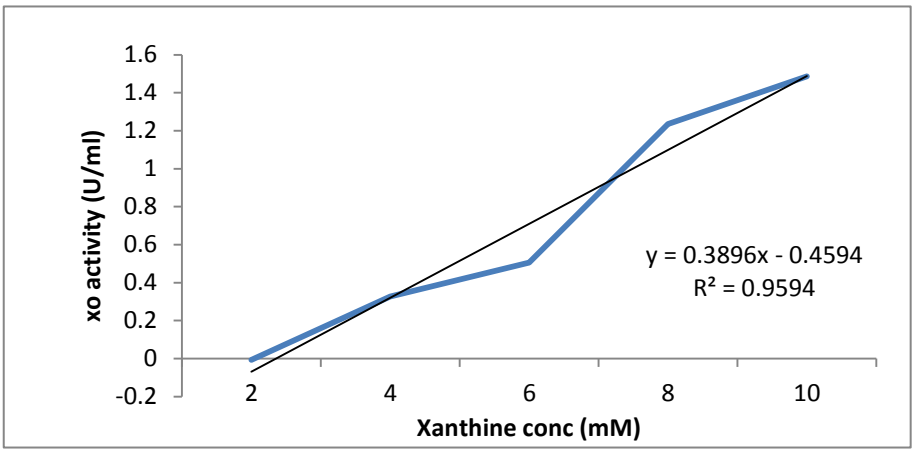
Table 14 : Kinetic parameters of purified XO.



Graph no. 13 : Line weaver Burk plot of purified XO

5.13 Effect of substrate concentration on enzyme activity.

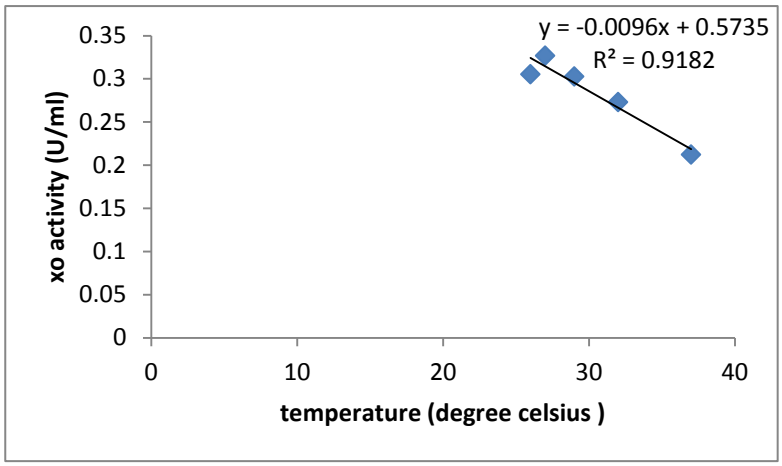
The effect of subtrate concentration was analysed by taking the absorbance of a reaction mixture at 575 nm.The subtrate concentration deduced from the standard curve was divided by the reaction time to calculate the enzymatic activity. Although , during optimization , maximum XO activity was obtained at 0.6 mM xanthine but Purified XO showed increase in its activity with increasing the substrate concentration from 2mM -10 mM.This explains that saturation level of the enzyme has not been attained yet and enzyme shows high affinity for substrate.



Graph no 14 : The effect of Xanthine concentration on purified XO activity

5.14 Effect of temperature on enzyme activity

Temperature is a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzymes to be active and stable at higher temperature. Assay mixture was incubated at different temperature ranging from 26-37°C and enzyme activity was maximum at 27°C (Li *et al*.,1997) reported that alkaline protease isolated from *Thermomyces lanuginose* P134 had a broad temperature optimum of 50°C.

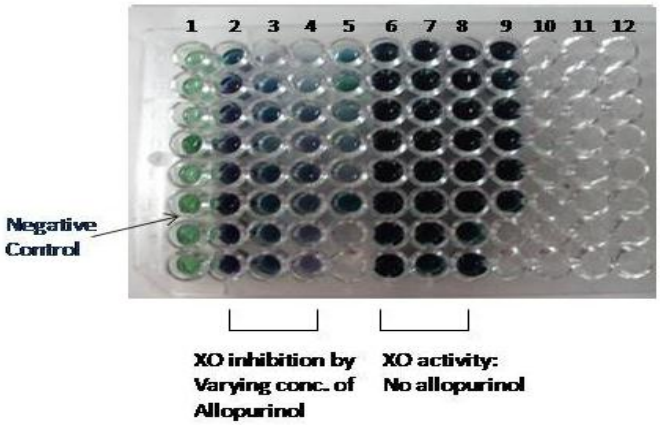


Graph no. 15 showing the effect of temperature on purified XO activity

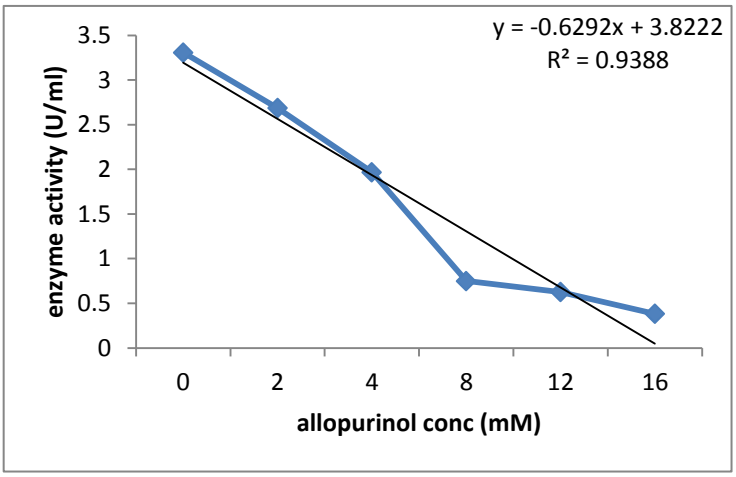
5.15 Effect of inhibitors on enzyme activity.

Allopurinol, or 1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one have inhibitory activity on XO, lowering down both urinary and serum uric acid levels. The purified fraction of XO showing maximum enzyme activity was evaluated for inhibition by Allopurinol so as to confirm the presence of Xanthine oxidase. It was found that the enzyme activity declined sharply with increasing concentration of Allopurinol (Graph no.16) and in the absence of standard XO

Fig 13: showing the microtiter plate showing XO inhibition by allopurinol in purified enzyme.

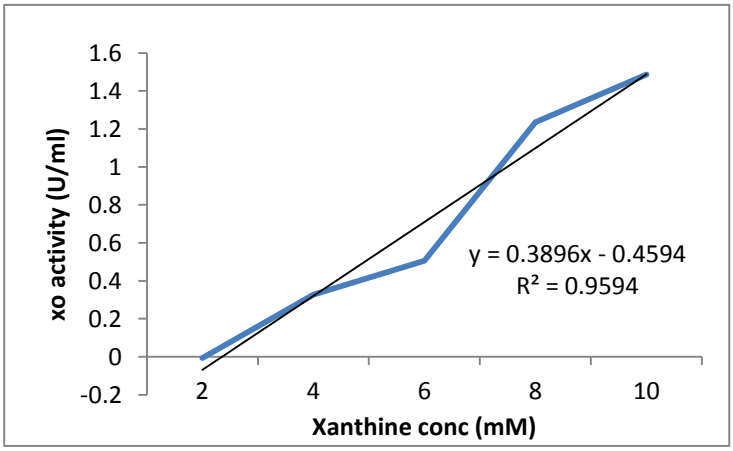


inhibitor the enzymatic activity was increasing exponentially (Graph no.17) thereby confirming the presence of xanthine oxidase in the purified fraction.



Graph no 16: XO activity in presence of Allopurinol

The XO activity is found to be relatively greater in absence of allopurinol is found to be greater than in the presence of it. Moreover, the XO activity is found to increase with the substrate concentration



Graph: 17: XO activity in presence of allopurinol.

Chapter 6

Conclusion

Conclusion

The current study revealed that endophytic fungal strain # 19 NOBASVNP, producer of Xanthine oxidase, was producing maximum yield of Xanthine Oxidase at an optimized media in which all the nutritional factors like substrate concentration, sucrose concentration and potato extract volume as well as physical parameters i.e. temperature, pH, rpm was optimized .The optimization was done by a conventional method involving optimization of a parameter one at a time and by Design Expert Version8. The Placket Burman design was used to deduce negative and positive factors involved responsible for XO activity. The optimum values of the positive factors were then deduced by Response Surface methodology.

The enzyme was isolated and purified by acetone precipitation and by application of enzyme to Gel filtration column consisting of Sepharose 6B. The Purification fold of about 34 % was obtained. The enzyme kinetics was studied and various kinetic parameters such as V_{max} and K_m were deciphered. The effect of substrate concentration, temperature and inhibitors like allopurinol on the enzme activity was deduced.

Further study is required to fully purify the enzyme and characterize the purified enzyme. The protein sequence also need to be deciphered in order to allow its comparison with already sequenced Xanthine Oxidase in the protein database.

Chapter 7

References

Woolfolk CA and Downard JS (1978): Bacterial Xanthine Oxidase from *Arthrobactor S-2*: *Journal of Bacteriology*; 135: 422-428.

Agarwal A and Banergee U C (2009): Screening of Xanthine Oxidase producing using Nitroblue Tetrazolium Based Calorimeteric Assay method :*The Open Biotechnology journal* ; 3: 46-49.

Allam AM and Elzainy TA.(1969): Degradation of Xanthine by *Penicillium chrysogenum*: *J.gen.Microbiol* ;56:293-300.

Carroll JJ , Coburn HD , Roberta and Arthur L (1971): A simplified Alkaline Phoshpotugstate assay for uric acid in serum : *Clinical chemistry* ;Vol 17:3.

Dietrich LS and Borries E (1953): On determination of Xanthine Oxidase activity in animal tissues: *J.Biol.Chem*; 140:725

Gokulakrishnan S, Chandraraj K, Gummmadi, SN (2005): Microbial and enzymatic methods for removal of caffeine : *Enzyme Microb Technol* ;37: 225-232.

Hille R and Nishino T (1995): Xanthine Oxidase and Xanthine dehydrogenase : *The Faseb journal;* 9:995-1003.

Hellsten Y, Frandsen U, Orthenblad N, and Richter EA (1997):Xanthine Oxidase in human skeleton muscle following eccentric exercise ; a role in inflammation : *Journal of Physiology ;* 498:239-248.

Hashimoto S (1974). A new spectrophotometer assay method of Xanthine Oxidase in crude tissue homogenate : *Analytic Biochem* ; 62:426-435.

Fridovich I and Handler P (1958): Xanthine Oxidase: III sulphite oxidation as an ultrasensitive assay: *Journal of Biological Chemistry*; 233:1578-1580.

Battelli MG and Stripe F (1972): Milk Xanthine Oxidase Type D (Dehydrogenase) and Type o (Oxidase) : *Biochem.J*;131:191-198.

Yamaoka –yano DM and Mazzafera P (1999) :Catabolism of caffeine and Purification of a Xanthine Oxidase responsible for methyluric acids production in *Pseudomonas putida :Revista de microbiologia* ; 30 :62-70.

Ozer N. , Muftuoglu M,Ataman D,Ercan A,Ogus H (1999):Simple , high –yield purification of Xanthine Oxidase from bovine milk :*J.biochem.biophys,*39:153-159.

Mohapatra BR, Harris N, Norin R , Mazumder A (2006):Purification and characterization of a novel caffeine Oxidase from *Alcaligens species : Journal of biotechnology*; 125:319-327.

Khalidi UAS and Chaglasstan TH (1965): The species distribution of Xanthine Oxidase : *Biochem .J*;97:318-320.

Morpeth F, George GN and Bray, RC (1984) : Form amide as substrate of Xanthine Oxidase .*Biochem J* ;220: 235-242.

Desco MC, AsensiM, Marquez R,Valls JM, Vento M,Pallardo FV, Sastre J and Vina J.(2002): Xanthine Oxidase is involved in Free radical production in Type 1 diabetes :protection by allopurinol*: DIABETES*; 51:1118-1124.

Papapostolou I, Georgiou CD (2010): Superoxide radical is involved in the sclerotial differentiation of filamentous phytopathogenic fungi: Identification of fungal Oxidase : *Fungal Biology* ; 114:387-395.

Catignanai GL and Darby WJ (1974): Vitamin E deficiency :Immunochemical evidence for increased accumulation of liver Oxidase : *Proc.Nat.Acad.Sci.USA*;71:5.

Pacher P and Szabo C (2006): Therapeutic effects of Xanthine Oxidase inhibitors: *Pharmacol Rev;*58: 87-114.

Hille R, Massey V: Studies on oxidative half- reaction of Xanthine Oxidase : *the journal of biological chemistry* 132 :293-297.

Kunamneneni A ,Singh S (2005) :Response surface methodological approach to optimize the nutritional parameters for enhanced production of amylase in solid state fermentation by *Thermomyces lanuginosus* : *African Journal of Biotechnology* ; 4:708-716.

Woodlock, C (1985): Purification and properties of a novel Ferricyanide –Linked Xanthine dehydrogenase from *Pseudomonas putida* 40: *Journal of bacteriology*; 163: 600-609.

Bommarius A, and Wang IC (1995): Xanthine Oxidase reactivity in reversed micellar Systems: A contribution to prediction of enzymatic activity in organized media.*J.Am.Chem.Soc*: 117:4515-4523.

Tasharoffi N ,Fazeli M and Faramarzi MA (2011):Optimization of Chitinase production by Bacillus pumilus using Plackett –Burman Design and response surface methodology :*Iranain Journal of pharmaceutical Research* ; 10:759-768.

Otto F, Denis W (1912): On phosphotunstic-Phosphomolybdic compounds as color reagents: *The Journal of biological chemistry ;* 9:241-243.

Strobel, G. (2006): Harnessing endophytes for industrial microbiology: *Current opinion in Microbiology*; 9:240-244.

Strobel G (2003): Endophytes as source of bioactive products: *Microbes and infection* ;5:535-544.

Strobe G, Daisy B, and Cawestilo, U (2005): The biological promise of microbial endophytes and their natural products. *Plant Path.J*;4 :161-176.

Xiang Q. and Edmondson, DE (1996): Purification and characterization of Prokaryotic Xanthine dehydrogenase from *Comamonas acidovorans* : *Biochemistry* 35:5441 -5450.

Tokano Y , Aoki K ,Horiuchi H. , Zhao L,Kassahara Y ,Kondo S and Becker M (2004): Selectivity of Febuxostat , a novel non – purine inhibitor of Xanthine Oxidase /Xanthine dehydrogenase *: Life Sciences* ; 76:1835-1847.

Trivedi, R.C., Reber, L., Desai, K. and Strong, L. J. (1978): New ultraviolet (340 nm) method for assay of uric acid in serum or plasma :Clinical chemistry .24 (4):562-566.

Schulz and Boyle C.(2006): Microbial root endophytes : *soil biology* ; 9:1-13 .

Raghuvanshi R, Kaul A,Bhakuni P ,Mishra A and Misra MK (2007) :Xanthine Oxidase as a marker of myocardial infarction :*Indian journal of Biochemistry* ; 22 (2) :90-92.

Nishikawa Y, Fukomoto K and Watanabe F (1985) :Analysis of guanase by agarose gel electrophoresis and activity staining : *Enzym;*.33:143-146.

Richert D A and Westerfeld WW (1953) : Isolation and identification of the Xanthine Oxidase factor as molybdenum :*Journal of biotechnology* 34 :915-923.

Enroth C, Bryan TE, Pai EF (2000) : Crysatl structure of bovine milk Xanthine dehydrogenase and Xanthine Oxidase : structure based mechanism of conversion : *PNAS* ;97 :10723-10728.

Sedewitz B , and Gotz F,(1984) : Purification and biuochemical Characterization of Pyruvate Oxidase from *Lactobacillus plantarum* : *Journal of Bacteriology* ; 160 : 273-278.

Choi HS (1993) : Purification and Characterization of adenosine deaminase from *Aspergillus oryzae* : *Kor Jour Microbiol* ; 3 :54-62.