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SCREENING, INDUCTION AND CHARACTERIZATION OF ARYL ALCOHOL OXIDASE (AAO) FROM *Pleurotus sp*

PROJECT REPORT

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ABSTRACT

ABSTRACT

Screening of lignolytic and aryl alcohol oxidase (AAO) activity from twenty different fungal species were carried out. Among them, seven species showed lignolytic activity and three (*Pleurotus ostreatus, P.eous* and *P.platypus*) of them were found to be AAO positive. Maximal AAO activity was observed in batch cultures of *Pleurotus ostreatus* and was found to be induced by aromatic amino acids and aryl alcohols upto a level of 290 U/lit. Optimum pH and temperature for *Pleurotus ostreatus* was found to be around 6 and 40 ^{o}C respectively. The K_m value of AAO for oxidizing veratryl alcohol was determined to be 0.6 mM. Solid state fermentation was carried out to improve AAO production. Cheap agricultural byproducts rich in lignin content like grape seed was employed for the production of AAO. Results show that the production of AAO is significantly increased and accelerated in SSF when an organic support (grape seed) is used as compared with submerged fermentation (SmF). Grape seed resulted in maximum yield of AAO as compared to other substrates viz. rice bran, wheat bran, groundnut shell, and sago hampus after 6 d of incubation at 28^{o}C. The time to reach maximum enzyme production was considerably reduced in SSF (12 days of incubation) as compared to SmF (18 days). Moreover, AAO activity was 6.44-fold higher in SSF than in SmF. Purification of AAO was carried out by three phase partitioning (TPP). The enzyme was partially purified 10.19 fold by TPP with an overall recovery of 10.95 % with a specific activity of 1.58 units per mg of protein. Results of the study indicate *Pleurotus ostreatus, P.eous* and *P.platypus* to be good producers of AAO, and they could be employed as promising fungal species for biotechnological applications.

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ABBREVATIONS

AAD	Aryl alcohol dehydrogenase
AAO	Aryl Alcohol Oxidase
ABTS	2, 2'- azonobis (3-ethylbenzthiazoline-6-sulfonate)
DMBA	3, 4-dimethoxybenzyl alcohol
DNS	Dinitrosalicylic acid
E.C	Enzyme commission
FAD	Flavin adenine nucleotide
GMC	Glucose Methanol Choline
GNS	Groundnut shell
GS	Grape Seed
H	Hydrogen
H_2O_2	Hydrogen peroxide
HBT	1- Hydroxyl benzotriazole
LiP	Lignin Peroxidases
M	Molarity
MBA	Methoxysubstituted benzyl alcohol
MDBL	Manidharma biotech private limited

mM	Millimolar
MnP	Manganese Peroxidases
nm	Nanometer
O_2	Oxygen
PAH	Polycyclic aromatic hydrocarbons
PDA	Potato dextrose agar
PSGMC	PSG Medical College
RB	Rice bran
SH	Sago hampas
SmF	Submerged fermentation
SSF	Solid State Fermentation
Temp	Temperature
TEMPO	2, 2', 6, 6'-tetramethyl piperidine - *N*-oxyl
TMBA	3, 4, 5-trimethoxybenzyl alcohol
TNAU	Tamil Nadu Agriculture University
TPP	Three phase partitioning
VAO	Veratryl alcohol oxidases
VLA	Violuric acid
WB	Wheat bran

INTRODUCTION

1. INTRODUCTION

Wood and other lignocellulosic materials are a major source of renewable carbon in the biosphere. The hard wood consists of cellulose (40-50%), hemicellulose (25-35%), lignin (20-25%), pectin (1-2%) and trace amounts of starch. Lignin is a 3-dimensional polymer and is the most abundant renewable aromatic polymer in the biosphere. Lignin is composed of phenyl propanoid units interconnected by stable C-C and C-O bonds (Figure.1.1). The heterogeneity and complexity of its structure confers resistance to microbial attack. However lignin can be degraded slowly in nature, mainly by white rot fungi (Bourbonnais and Paice, 1988).

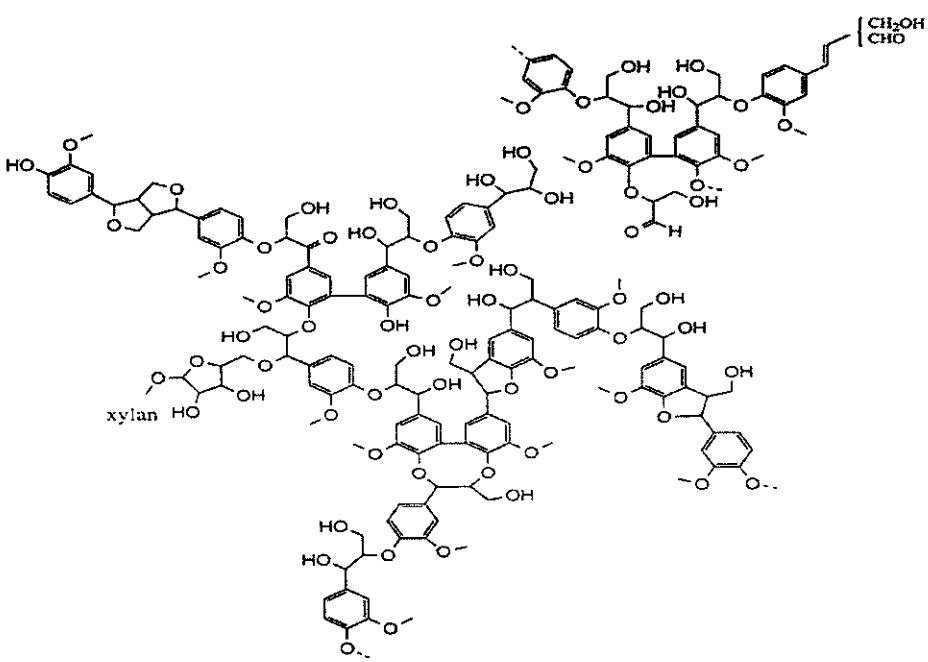


Fig. 1.1: Structure of lignin

About 940 million tons of lignite was produced worldwide each year. In India, the conservative reserves of lignite are about 130.3 million tons, accounting for 13% of total coal resources. Converting this low grade coal to useful materials poses a significant problem both in India and worldwide (Buddolla *et al.*, 2008).

1.1 Lignin and its Impacts in Paper Industry

Lignin comprises 20-25% of woody plant cell walls and gives strength and protection against biodegradation. Lignin provides mechanical strength to plants and its advantageous, however not to the industry especially to the paper industries. Lignin should be removed in order to produce fine papers. The present of lignin will contribute to problems such as acid gives off as the woods deteriorates. The same problem goes to paper and board which contain lignin. The structure of lignin is complex. It makes the cellulose and hemicellulose in wood indigestible. It contains benzene ring with a tail of three carbons. It also appears to have a lot of internal H bonds making them very resistant to degradation process naturally (Crawford *et al.*, 1983). Before being produced as papers, the timber will undergo certain treatments to remove lignin. Conventionally, the paper industries are using the cooking method in order to remove lignin. This method is limited to certain time and has to be stopped before all the lignin can be removed. This is because the cellulose would be gone too if the treatment is continued. To overcome this, they use bleaching method which will remove the remaining lignin. Bleaching method needs the usage of toxic chemicals such as chlorine. In order to get benefit for both industry and environment, many studies have been done.

1.2 Organisms that Capable of Degrading Lignin

Lignin degradation is a complex process that involves a wide variety of microorganisms related to wood rotting and plant-litter decomposition. As is well known, enzymes such as peroxidases (lignin and manganese peroxidase), laccase and other oxidases have repeatedly been claimed to take part in lignin transformation and degradation. However, it has been shown that lignocellulolytic enzymes cannot penetrate the wood structure except where the wood cell-wall is already partially decayed. Small molecules like activated oxygen species would be capable of diffusing into the wood cell-wall structure to initiate decay (Guillen *et al.* 1990).

The first step in lignin degradation is depolymerization, catalyzed by ligninolytic enzymes. Several enzymes secreted by the fungus during idiophasic metabolism are believed to participate in the degradation system. Some of the more important ones

include the lignin peroxidases (Tien, 1987), H_2O_2 generating enzymes (Philip and Kirk, 1987; Kelly and Reddy, 1986), and veratryl alcohol. Lignin peroxidases have been suggested to play an important role in the degradation of lignin and organopollutants (Bumpus *et al.*, 1985; Aust, 1990). Most active lignin degraders such as *Phanerochaete chrysosporium, Phlebia radiata, Trametes versicolor, Bjerkandera adjusta, Chrysonilia sitophila, Streptomyces badius* and *Streptomyces flavovirens* belong to the basidiomycetes (Kirk and Farrell 1987), though some of them are *Ascomycetes* (Duran *et al.* 1995) or *Actinomycetes* (Crawford *et al.* 1983). However most of the Enzymology of degradation have concentrated on one species, *Phanerochaete chrysosporium* and several enzymes involved in this process have been studied (Krik and Farrell 1987).

1.3 Enzyme Systems Involved in Lignin Degradation

Since white-rot fungi are the only organisms capable of efficient lignin degradation, their ligninolytic enzyme system has been studied extensively. Lignin polymer structure is irregular, which means that the degradative enzymes must show lower substrate specificity compared to the hydrolytic enzymes in cellulose or hemicellulose degradation. Three main fungal enzymes that affect lignin structures either directly or indirectly are Lignin Peroxidases (LiP), Manganese Peroxidases (MnP) and laccases. Peroxidase function requires extra cellular hydrogen peroxide, which is supplied by enzymes such as glyoxal oxidase, Aryl Alcohol Oxidase (AAO) and glucose-2-oxidase or by nonenzymatic systems via Fenton reaction. Table 1.1 shows the different types of enzyme which helps degrading lignin in different parts of its structure. Figure 1.2 shows scheme for lignin biodegradation including enzymatic reactions and oxygen activation.

1.3.1 Lignin Peroxidase (LiP)

Lignin peroxidases (EC 1.11.1.14) were first characterised by Tien and Kirk (1983) as "ligninases", but soon thereafter another ligninolytic peroxidase was found, that required manganese (Tien and Kirk, 1983). It degrades lignin by oxidation activity and this oxidative activity has been found occurred in pH range of 4.0 – 8.0. Acidic pH condition is the optimal for lignin mineralization. The mechanism of action of lignin

peroxidase is just the same as other mechanism of peroxidases enzymes. The reaction is single electron oxidation of aromatic nucleito produce unstable species called radicals. These radicals undergo a variety of further reaction, many of which do not involve the enzyme.

Table 1.1: Enzymes involved in the degradation of lignin

Lignolytic enzymes	**Substrate**	**Mediator**	**Main effect or reaction**
Lignin peroxidases	Veratryl alcohol	H_2O_2	Aromatic ring oxidized to cation radical
Manganese peroxidases	Mn, Organic Acid as chelators,	H_2O_2 , Thiols, Unsaturated lipids	Mn(II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reaction in the presence of additional compounds
Laccase	Hydroxyl benzatriazole. Guiacol.	O_2	Phenol or oxidized to phenoxyl radicals; other reaction in the presence of mediators.
Glyoxal oxidase	Glyoxal, Methyl glyoxal	O_2	Glyoxal oxidized to glyoxicillic acid; H_2O_2 production.
Aryl alcohol oxidase (AAO)	Aromatic alcohols	O_2	Aromatic alcohols are oxidized to aldehydes, H_2O_2 production.
Other H_2O_2 producers	Many organic compounds	O_2	O_2 reduced to H_2O_2.

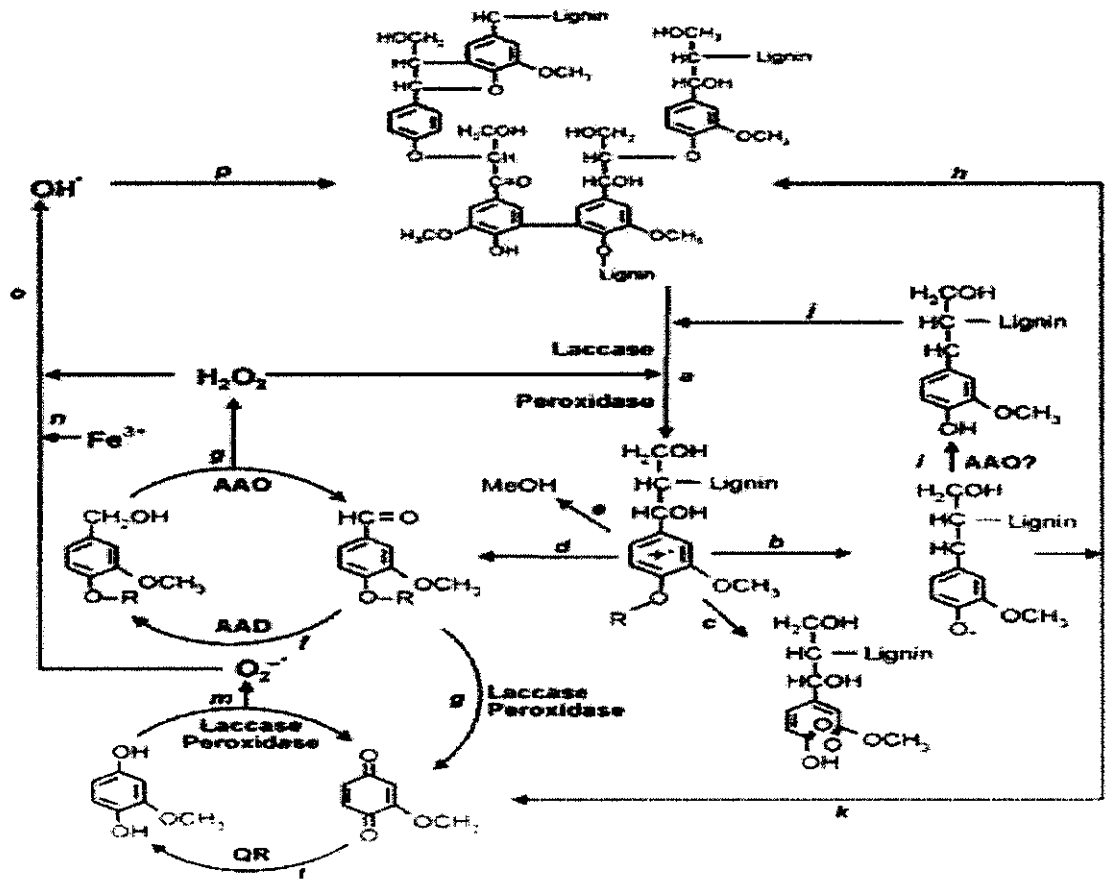


Fig. 1.2: A scheme for lignin biodegradation including enzymatic reactions

1.3.2 Manganese peroxidase (MnP)

Manganese peroxidase (E.C. 1.11.1.13) has a similar catalytic cycle as LiP, but it utilises Mn (II) as a substrate. Manganese peroxidase is a heme containing enzyme and it has been one of the important in delignification of kraft pulps. It increases the brightness of hardwood kraft pulp. Manganese peroxidase requires H_2O_2 as a cosubstrate and catalyzes the oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} complexed with an organic acid acts as a primary agent in lignolysis. Manganese peroxides could degrade residual lignin in kraft pulp. Also LiPs have been shown to be able to oxidise chelated Mn (II) in the presence of H_2O_2. Moreover, MnP is able to liberate CO_2 directly from lignin substructures. It can also produce H_2O_2 in O_2-requiring oxidation of NAD(P)H, glutathione and dithiotreitol.

1.3.3 Laccases

Fungal laccases (E.C.1.10.3.1) are implicated in the degradation of lignin. The enzyme is blue copper-containing polyphenol oxidase. It catalyses the reduction of molecular oxygen to water by four consecutive 1-electron oxidation steps of a phenolic substrate, or aromatic amines and other electron-rich substrates. Laccases are multicopper enzymes belonging to the group of oxidoreductases which oxidize diphenols and allied substances and use molecular oxygen as an electron acceptor (Thurston, 1994). It has been reported that the inclusion of a mediator such as ABTS (2, 2'- azonobis (3-ethylbenzthiazoline-6-sulfonate), HBT (1- Hydroxyl benzotriazole), VLA (violuric acid), and TEMPO (2, 2', 6, 6'-tetramethyl piperidine - *N*-oxyl) can extend the substrate range of laccase to non-phenolic subunits of lignin. The degradation by laccase was accomplished when surfactants were added to the system. The type of laccase, redox mediator and surfactant affected the extent of degradation.

1.3.4 Hydrogen Peroxide Generating System

Hydrogen peroxide (H_2O_2) generating systems form an important part of the fungal delignification system, as peroxides are required for peroxidase function. Extracellular enzymes which produce hydrogen peroxide include glyoxal oxidase (Tien and Kirk, 1983), AAO (Farmer *et al.*, 1960) and glucose- 2-oxidase (Daniel *et al.*, 1994). Intracellular methanol oxidase catalyses the reaction from methanol to formaldehyde and H_2O_2. Extracellular formaldehyde and acetaldehyde, formed in ethanol oxidation, can then be oxidised by glyoxal oxidase to produce more H_2O_2. Extracellular and intracellular oxidases have been found in lignolytic cultures of *Phanerochaete chryosporium*, producing hydrogen peroxide from fatty acyl coenzyme A, glucose, methanol, glyoxal and related compounds. Some fungi also produce extra-cellular aryl alcohol oxidase. Several oxidases have been reported as being potentially involved in hydrogen peroxide generation by ligninolytic fungi. However, some of them can be discounted because of their intracellular location, and only extracellular glyoxal oxidase, pyranose-2-oxidase and aryl-alcohol oxidase (AAO) are currently considered to be involved in lignin biodegradation.

OBJECTIVES

2. OBJECTIVES

The present study has the following objectives:

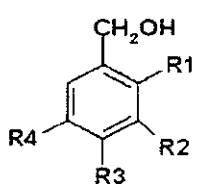
1. To screen the lignolytic and aryl alcohol oxidase (AAO) activity in different fungi.
2. To optimize the process and medium parameters to increase production of AAO in solid state and submerged fermentation.
3. To purify AAO using three phase partitioning.
4. To characterize the AAO activity with respect to pH, temperature, substrates and stability.

LITERATURE REVIEW

3. LITERATURE REVIEW

3.1 Aryl Alcohols

Methoxyl-substituted aromatic substrates are of interest considering that lignin is formed mainly from two methoxylated precursors, coniferyl alcohol and sinapyl alcohol (Brunow *et al.*, 1999). The basic structure of aryl alcohols was shown in Figure 3.3. In addition, 3, 4-dimethoxybenzyl alcohol (DMBA; veratryl alcohol) is produced as a secondary metabolite by lignin-degrading fungi (Lundquist and Kirk 1978). The list of other methoxyl substituted aromatic substrates are benzyl alcohol (Phenyl methanol), 2-methoxybenzyl alcohol, 3-methoxybenzyl alcohol, p-anisyl alcohol (4-methoxybenzyl alcohol), 2, 4-dimethoxybenzyl alcohol, veratryl alcohol, 3,5-methoxybenzyl alcohol, 3,4,5-trimethoxybenzyl alcohol (TMBA), 3- Hydroxy benzyl alcohol, 4- Hydroxy benzyl alcohol, 3-Hydroxy-4-methoxybenzyl alcohol, 4-Hydroxy -3- methoxybenzyl alcohol, cinnamyl alcohol, Allyl alcohol, 2,4 Hexadien-1-ol. The investigation of the oxidation of methoxysubstituted benzyl alcohols (MBAs) is therefore relevant for the elucidation of lignin biodegradation in nature.



	R1	R2	R3	R4
2,3-DMBA	OCH_3	OCH_3	H	H
2,5-DMBA	OCH_3	H	H	OCH_3
3,4-DMBA	H	OCH_3	OCH_3	H
2,3,4-TMBA	OCH_3	OCH_3	OCH_3	H
2,4,5-TMBA	OCH_3	H	OCH_3	OCH_3
3,4,5-TMBA	H	OCH_3	OCH_3	OCH_3

Figure.3.3: Basic structure of aryl alcohols

3.1.1 Benzyl Alcohol

Benzyl alcohol is a natural constituent of a number of plants. It occurs, for example, in some edible fruits (up to 5 mg/kg) and in green and black tea (1-30 and 1-15 mg/kg, respectively) (CoE, 1992). The Food application of Benzyl alcohol is added as a

flavouring substance to some foods and beverages at a level up to about 400 mg/kg (chewing gum 1254 mg/kg) (Fenaroli, 1994). The quantity which is requested to be used as a carrier solvent for flavouring substances to food and beverages is up to 300 mg/kg in the final food as consumed. Other applications are it is used as local anaesthetic, pharmaceutical aid, and in perfumery. It is an indirect food additive for use as a component of resinous and polymeric coatings. Benzyl alcohol is used in a wide variety of cosmetic formulations as a fragrance component, preservative, solvent, and viscosity-decreasing agent. In 1998, benzyl alcohol was reported by the US Food and Drug Administration to be used in 322 cosmetic formulations belonging to 43 cosmetic-product categories (CIR, 2001).

3.1.2 Veratryl Alcohol

Veratryl (3, 4-dimethoxybenzyl) alcohol, a secondary metabolite synthesised de-novo by several fungi (De Jong *et al.* 1994), encodes the transcription of genes for laccases and manganese peroxidases (Scheel *et al.* 2000), but not of LiP (Cancel *et al.* 1993), in certain basidiomycetes, and as an inducer of laccases in the ascomycete, *Botryosphaeria* sp. (Barbosa *et al.* 1996, Dekker and Barbosa 2001). This alcohol has also been ascribed to play specific roles as a physiological cofactor of LiPs, a redox mediator between LiPs and lignin degradation, and for the protection of LiPs during the redox cycle from metabolically-produced H_2O_2. The biological roles of veratryl alcohol are, Extracellular laccase induction at high veratryl alcohol concentrations in three botryosphaeriaceous fungi (Dekker and Barbosa, 2001), Production of intracellular laccases by all three *botryosphaeriaceous* fungi at enzyme levels lower than in un-induced cultures, A general reduction in biomass due to veratryl alcohol becoming toxic to *Botryosphaeria* sp. at concentrations >30 mM. The fungus, nevertheless, still could grow on 100 mM veratryl alcohol (Barbosa *et al.* 1996; Dekker and Barbosa, 2001), Derepression of laccase synthesis in the presence of cycloheximide. Repression of synthesis of amylase, pectinases, xylanase and cellulase (Dekker and Barbosa, 2001), Stimulation of total extracellular protein synthesis, aryl alcohol oxidase (AAO) and laccase (Dekker and Barbosa 2001; Asada *et al.*, 1995; Kumar and Rapheal, 2010), Reduction in the production of exopolysaccharide (ß-glucan) (Dekker and Barbosa,

2001), *In-vitro* inhibition of laccase-catalysed polymerisation of 2,6-dimethoxyphenol (DMP) (Dekker and Barbosa, 2001).

3.2 Aryl Alcohol Oxidase (AAO)

Aryl alcohol oxidase (EC 1.1.3.7) belongs to the family of oxido reductase specifically those acting on the alcohol group of donor with O_2 as acceptor (Ander and Marzullo, 1997). Following the discovery of lignin peroxidases (Tien and Kirk, 1983), the search for enzymatic sources for hydrogen peroxide (H_2O_2) production has been undertaken. H_2O_2 plays an important role in lignin degradation as cosubstrate of ligninolytic peroxidases (Kirk and Farrell, 1987). Since lignin degradation is an extracellular process, extracellular glyoxal oxidase and aryl-alcohol oxidase (AAO) seem to be the main H_2O_2 generating enzymes involved in lignin degradation. AAO participates in fungal degradation of lignin, by providing the H_2O_2 required by the lignolytic peroxidases. The H_2O_2 is generated by the redox cycling of aromatic fungal metabolites like p-anisaldehyde.

Fungal peroxidase catalyzes the oxidative degradation of lignin using the hydrogen peroxide generated by AAO system. It is a flavoenzyme in the (glucose methanol- choline oxidase) GMC oxidoreductase family. These enzymes may be categorized into alcohol oxidase. on the basis of the mechanisms involved in the catalytic reactions. The aromatic alcohol oxidase (AAO), also known as aryl alcohol oxidase, such as, veratryl alcohol oxidase (EC 1.1.3.7) and vanillyl alcohol oxidase (EC 1.1.3.38). The enzymes are also specified veratryl alcohol oxidase (VAO) in the literature.

3.3 Mechanism of Aryl Alcohol Oxidase (AAO) Action

Aryl alcohol oxidase catalyzes the oxidation of aryl α and α–β-unsaturated γ-alcohols to the corresponding aldehydes with concomitant reduction of O_2 and H_2O_2. The metabolic role proposed for these enzymes has been that of providing H_2O_2 to be used by peroxidases, which have been assumed to be principally responsible for lignin degradation. The enzyme reaction is as follows,

Aromatic primary alcohol + O_2 ⟶ Aromatic aldehyde + H_2O_2

In particular, *P. eryngii* AAO conserves two histidine residues, His-502 and His-546, involved in catalysis in different members of this family (the second residue being an asparagine in some of them) (Varela *et al.*, 2000). Non glycosylated *P. eryngii* AAO expressed in *Escherichia coli* (Ruiz *et al.*, 2006) is used for further characterization studies. The enzyme catalyzes the oxidative dehydrogenation of unsaturated alcohols with a primary hydroxyl at Ca, exhibiting broad substrate specificity. In addition to benzyl alcohols, its active site also binds and oxidizes aliphatic polyunsaturated primary alcohols (such as 2,4- hexadien-1-ol), naphthyl and cinnamyl alcohols, and shows low activity on some aromatic aldehydes (Ferreira *et al.*, 2005).

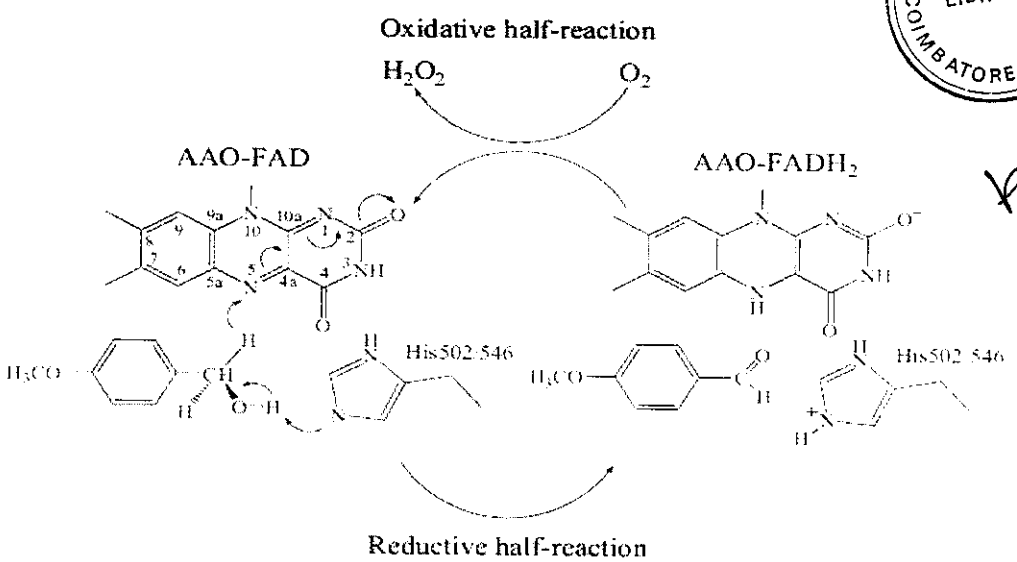


Fig. 3.4: Mechanism of action of AAO

Methanols, and other saturated alcohols, are not AAO substrates, and the monounsaturated allyl alcohol is very slowly oxidized (Guillen *et al.*, 1990). It is suggested that the AAO catalytic mechanism proceeds *via* electrophilic attack and direct transfer of a hydride to the flavin (Ferreira *et al.*, 2005). A recent mutational study confirmed the strict requirement for catalysis of His-502 and His-546 located near the

isoalloxazine ring of FAD (Figure 3.4), as well as the involvement of two aromatic residues (Ferreira *et al.*, 2006).

3.4 AAO Producers

Aryl alcohol oxidases (AAO) have so far been detected in the culture medium of only a small number of fungal species and their physiological function has been less investigated than the other enzymes (Ander and Marzullo, 1997). In all the species examined, these enzymes have been identified in the culture broth in the later stage of growth when secondary metabolite pathways are presumably switched on by starvation. The first AAO activity was detected by Farmer *et al.*, (1960) in *Polystictus versicolor* also called as *Trametes versicolor*, and similar enzymatic activities have now been identified in nine *Pleurotus* species (Bourbonnais and Paice, 1988; Guillen *et al.*, 1990; Sannia *et al.*, 1991; Guiterrez *et al.*, 1994; Eichlerova *et al.*, 2006) and in *Bjerkandera adusta* (Muheim *et al.*, 1990). These enzymes, named Veratryl alcohol oxidases (VAO) according to Bourbonnais and Paice (1988), have a very similar catalytic behaviour and are quite different from other AAO detected in *Fusarium solani* (Saparrat *et al.*, 2000), *Geotrichum candidum* (Kim *et al.*, 2001), *Phanerochaete chrysosporium* ME-446 (Asada *et al.*, 1995), *Fusarium proliferatum* (Regalado *et al.*, 1999).and in the non-ligninolytic fungus *Penicillium simplicissimus* (Mattevi *et al.*, 1997). These AAO show a narrower substrate specificity and in *Penicillium* also an intracellular localization. This is a typical extracellular enzyme in *Pleurotus*-species and *Bjerkandera adusta*, but has been found also from *T. versicolor* (De Jong *et al.*, 1994) and as an intracellular enzyme from *P. chrysosporium* (Asada *et al.*, 1995). Recently, the gene *aao* has been cloned and sequenced in *Pleurotus eryngii* (Varela *et al.*, 1999).

3.5 Production Conditions for AAO

Historically, there are only a few reports on the microbial production of AAO. AAO is produced by many microorganisms, as noted in Table 3.2. Microbial AAO may be produced both by submerged culture and solid-state fermentation processing, as discussed.

Table. 3.2: List of AAO producing fungal strains

Species	Inducer	Activity (U/lit)	Conditions
Pleurotus sajorcaju	-	1000	25°C, 24 days, 200 rpm
	2,4 DCP	10-20	28°C, 160 rpm
	2,4 DCP	ND	28°C
Pleurotus eryngii	Alkali lignin, Wheat straw	10	15 days, 200 rpm, 28°C
	Wheat straw	25	40 days, 200 rpm, 28°C
Pleurotus ostreatus	Tyrosine	313	18 days, static, 30°C
Pleurotus pulmonaris	Alkali lignin, Wheat straw	870	6 days, 200 rpm, 28°C
Fusarium proliferatum	Kraft lignin, Benzyl alcohol	7 nmol/ min mg	30 days,
Fusarium solani	-	57	21 days, 25°C, 150 rpm
Pleurotus comucopiae, P. floridanus	Alkali lignin, Wheat straw	110	15 days, 200 rpm, 28°C
Phanerochaete chryosporium	Benzyl, anisyl and vannilyl alcohol	2180	28°C, Shaking
Polystictus versicolor	-	1500	10 days,
Geotrichum candidum	-	55	120 rpm, 30°C
Pleurotus calyptratus, P.citrinopileatus,	-	24, 46	27°C, 20 days
Bjerkandera sp.	-	410	Static, 5 days, 30°C.

3.5.1. Submerged Fermentation

The majority of fermentation plants around the world known for commercial production of enzymes and pharmaceutically important products use a technology called submerged fermentation .The microorganisms was cultivated in a liquid medium in a large tank, where it is supplied with all the necessary growth ingredients. Submerged fermentation is more useful for large volume production. Submerged fermentation in addition to being completely automated and non labor intensive is also by virtue of the process "a contained process". Contained means that the exposure of the production micro organisms and the product to the outside environment can be controlled and restricted, thus ensuring better the integrity of the process and the safety of the environment.

A large number of microorganisms have been screened for their AAO producing ability. The AAO from *Phanerochaete* is produced in high nitrogen medium during primary metabolism and is to be inducible by aryl alcohols. About 4.0, 2.6 and 2.2 fold increases in the specific activity of intracellular AAO were observed when *Phanerochaete chryosporium* was grown in media containing 0.1% vannilyl alcohol, veratryl alcohol and benzyl alcohol respectively (Asada *et al.*, 1995). The specific activity of AAO reached a maximum 5 days after inoculation. The maximum production of VAO is reached in the late-stage of growth under nutrient-limited conditions and seems to be particularly related to carbon source (glucose) depletion. Many ligninolytic basidiomycetes fungi only secrete oxidative lytic enzymes under some nutrient- limited conditions. These strains generally require long incubation times, 15-20 days or even more, to complete their growth and reach the idiophase (Pal *et al.* 1980).

VAO produced in nitrogen-rich agitated cultures of *Pleurotus sajorcaju* strain 405 (Bourbonnais and Paice, 1988). *Pleurotus ostreatus* K16-2 was cultured statically, addition of l-tyrosine to the medium (pH 6.3) markedly enhanced AAO production (Okamoto *et al.* 2002) at 30°C for 18 days. De Jong and coworkers (1994) found that 3-chloro *p*-anisyl alcohol and 3, 5-dichloro-*p*-anisyl alcohol serve as even better substrates

for H_2O_2 production by AAO than do the other nonchlorinated aryl metabolites in *Bjerkandera* sp. strain BOS55.

Fusarium proliferatum (MUCL 31970) were supplemented with the polymeric fraction of kraft lignin (1g/l) or with benzyl alcohol (0.5 mM). AAO was induced in cultures with benzyl alcohol, reaching 7 nmol min^{-1} ml^{-1} after 30 days of incubation respectively (Regaldo *et al.*, 1999). The nitrogen limited glucose medium with straw alkali-lignin (0.1%) or milled wheat straw (1%) used for studying the production of enzymes. After 15 days of growth, the following AAO activities *P.comucopiae*, 40 U $liter^{-1}$ in *P. ostreatus*, 10 U $liter^{-1}$ in *P. eryngii, P. sajor-caju*, and *P. floridanus*. The AAO activity in *P. pulmonarius* was higher in the lignin medium (170 U $liter^{-1}$) and, especially, in the straw medium (870 U $liter^{-1}$). In *P.pulmonaris*, AAO activity occurred first in the mycelium and later in the extracellular medium, attaining nearly 500 U $liter^{-1}$ after 9 days.

Polystictus versicolor was the first organism reported for the production of AAO. Intracellular activity of AAO in growth solutions reached a maximum after 10 days. Kim *et al.*, 2001 showed *Geotrichum candidum* grown in Maltose BIII medium and the AAO activity was observed clearly from the 8^{th} day and increased even after the sugar in the broth. VAO from *P. simplicissimum* is an inducible intracellular octameric flavoprotein oxidase. These features may indicate that this oxidase is a peroxisomal enzyme like the alcohol oxidases from methylotrophic yeasts.

3.5.2 Solid State Fermentation

Solid-state fermentation (SSF) has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism (Sanghi *et al.*, 2008). These fermentation systems, which are closer to natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites, which usually will not be produced or will be produced only at low yield in submerged cultures (Babu and Satyanarayana, 1995; Pandey *et al.*, 2000; Jecu 2000).

SSF offers distinct advantages over submerged fermentation including economy of space, simplicity of media, no complex machinery, equipment and control systems, greater compactness of the fermentation vessel owing to lower water volume, greater product yields, reduced energy demand, lower capital and recurring expenditure in industry, easier scale-up of processes, smaller volume of solvent needed for product recovery, superior yields, absence of foam build-up and easier control of contamination due to the low moisture level in the system (Babu and Satyanarayana, 1995; Pandey *et al.*, 2000; Jecu 2000; Sanghi *et al.*, 2008).

In recent years, there has been an increasing trend towards efficient utilisation and value-addition of agroindustrial wastes in solid state cultivation. Not only the utilisation of these wastes in bioprocesses provides alternative substrates, but also helps in solving pollution problems. The microorganisms in solid state cultures grow under conditions close to their natural habitats, due to which they may be more capable of producing certain enzymes and metabolites, which usually will not be produced or will be produced only at low yield in submerged cultures (Pandey *et al.*. 2000). Various agricultural substrates / byproducts have been used successfully in solid state fermentation for AAO enzyme production (Barassa *et al.*, 1998). Most of these compounds are expensive. making their application at industrial scale impracticable. So then, studies based on the search of environmentally and economically feasible compounds having a stimulatory effect on AAO production are necessary. Lignocellulosic materials comprise a broad range of wastes from agricultural, food and forest industry that can stimulate AAO production. Moreover, these materials can also provide some of the necessary nutrients to the fungi, which imply a considerable reduction in AAO production costs.

In *Bjerkendra adjusta*, perlite (10 g) or wheat bran (15 g) were examined for the AAO production, from that AAO activity reached 1400 U kg^{-1} support after 5 days of cultivation with 66% moisture (Lapadatescu and Bonnarme, 1999). They also mentioned AAO activity was associated with benzaldehyde biosynthesis. The differences in metabolite production levels between both supports can be attributed to the basic support structure: wheat bran acts simultaneously as a support and a substrate for growth, while

Perlite is only an inert support (Durand *et al.*, 1996). The highest AAO activity was 1260 U kg^{-1} support for an initial L-phenylalanine concentration of 30 g kg^{-1} support.

In *Pleurotus eryngii*, the influence of wheat straw under SSF conditions was studied in 100 ml flasks with 2 g of sterilized wheat straw (5 to 20 mm long; autoclaved at 120°C for 15 min) and 6 ml of water. The absence of straw supplementation, AAO attained its highest levels at the end of the incubation period. Straw addition resulted in rapid ammonium exhaustion after 9 days and earlier production of the maximal AAO level (23 U/l). Study shows that the presence of wheat straw stimulated AAO production and provides the first evidence of AAO localization during lignocellulose degradation (Barassa *et al.*, 1998). List of elicitors reported so for AAO production are, L-Phenyl alanine and Benzyl alcohol (Lapadatescu and Bonnarme, 1999) and wheat straw (Barassa *et al.*, 1998).

3.6 Properties of AAO

VAOs have a molecular mass ranging from 71 to 78 kDa and are probably modified by glycosylation as demonstrated in *Pleurotus* species. Two VAO isoenzymes have been identified in *P. sajorcaju* and in *B. adjusta* on the basis of their slight difference in pI. Three AAO enzymes I, II and III have been identified in *Pleurotus ostreatus* and has the same molecular mass 70-72 KDa in gel filtration chromatography and SDS-PAGE respectively. In particular, *P. eryngii* AAO conserves two histidine residues, His-502 and His-546, involved in catalysis in different members of this family (the second residue being an asparagine in some of them. The characteristics of AAO from different microbial species were listed in Table.3.3.

Moreover, *N*-terminal sequence analysis of the enzyme purified from *P. ostreatus* showed heterogeneity: although the proteins behaved as a single protein to SDS-gel and IEF electrophoresis, protein sequence analysis showed two overlapping *N*-terminal sequences differing only for the presence of three protruding amino acids. VAO N-terminal sequences from *P. ostreatus* and *P. eryngii* are highly conserved (14 out of 15 amino acids match in both enzymes) and contain the consensus sequence Gly-Xaa-Gly-

Xaa- Xaa-Gly that is characteristic of dinucleotide binding proteins (Wierenga *et al.*, 1986). In fact spectroscopic evidences and direct analysis have demonstrated that VAOs are flavoproteins containing one molecule of FAD per molecule of protein. This coenzyme acts as a redox center in the transfer of two hydrogen atoms from aryl alcohols to molecular oxygen, although the details of the reaction mechanism have not yet been fully investigated.

Table.3.3: List of characterization of AAO from microbial strains

Microbial Strains	pH	Temp (°C)	Characteristic	Kinetic constants	Reference
Pleurotus sajorcaju	5.0	-	2 Isoenzymes, 71,000 KDa, pI 3.8-4.0	0.41-0.46 mM,3869-4930U/mg	Bourbonnais and Paice, (1988)
Pleurotus eryngii	5.0	55	72.6 KDa	-	Guillen *et al.*, 1990
Pleurotus ostreatus	5-6	40	3 Isoenzymes, 70-72 KDa, pI 3.5.	-	Okamoto and Yannase., 2002
*Pleurotus platypus**	6.0	35	-	0.57 mM, 2.8 U/mg	Kumar and Rapheal, (2010)
*Pleurotus eous**	5.5	50	-	0.81 mM, 1.14 U/mg	Kumar and Rapheal, (2010)
Phanerochaete chryosporium	6.0 - 7.0	45	78 KDa, pI 5.35	-	Asada *et al.*, 1995
Comomonas sp	3.0	65	66 KDa	2.5 mM	Jadhav *et al.*, 2009
Polystictus versicolor	6.0 - 6.5	45	-	1.3 mM	Farmer *et al.*, 1960
Geotrichum candidum	5.5	45	65.3 KDa	1.7mM, 4.2 U/mg	Kim *et al.*, 2001

*** Present study**

3.6.1 Spectral Properties of AAO

Purified AAO showed absorption maxima in the visible region at 385 nm and 463 nm (with troughs at 414 nm and 317 nm) and an *A*280/*A*463 ratio of 10. After heat treatment (70°C for 10 min), the AAO protein was precipitated, and the cofactor absorption spectrum lost the 463 nm maximum, which was replaced by the 450 nm maximum of free flavin, indicating that the flavin was not covalently bound to the protein. From these spectral changes, the molar absorption coefficient of the recombinant enzyme (ε_{463} 10280 $M^{-1}.cm^{-1}$) was estimated (Ferraria *et al.*, 2005).

AAO does not react with sulphite, since no spectral changes were observed in the presence of up to 20 mM sodium sulphite. In anaerobic reduction of the enzyme, decrease in the 463 nm absorbance was observed during *p*-anisyl alcohol oxidation, revealing the participation of the flavin cofactor in the catalytic activity of AAO. An isosbestic point was observed at 330 nm, indicative of a two-electron reduction of the cofactor. Excess of *p*-anisyl alcohol (5–6-fold) was required for complete reduction of AAO. During AAO reduction, stoichiometric amounts of *p*-anisaldehyde (calculated from its absorbance at 298 nm) were formed (Ferraria *et al.*, 2005). The anaerobic reduction of AAO by *p*-anisyl alcohol was also followed using the stopped flow equipment coupled to a photodiode array detector. The spectral changes observed in the 350–650 nm range showed the reduction of the oxidized flavin to the hydroquinone form, without formation of an observable semiquinone intermediate. Anaerobic titration of AAO with dithionite gave similar spectra, where only two chemical species (corresponding to reduced and oxidized enzyme) could be identified. Dithionite reduction appeared as amonophasic process when analysed using the stopped flow equipment. In both anaerobic reduction experiments (using *p*-anisyl alcohol and dithionite), reoxidation with air restored the spectral properties of the oxidized form of AAO. Photoreduction at pH 6 and pH 9 did not show semiquinone formation, and only fully oxidized and reduced species were observed. Reoxidation completely restored the original spectrum.

Table.3.4: Relative rate of oxidation of various substrates by AAO from *Pleurotus sp*

Relative rate of oxidation of various substrates by VAO from *Pleurotus* species. Relative activities are expressed as the percentage of activity observed with benzyl alcohol (Sannia et al., 1991; Guillén et al., 1990; Bourbonnais and Paice, 1988)

Substrate	*P. ostreatus*	*P. eryngii*	*P. sajor-caju*[a]
Benzyl alcohol	100	100	100/100
2-Methoxybenzyl alcohol	36	<5	<5
3-Methoxybenzyl alcohol	327	100	63/64
4-Methoxybenzyl alcohol	936	571	667/1064
2,4-Dimethoxybenzyl alcohol	809	177	167/300
Veratryl alcohol	909	326	333/400
3,5-Dimethoxybenzyl alcohol	273	—	23/32
3,4,5-Trimethoxybenzyl alcohol	—	5	<5
3-Hydroxybenzyl alcohol	—	<5	<5
4-Hydroxybenzyl alcohol	—	<5	<5
3-Hydroxy-4-methoxybenzyl alcohol	1309	319	207/284
4-Hydroxy-3-methoxybenzyl alcohol	227	<5	<5
Cinnamyl alcohol	1973	451	257/220
Coniferyl alcohol	400	<5	<5
Allyl alcohol	54	<5	<5
2,4-Hexadien-1-ol	—	531	—

[a]The two reported values refer to VAO I and VAO II isoenzyme activities.
[b]Not measured.

3.6.2 Catalytic Properties of AAO

Studies on the catalytic properties of VAOs have shown that they have in common wide substrate specificity towards differently substituted methoxy phenols (Table.3.4). However, from the comparison of relative activities of the enzymes towards the tested substrates it is possible to recognize some critical features which influence their reactivity. The substrate specificity of VAO from *Pleurotus* species shows that the presence of electron donors on the aromatic ring and the number of conjugated double bonds influence enzyme activity. Particularly, the methoxy group in para position significantly increases the reactivity of benzyl alcohols, while the hydroxy group in the same position makes the same molecules rather inert to the enzymatic oxidation. Moreover the effect of the number of conjugated double bonds is exemplified by the higher reactivity of cinnamyl alcohol compared to benzyl alcohol or of 2, 4-hexadien-1-ol as compared to allyl alcohol.

3.7 Application of AAO

The redox enzymes that catalyze the oxidation of alcohol to the corresponding aldehyde have potential application in the pharmaceutical, food, clinical, pulp, and other industries (Azevedo *et al.*, 2005).

3.7.1 AAO Involved in Lignin Degradation

H_2O_2 plays an important role in lignin degradation as cosubstrate of ligninolytic peroxidases (Kirk and Farrell, 1987). AAO is an extracellular FAD-containing enzyme (Guillen *et al.*, 1990) that, in collaboration with myceliar aryl-alcohol dehydrogenases participates in fungal degradation of lignin, a process of high ecological and biotechnological relevance, by providing the hydrogen peroxide required by ligninolytic peroxidases (Ander and Marzullo, 1997) (Figure.3.5).

VAO is probably only induced by glucose and not by cellulose. (AAD, Aryl alcohol dehydrogenase). Aryl alcohol; Aryl aldehyde; Lignin derived radicals or quinones and their reduced forms; Aryl alcohol radical cation; Metal chelating agents (e.g. oxalic acid). In *Pleurotus* species, this peroxide is generated in the redox cycling of *p*-anisaldehyde, an extracellular fungal aromatic metabolite (Gutierrez *et al.*, 1994; Guillen *et al.*, 1990). Fungal high redox-potential peroxidases catalyze the oxidative degradation of lignin by this extracellular peroxide (Ruiz *et al.*, 2006). Since lignin degradation is an extracellular process, extracellular AAO reported for the first time in *P. chrysosporium* and *Trametes versicolor*, respectively (Farmer *et al.*, 1960; Asada et al., 1995), seem to be the main H_2O_2-generating enzymes involved in lignin degradation. *Pleurotus* species as belonging to a special laccase-AAO group, thus recognizing a role for the aryl alcohol oxidases in lignin degradation.

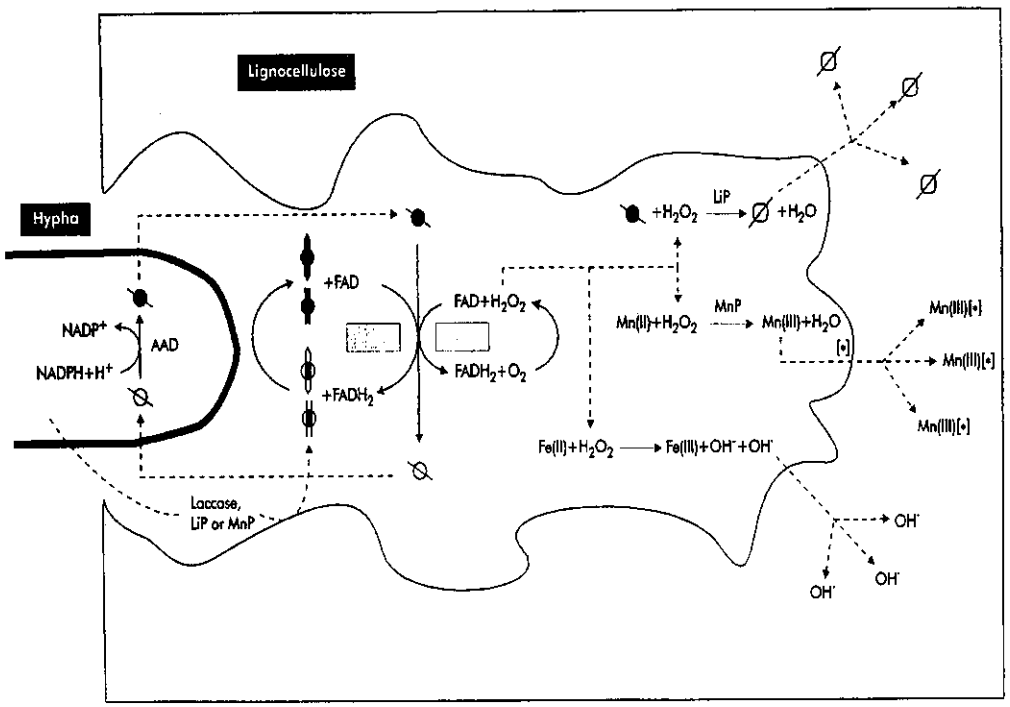


Figure. 3.5: Interaction of VAO with ligninolytic enzymes produced by fungal hyphae during lignocellulose degradation

3.7.2 Degradation of Phenolics Aromatic Pollutants

Peroxidase catalyzes a variety of oxidation reactions. Oxidases are capable of oxidizing a variety of xenobiotic compounds including polycyclic aromatic hydrocarbons (PAH), polychlorinated phenols, nitroaromatics, and azo dyes. *Pleurotus* species are able to degrade PAH and chlorinated biphenyls. During degradation of 2,4-DCP in liquid cultures, similar activity levels were produced by *P. eryngii, P. ostreatus* and *P. sajor-caju* (400–700 mU/ml peroxidase, 10–20 mU/ml AAO and 10–30 mU laccase). Although *P. pulmonarius* produced similar levels of peroxidase and laccase, this fungus secreted very high levels of AAO (reaching 1200 mU/ml after 24 h incubation). During degradation of benzo pyrene by these fungi, which required several days, the three enzymatic activities were also detected in the four cultures (Rodriguez *et al.*, 2004).

3.7.3 Decolourization of Textile Dyes

Purified veratryl alcohol oxidase (VAO) from *Comamonas sp.* UVS and its role in decolorization of structurally different groups such as azo, thiazin, heterocyclic, and polymeric dyes were studied by Jadhav *et al.*, 2009. The purified enzyme was able to decolorize textile dyes, Red HE7B (57.5%) and Direct Blue GLL (51.09%) within 15 h at 40 µg/mL concentration. Eichlerova *et al.*, 2006 explains *Pleurotus* species able to decolorize both RBBR (85%) and Orange G dye (91%) produced mostly low amounts of MnP, higher production of Laccase and AAO but they did not reveal any correlation between the overall H_2O_2 production and the decolorization properties of *Pleurotus* species. They found a very high production of H_2O_2 in the species with no (*P. citrinopileatus*) or very low (*P. dryinus*) decolorization ability and also in the species with a high decolorization capacity *(P. ostreatus*). This suggests the synergistic action of different enzyme types in the decolorization process. It is well known that also different mediators, radicals, H_2O_2 etc. take part in decolorization processes [Tanaka *et al.*, 1999]. The strains producing H_2O_2 at a concentration of 1.0–1.5 µM exhibited the most efficient decolorization; higher or lower H_2O_2 concentration reduced this ability.

3.7.4 Other Applications

Aryl alcohol oxidase (AAO) has other important applications and potential applications besides lignin degradation. Some of the applications are in industrial processes, such as bioethanol production and paper pulp manufacturing, where the removal of lignin is a previous and essential step to use the cellulose present in plant biomass as a source for renewable fuels, chemicals and materials (Ferriera *et al.*, 2009).

3.8 Predicted Molecular Structure of AAO

The AAO molecular model [Varela *et al.*, 2000] has an FAD-binding domain formed by two main β-sheets and a variable number of α-helices, whose structure is conserved in the members of the GMC family whose structure has been solved [Ferraria *et al.*, 2006], and a substrate- binding domain including a large β-sheet and several α-helices, whose general structure and architecture of the catalytic site is more variable, in agreement with the different types of substrate of GMC oxidoreductases [Kiess *et al.*,

1998]. The enzymes are also specified veratryl alcohol oxidase (VAO) in the literature. It is a flavoenzyme in the (glucose methanol choline oxidase) GMC oxidoreductase family.

Figure.3.6: Predicted molecular structure of AAO

This family, named after the initial members glucose oxidase, methanol oxidase and choline dehydrogenase [Coverner, 1992], currently consists of more than 500 protein sequences. AAO shares the highest sequence identity (28% identity) with glucose oxidase from *A. niger* [Frederick *et al.*, 1990], and some hypothetical proteins such as choline dehydrogenase from *Vibrio vulnificus* (up to 34% identity) [Chen *et al.*, 2003]

3.9 Reported Molecular Model of AAO

AAO was cloned for the first time in *Pleurotus eryngii* (Varela *et al.*, 1999), a fungus of biotechnological interest because of its ability to degrade lignin selectively (Martinez *et al.*, 1994). The AAO amino-acid sequence revealed moderate homology with glucose oxidase from *Aspergillus niger* (Frederick *et al.*, 1990), a flavoenzyme in the glucose methanol- choline oxidases (GMC) oxidoreductase family. The reported molecular model of AAO, based on the glucose oxidase crystal structure (Hecht *et al.*,

1993), showed common features with the overall structural topology of bacterial choline oxidase and almond hydroxynitrile lyase (a lyase with oxidoreductase structure), as well as with other members of the GMC family; such as the extracellular flavoenzymes pyranose-2 oxidase and cellobiose dehydrogenase from white-rot basidiomycetes, and bacterial cholesterol oxidase (Quaye *et al.*, 2007) (Figure.3.6).

3.10 Three Phase Partitioning

In the past, various methods were used to purify AAO by conventional or special chromatographic methods such as anion exchange column (DEAE-Toyopearl 650 M & Butyl-Toyopearl 650 M) and gel filtration (Sephacryl S-100). These separation techniques are expensive and require pretreatment of samples [Okamoto and Yannase 2002]. Hence, the trend has been to develop protocols with limited number of steps for protein purification to make the process economical. The problems with conventional purification methods include small feed volumes at a time and high cost of upto 60–90% of the downstream processing. Three phase partitioning (TPP) provides an alternative, efficient, economical and scalable technique for purification of proteins, carbohydrate polymers and extraction of oils (Sharma and Gupta 2001; Dennison and Lovrein 1997). It has been used both for upstream and downstream protein purification processes and has same times been used as a one-step purification protocol (Sharma and Gupta 2001). Table.3.5 listed out that purification and extraction of proteins, enzymes, oils and carbohydrate polymers using three phase partitioning.

TPP is a non-chromatographic separation technique, which need to be tested for its wider range of enzymes for its practical applications. TPP can be successfully utilized for purification of biomolecules provided that the following conditions are satisfied: (i) Phase constituents (solvents and salts) in the concentration used, must not be toxic or inhibitory to the biomolecules, (ii) Biomolecules must partition wholly or predominantly into one of the phases, and (iii) The system must be cost effective.

3.10.1 Preliminary Work in Developing TPP

Preliminary work in developing TPP to first determine if it may work, then be optimized, generally should include the following steps, after total protein and bioactivity (Enzyme, Hormone, etc.) assays are calibrated:

(i) Protecting agents, especially sulfate in various concentrations should be added first. (At some point of course, protein precipitates may form without *t*-butanol, which equates to conventional salting out.) Roughly half to three fourths as much ammonium sulphate as added.

(ii) Adjust pH, using buffers adequate to over come the buffering capacity of the sample. If the iso ionic point of a sought for protein is known, first investigate a pH range 2 to 4 units below the isoionic point. In case of crudes containing proteins with divergent isoionic points, one may try four or five different pHs, ranging between 3 and 7, using bioactivity assays (eg., enzymatic activities) to monitor the course of TPP precipitation of the sought-for protein. The aqueous (lower) layer should also be scanned, for situations where proteins first precipitated are unwanted or foreign proteins with the sought-for protein are pushed into the aqueous layer.

(iii) There is not a clear need to use cold temperatures for TPP, where temperature variation has been investigated. It may be strategic, however, to experiment with low and high temperatures for unknown systems.

(iv) The protective capacity of sulfate and of *t*-butanol may be illustrated by inverting the above-recommended sequence of addition with respect to pH. If proteins are damaged by moderate or severe pH conditions, e.g., acid pHs below 4 or 3.5, exposure before addition of sulfate or of *t*-butanol may denature them. They are likely to partition out in a denatured state but may lose activity.

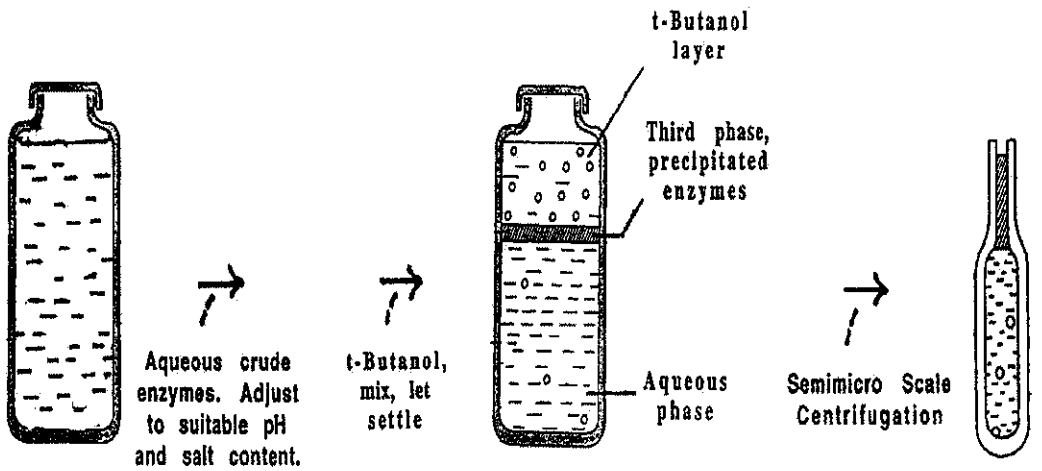


Fig.3.7: TPP on a Semimicro scale

Figure.3.7 summarizes three main points regarding sulfate's function not only in TPP but also in other protein separation–isolation uses.

(i) Sulfate ion binding to proteins tends to tighten protein conformation, which is clearly helpful in sulfate's long established, general ability to protect proteins, used in large enough (salting out) concentrations.

(ii) The pH dependence (often a rather sharp dependence) of sulfate-promoted precipitation and coprecipitation of proteins in relation to each protein's isoionic point, i.e., to the numbers of positive charges borne on proteins, indicates that sulfate binding is largely electrostatic in nature.

(iii) Sulfate binding to discrete sites of proteins, when it occurs, usually is completed when about 0.1 to 0.2 sulfate concentrations are reached. However, 0.1 to 0.2 M sulfate concentrations are well below concentrations where kosmotropy, exclusion, cavity surface tension, etc., operate. They usually do not come into play until 0.4 to perhaps 3 or 4 M concentrations are reached. By the time overt precipitation is reached, the "precipitates" frequently are actually coprecipitates of protein with bound sulfate ions.

Table.3.5: Purification of Biomolecules Using Three Phase Partitioning

Enzyme	Source	References
Cellulase	*Trichoderma ressei*	Odegaard *et al.*, 1984
Peroxidase	Horse radish, Ipomea leaves	Lovrien *et al.*, 1987; Narayan *et al.*, 2008
Pepsin	Procine stomach	Lovrien *et al.*, 1987
Protease	*Bacillus subtilis*	Lovrien *et al.*, 1987
α-Amylase	Barley malt, *Aspergillus oryzae, Bacillus amyloliquefaciencs, B. licheniformis.*	Lovrien *et al.*, 1987
Invertase	*Saccharomyces cervisiae*	Lovrien *et al.*, 1987
β-Galactosidase	*Aspergillus oryzae*, Almond	Lovrien *et al.*, 1987
Amyloglucosidase	*Rhizopus* sp	Lovrien *et al.*, 1987
Lipase	*Candida cylindracea, C. rugosa*	Lovrien *et al.*, 1987
B/B proteaseinhibitor	Soyabean	Lovrien *et al.*, 1987
Cathespin D,L, S	Bovine spleen, Sheep liver,	Pike and Dennison, 1989;
Mannitol dehydrogenase	*Aspergillus parasiticus*	Niehaus and Dilts, 1982
Carbonic anhydrase, Catalase, Superoxide dismutase	Human erythrocytes	Pol *et al.*, 1990
Trypanoapin – tb protease	Trypanosomes	Troeberg *et al.*, 1996
Trypsin inhibitor	Soyaflour (defatted)	Roy and Gupta, 2002
Alkaline phosphatase	Chicken intestine	Sharma *et al.*, 2000
Sodium alginate	Commercial source	Sharma and Gupta, 2001a
Chitosan	Commercial source	Sharma *et al.*, 2003
Green fluorescent protein	*E.coli*	Penna *et al.*, 2003

Pectinase	Tomato, Pectinex Ultra SP-L	Sharma and Gupta, 2001b
Almond, apricot, rice bran oil	Almond, apricot seeds and rice bran powder	Sharma and Gupta, 2004
Phospholipase	*Dacus carota*	Sharma and Gupta, 2001c
Polygalacturonase	*Aspergillus oryzae*	Dogan and Tari, 2008
Protease/amylase inhibitor	Wheat germ	Sharma and gupta, 2001d
Soy oil	Soyabean	Sharma *et al.*, 2002
Jatropha oil	Jatropha seeds	Shah *et al.*, 2004
Xylanase	*Aspergillus niger*	Sharma and gupta, 2002

3.10.2 Role of Ammonium Sulfate in TPP

The sulfate ion has been viewed in five principal ways, concerning how it salts out proteins: ionic strength effects, kosmotropy, cavity surface tension enhancement, osmotic stressor (dehydration), and as an exclusion crowding agent. Possibly all five such factors, or mixtures of them, operate in various proportions, depending on sulfate concentration and the protein molecule charge (denoted Z_H^+) in response to pH. There is a sixth matter which has been more slowly emerging, but has equal weight now. Namely, that the divalent sulfate anion SO_4^{2-} tends to bind into a few cationic sites of many proteins when proteins have a net positive charge Z_H^+. It was observed that, other salts cannot approach ammonium sulfate performance in TPP (pulling and pushing effect combined by *t*-butanol) [Dennison and Loverein, 1997]. In this TPP based protocol for protein separation, *t*-butanol has been found to consistently perform better than all other organic solvents [Dennison and Loverein, 1997; Sharma and Gupta. 2000]. It is believed that because of its size and branched structure, *t*-butanol does not easily permeate inside the folded protein molecules and hence does not cause denaturation [Dennison *et al.* 2000]. Temperature is also an important parameter in the partitioning of proteins.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Chemicals and Fungi

Guaiacol, p-anisyl and veratryl alcohol were obtained from Himedia, Mumbai, India. Benzyl alcohol was obtained from S.D fine Chemicals, Mumbai India. All other reagents and chemicals used were of analytical grade. The fungal species used for the study are listed in Table 1 and were maintained on potato dextrose agar slants.

4.2 Laccase and AAO Screening Assay

Screening of laccase producing organisms was done on plates using potato dextrose agar (PDA) supplemented with 0.02% guaiacol. Twenty different fungal species were inoculated in sterile petri plates containing the above media and were incubated at 30°C for a period of 7 days. Lignolytic fungi produce laccase that converts guiacol in the medium to reddish brown colour [Budallo *et al.*, 2008]. Cultures showing definite color changes were considered lignolytic. AAO quantitative screening work was done on 250 ml Erlenmeyer flasks containing 80 ml of culture medium (pH 6.3) contains of malt extract 1 %; yeast extract 0.4 %; glucose 0.4 % and 5 mM l-tyrosine as previously reported [Okamoto and Yanase, 2002]. The media was inoculated with seven day old sporulated culture suspension prepared from the cultures grown in petriplates. For growing basidiomycetes in suspension, inoculum was prepared by homogenizing the six day old mycelium obtained from the petriplates. The flasks were then incubated at 30°C for upto 24 days. Culture filtrate was estimated for pH, reducing sugar, protein and AAO activity.

4.3 Induction Studies

The compounds tested as inducers were sterilized by filtration. The AAO producing fungi were cultured for upto 24 days on above mentioned media in the presence of inducers (phenyl alanine and tyrosine (5 mM), veratryl alcohol (7 mM), benzyl alcohol (10 mM) and anisyl alcohol (8 mM)). AAO activity was determined at 3

day intervals, by centrifuging the culture at 10,000 g for 30 min at 4°C and the supernatant was filtered through Whatman No. 1 filter paper and used for the assay.

4.4 Solid State Fermentation

4.4.1 Selection of Solid Substrate

Five different agro industrial wastes like grape seed (GS), groundnut shell (GNS), rice bran (RB), wheat bran (WB) and Sago hampus (SH) were procured from the local market and chopped into small pieces and dried in oven at 60°C to 5% (w/w) moisture content and processed using standard sieve set to obtain mean particle size (40 mm). To determine the amount of reducing sugars released during sterilization, 1 g of each of the different substrates was taken in a 50 ml conical flask with 20 ml distilled water and autoclaved at 121°C for 15 min. After sterilization the clear solution was separated from the solid substrates and the amount of reducing sugars released from the substrates was determined by DNS method.

4.4.2 Production of AAO

The cultures were carried out in petri dishes incubated in a tray chamber (50x50x70 cm) with room temperature (Elinabaum *et al.*, 2002). WB, RB, SH, GS and washed GNS were used as solid substrates. The solid materials were moistened with 10 ml of a nutrient solution containing malt extract 1%; yeast extract 0.4%; glucose 0.4%, 5 mM l-tyrosine and the initial pH of the cultures were 6.3. The appropriate quantity of solids that absorb 10 ml of the nutrient solution without drainage was determined previously for each material, being 5 g for GNS and 5 g for SH. In order to avoid any nutrient limitations when using WB, GS and RB it was moistened with same nutrient solution as the other supports, 10 g solid was moistened with 10 ml solution. For growing basidiomycetes in suspension, inoculum was prepared by homogenizing the six day old mycelium. At selected times duplicate dishes were taken out. 25 ml of buffer was added to complete 5 g of solid residues, the suspensions were stirred and centrifuge at 10000 rpm for 10 min and the pH, reducing sugar, protein and enzyme activities determined in the filtrate.

4.5 Analytical Methods

The culture supernatant obtained after centrifugation was used for the following analyses.

4.5.1 pH

The pH of the culture filtrate was measured using pH meter (Elico LI 120). pH of the culture filtrate were adjusted with 0.1N Hydro chloric acid or 0.1N Sodium hydroxide.

4.5.2 Protein Estimation

Protein was determined by Lowry's method (Lowry *et al.*, 1951). The basis of the method was the biuret reaction of proteins with copper under alkaline conditions and the Folin Ciocalteau phosphomolybdic phosphotungstic acid reduction to heteromolybdenum blue by the copper catalyzed oxidation of aminoacids. The protein standard was prepared with Bovine serum albumin.

4.5.3 Reducing Sugar Estimation

This was done by the Dinitrosalicylic acid (DNS) method (Miller, 1959). Reducing sugar was estimated using a standard graph prepared with glucose. On reaction with glucose 3, 5 – DNS gets reduced to 3 - amino 5 - nitro salicylic acid while the sugar gets oxidized to gluconic acid.

4.5.4 Laccase Assay

Laccase activity was measured based on guaiacol oxidation (Buddolla *et al.*, 2008). Enzyme activity was assayed at 30°C by using 10 mM guaiacol in 100 mM acetate buffer containing 10% (v/v) acetone. The changes in absorbance of the reaction mixtures containing guaiacol were monitored at 460 nm for 5 min of incubation. The enzyme activities were calculated using an extinction coefficient of 6,740 / M.cm and expressed as U/ml.

4.5.5 AAO Assay

AAO activity was assayed spectrophotometrically (Elico SL159) using veratryl alcohol as a substrate. The reaction mixture of the standard assay contained 1 mM veratryl alcohol, 50 mM potassium phosphate buffer (pH 6.0), and a suitable enzyme in a total volume of 1.0 ml. Oxidation of the substrate at 30°C was monitored based on an absorbance increase at 310 nm resulting from the formation of veratraldehyde (ε_{310}-9300 / M.cm). One unit of the enzyme activity was defined as the amount of enzyme required to produce 1µmol of veratraldehyde per minute under the assay conditions (Okamoto and Yannase, 2002).

$$\textbf{1 Unit} = \Delta E/\Delta t \times V / \epsilon \times v \times t$$

$\Delta E/\Delta t$ = Increase in absorbance per minute; V – Volume of aliquot solution; ϵ - Extinction coefficient; v – Volume of sample; t - Time in minutes

4.5.6 Protease Assay

Protease assay was carried out using casein as substrate. Two eppendorf tubes marked 'test' and 'control' were taken. 2.0 ml of buffer was added to each of the tubes. 1.0 ml of substrate was added to both the tubes. 1.0 ml of tri chloro acetic acid (TCA) solution was added to the tube labeled 'control'. 1.0 ml of enzyme was added to both the tubes. The tubes were then incubated at 50°C for 20 min. 1.0 ml of TCA solution was added to the tube labeled 'test'. The tubes were then centrifuged at 10,000 rpm for 10 min. Then 0.05 ml of the supernatant was transferred into two test tubes labeled 'control' and 'test'. The volume was then made upto 1.0 ml with distilled water. 5.0 ml of freshly prepared alkaline copper reagent was added to the test tubes. 0.5 ml of Folins Ciocalteau reagent (FCR) was added to the test tubes and the contents. The test tubes were then incubated at room temperature for 30 min. The absorbance was measured at 660 nm. The graph was plotted using tyrosine (10-60 µg) as standard. The protease activity was calculated using the following equation.

$$\textbf{Activity (U/ml)} = \frac{\textbf{Amount of tyrosine released} \times \textbf{Volume of stopped reaction}}{\textbf{Volume of enzyme} \times \textbf{Reaction time} \times \textbf{Total volume}}$$

4.6 Characterization of Crude AAO

The cell free extract of the fungus was taken as the crude AAO preparation. While studying each parameter, the other reaction conditions were kept constant.

4.6.1 Effect of Veratryl Alcohol Concentration on AAO activity

The enzyme activity was assayed at different veratryl alcohol concentrations ranging from 0.1 mM to 2 mM at pH 6.0 and Temperature 30°C.

4.6.2 Effect of pH on Activity and Stability of the Crude AAO

The effect of pH on the activity of the crude AAO was determined by assaying the enzyme activity at different pH values ranging from 2.0 to 10.0 using 0.01M of the following buffer systems: Acetate (2.0, 3.0, 4.0, and 5.0), Phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 8.0, 9.0, and 10.0) Buffer systems (Atlas *et al.*, 1995). The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage. The pH stability of *Pleurotus* AAO was investigated in the pH range of 3.0–10.0. Therefore, 1 ml of the crude enzyme was mixed with 1ml of the buffer solutions mentioned above and incubated at 30°C for 2 h. Afterwards aliquots of the mixtures were taken to measure the residual AAO activity.

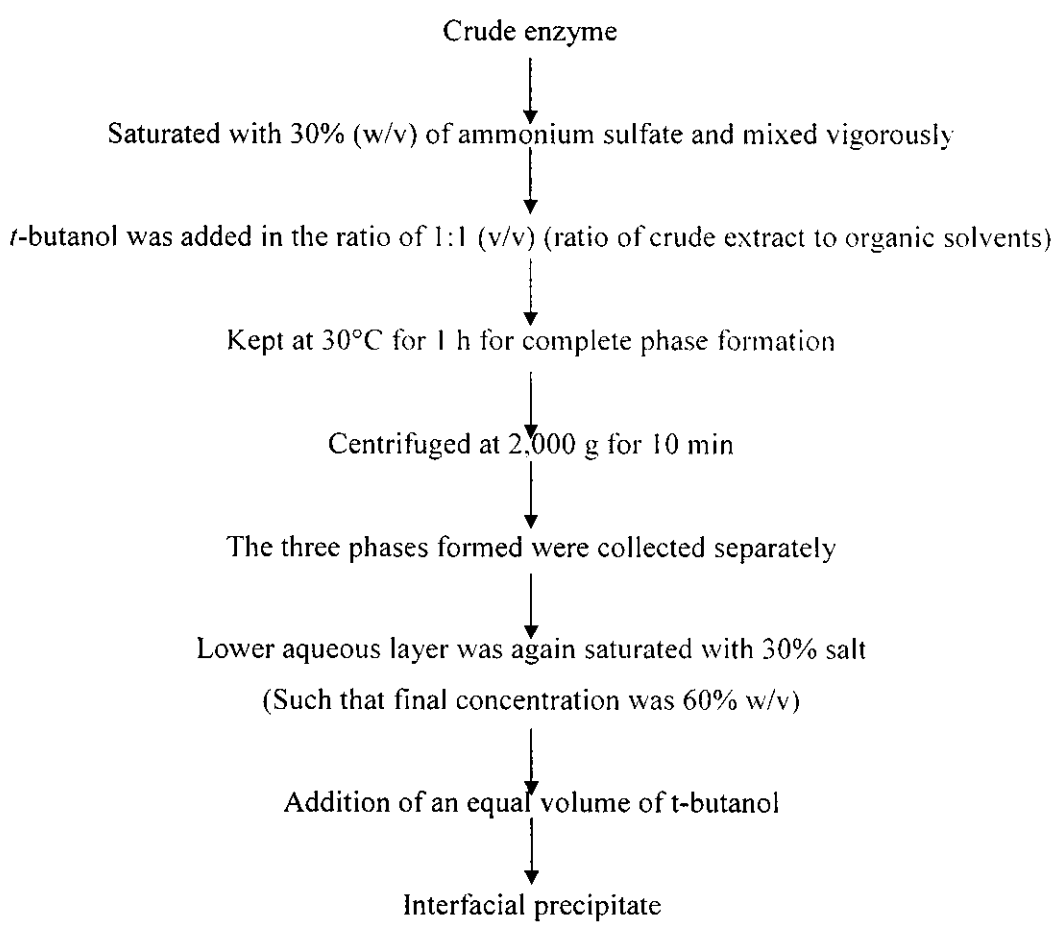
4.6.3 Effect of Temperature on Activity and Stability of the Crude AAO

The effect of temperature on the activity of the crude AAO was determined by performing the standard assay procedure at different temperatures ranging from 25 to 70°C (25, 37, 40, 50, 60, and 70°C). Before the addition of enzymes, veratryl alcohol was preincubated at the respective temperature for 10 min. The relative activities were expressed as the ratio of the AAO activity obtained at certain temperature to the maximum activity obtained at the given temperature range. The thermostability of the crude AAO was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 4 to 70°C (4, 28, 37, 45, 50, 60 and 70 °C) for 60 min.

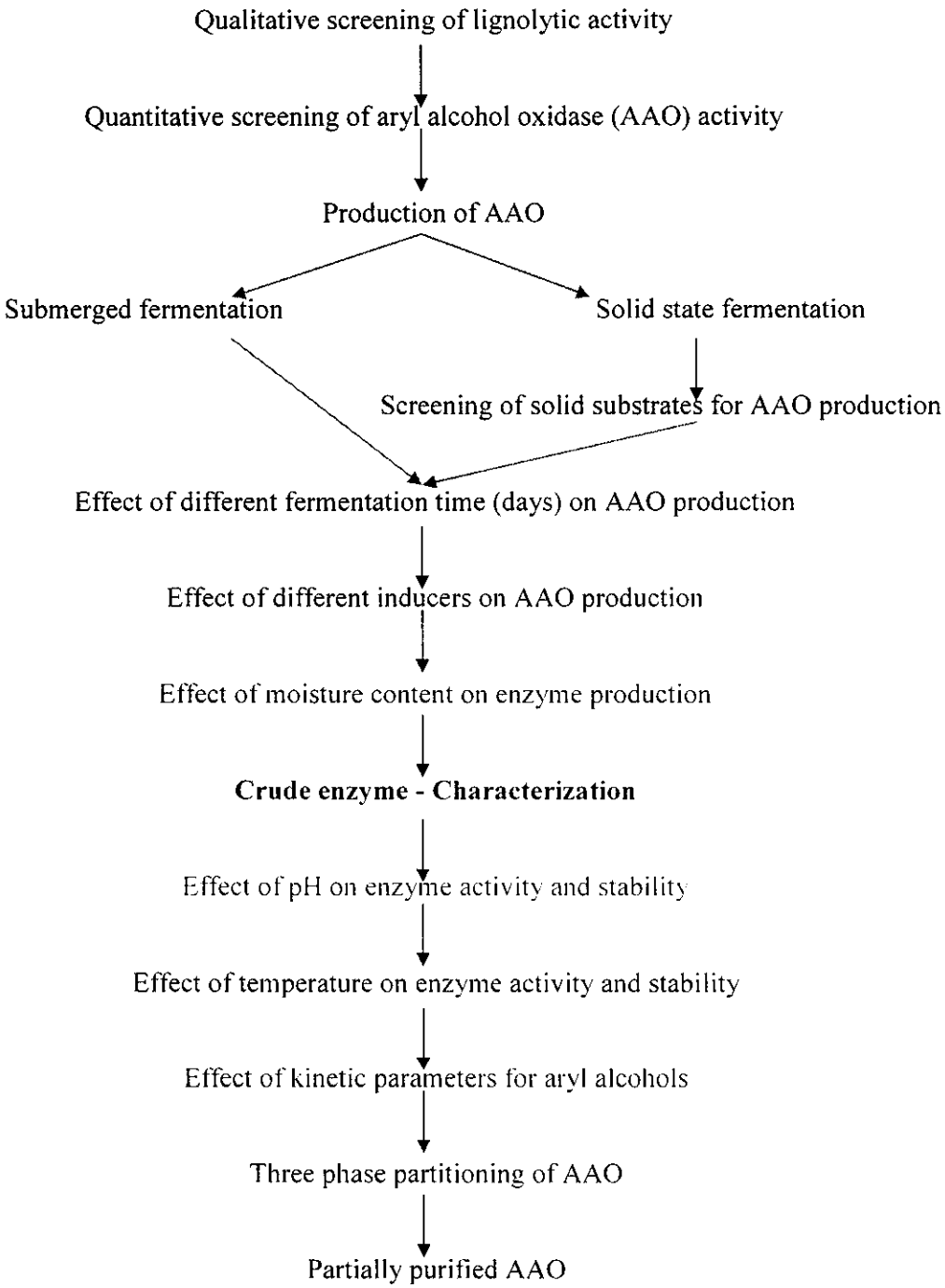
4.7 AAO Purification

4.7.1 Three Phase Partitioning (TPP)

TPP was carried out by the method of Sharma and Gupta (2000). The crude enzyme solutions was saturated with 30% (w/v) of ammonium sulfate and mixed vigorously to and *t*-butanol was added in the ratio of 1:1 (v/v) (ratio of crude extract to organic solvents) and the tubes were kept at 30°C for 1 h for complete phase formation. The tubes were centrifuged at 2,000 g for 10 min and the three phases formed were collected separately. The enzyme is usually precipitated in the middle layer. To enrich the enzyme concentration, lower aqueous layer obtained in the first stage was again saturated with 30% salt (such that final concentration was 60% w/v) followed by addition of an equal volume of t-butanol and the tubes were centrifuged and interfacial precipitate was separated as described previously.



Overview of the present study



RESULTS AND DISCUSSIONS

5. RESULTS AND DISCUSSION

5.1 Screening of AAO Producing Fungi

Primary and secondary screening was carried out for the AAO producing fungi. Initially organisms were screened for lignolytic activity, using guaiacol as indicator compound. Intense reddish brown colour was produced in the medium around the fungal colonies and was taken as positive reaction for the presence of lignolytic enzyme activity as previously reported [Budallo *et al.*, 2008]. Out of twenty fungal species, intense brown colour zone was formed around the colonies of six species *Pleurotus ostreatus, Pleurotus platypus, Fusarium solani, Pleurotus eous, Agaricus bisporus* and *Penicillium chrysogenum* was found to be lignolytic (Table.5.6; Figure.5.8).

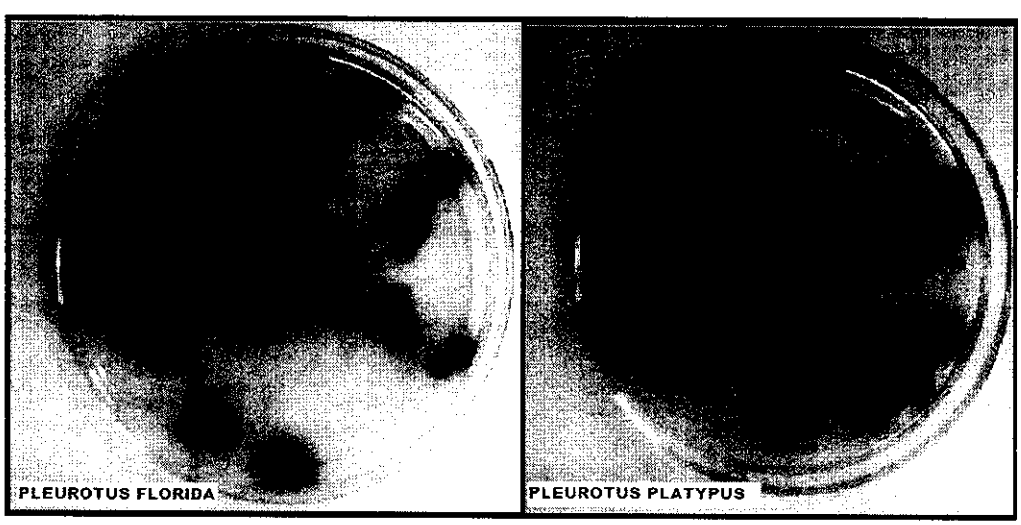


Fig.5.8: Screening for lignolytic activity

These fungal cultures were further screened for AAO production, and only three species viz. *Pleurotus ostreatus, P.eous* and *P.platypus* were found to produce extracellular AAO (Table.5.6). AAO activities of the fungi grown on defined medium at 18 days growth period were found to be as follows: *Pleurotus ostreatus* (40 U/lit), *Pleurotus platypus* (32 U/lit), *Pleurotus eous* (18 U/lit). In similar studies carried out by Eichlerova *et al.*, 2006 screened eight *Pleurotus* species, out of which *P. calyptratus, P. citrinopileatus, P. eryngii, P. ostreatus* and *P. pulmonarius* showed AAO activity.

Table. 5.6: Presence of lignolytic and AAO activity among the different fungal species

S.No	Species	AAO	Lignolytic
1	*Agaricus bisporus* [b]	-	+
2	*Aspergillus niger* MTCC 1344 [c]	-	-
3	*Aspergillus terrus* MTCC 2580 [c]	-	-
4	*Aspergillus flavus* [a]	-	-
5	*Beauveria bassiana* MTCC 6100 [c]	-	-
6	*Beauveria feline* MTCC 6294 [c]	-	-
7	*Fusarium solani* [b]	-	+
8	*Metarhizium anisopilae* MTCC 892 [c]	-	-
9	*Paecilomyces fumosoroseus* MTCC 6292 [c]	-	-
10	*Paecilomyces lilacinus* MTCC 1422 [c]	-	-
11	*Penicillium decumbens* [a]	-	-
12	*Penicillium chrysogenum* [a]	-	+
13	*Penicillium sp* [b]	-	-
14	*Pleurotus eous* [b]	+	+
15	*Pleurotus floridanus* [b]	-	+
16	*Pleurotus ostreatus* [b]	+	+
17	*Pleurotus platypus* [b]	+	+
18	*Trichoderma harzianum* MTCC 936 [c]	-	-
19	*Trichoderma viridie* MTCC 2535 [c]	-	-
20	*Verticillium lecani* [b]	-	-

+ - Present; - - Absent.

*The fungal isolate were obtained and maintained as pure cultures from the following culture collection centers: [a] PSGMC = PSG Medical College, Coimbatore, India; [b] TNAU = Tamil Nadu Agriculture University, Coimbatore, India; [c] MDBL = Manidharma biotech private limited, Chennai, India.

In the earlier reports, Southern blot method was applied for the screening of AAO gene in 30 basidiomycetes. However only *P. floridanus, P. sajorcaju, P. eryngii, P. ostreatus* and *P. pulmonarius* and *Bjerkendra adjusta*, showed positive results [Varela *et al.*, 2000]. They also mentioned the occurrence of AAO gene in *P. floridanus*, but we failed to detect AAO production in this fungus. This may be due to the fact that the composition of the culture medium is known to substantially affect the production of this enzyme.

5.2 Induction of AAO activity in *Pleurotus ostreatus*

The three AAO producing *Pleurotus* species were grown separately on defined medium containing one of the following inducers: Veratryl alcohol, Benzyl alcohol, Anisyl alcohol, Tyrosine and Phenyl alanine. Compared with the control, inducers increased the AAO production in *Pleurotus ostreatus* in the following order: tyrosine > veratryl alcohol > benzyl alcohol > phenyl alanine > p-anisyl alcohol. In the presence of tyrosine, maximum AAO activity was obtained on the 18th day in *Pleurotus ostreatus, P. eous* and *P. platypus*, 220 U/lit, 83 U/lit and 208 U/lit respectively (Table. 5.7, 5.8, 5.9 and Figure. 5.9, 5.10, 5.11). Veratryl alcohol enhanced AAO production to the maximum and also shortened the time for AAO production. Effect of different inducers on the AAO production in three *Pleurotus sp* is given in Table.5.10 and Figure.5.12. The findings emphasize the importance of tyrosine and veratryl alcohol in the AAO production and *Pleurotus ostreatus* consistently showed maximal AAO activity.

Other studies have also came to similar conclusions in the AAO production (Okamoto and Yanase, 2002; Asada *et al.*, 1995). In *Pleurotus ostreatus*, all inducers enhance AAO production, whereas benzyl and anisyl alcohol does not induce AAO production in *P.eous* and *P.platypus*. In aromatic amino acids, tyrosine induces AAO production in all *Pleurotus sp*, whereas phenyl alanine induces effectively in *Pleurotus platypus*. Among the aryl alcohols, veratryl alcohol is the promising inducer for enzyme production. Combination of tyrosine and veratryl alcohol yielded maximum amount of AAO from *Pleurotus ostreatus* (289 U/lit), *Pleurotus platypus* (241 U/lit), *Pleurotus eous* (103 U/lit). In the biosynthetic pathway of veratryl alcohol from tyrosine, final product

was veratryl alcohol, so more accumulation of veratryl alcohol in the fermentation medium results in high AAO production.

Table.5.7: Effect of different inducers during growth on enzyme production in *P. ostreatus.*

S.no	Inducer	Incubation time (Days)							
		3	6	9	12	15	18	21	24
1	**Control**	ND	ND	ND	21	38	40	33	6
2	**Tyrosine (5 mM)**	ND	ND	ND	20	80	220	137	128
3	**Phenyl alanine (5 mM)**	ND	ND	ND	12	59	76	51	24
4	**Veratryl alcohol (7 mM)**	ND	26	49	117	156	140	121	100
5	**Benzyl alcohol (10 mM)**	ND	ND	30	51	93	113	98	65
6	**Anisyl alcohol (8 mM)**	ND	ND	13	27	71	79	28	12
7	**Tyr (5 mM)+Ver.alc (7mM)**	ND	31	51	173	289	281	176	81

Control cultures did not contain any added inducers. ND – not detectable.

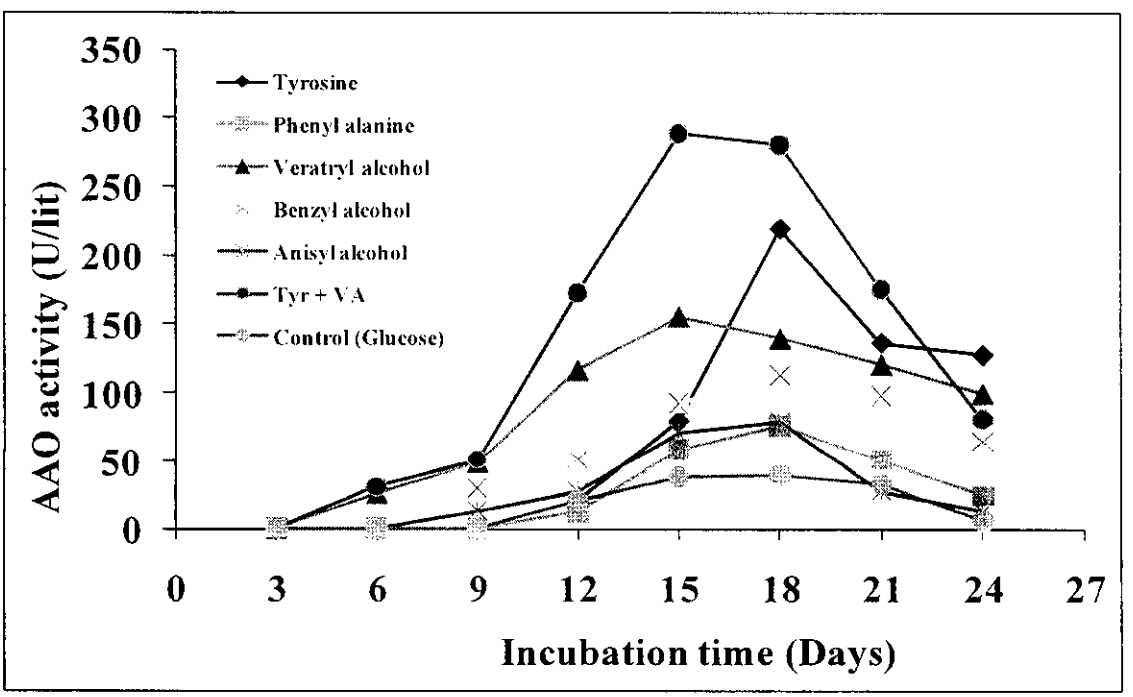


Fig.5.9: Effect of different inducers during growth on enzyme production in *P.ostreatus*

Table.5.8: Effect of different inducers during growth on enzyme production in *P.eous*

S.no	Inducer	Incubation time (Days)							
		3	6	9	12	15	18	21	24
1	**Control**	ND	ND	ND	ND	13	**18**	17	14
2	**Tyrosine (5 mM)**	ND	ND	6	39	80	**83**	29	14
3	**Phenyl alanine (5 mM)**	ND	ND	ND	11	31	**39**	30	21
4	**Veratryl alcohol (7 mM)**	ND	19	51	67	**89**	**89**	55	38
5	**Benzyl alcohol (10 mM)**	ND	ND	27	39	57	62	67	22
6	**Anisyl alcohol (8 mM)**	ND	ND	ND	ND	19	20	**22**	19
7	**Tyr (5 mM)+Ver.alc (7mM**	ND	11	49	68	97		83	69

Control cultures did not contain any added inducers. ND – not detectable.

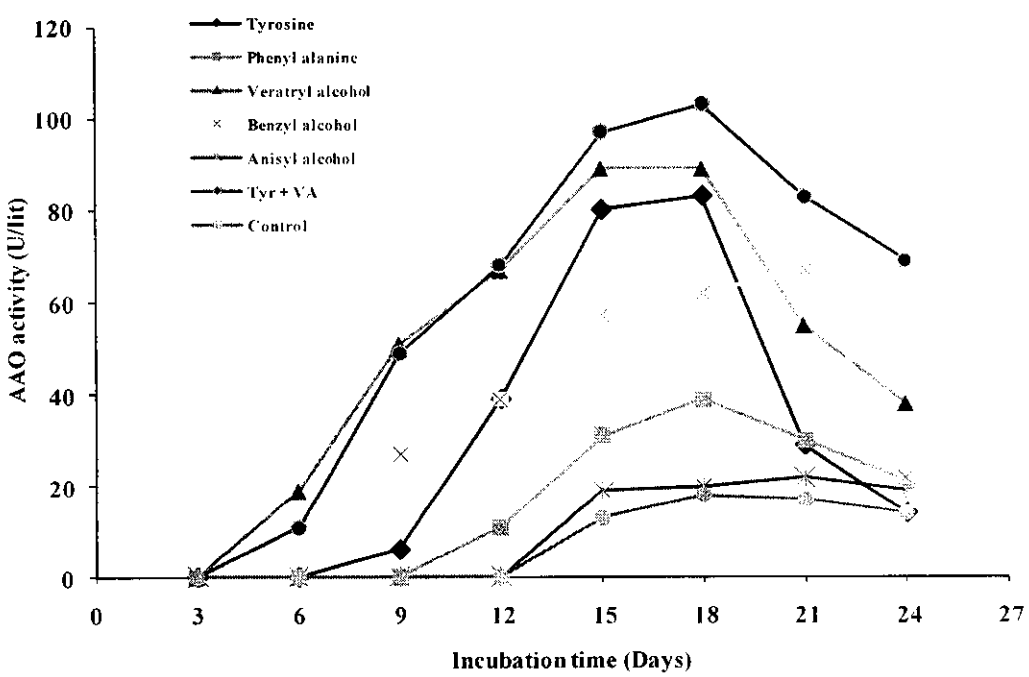


Fig.5.10: Effect of different inducers during growth on enzyme production in *P. eous*

Table.5.9: Effect of different inducers during growth on enzyme production in *P. platypus*

S.no	Inducer	Incubation time (Days)							
		3	6	9	12	15	18	21	24
1	**Control**	ND	ND	3	17	29	32	29	19
2	**Tyrosine (5 mM)**	ND	89	157	189	206	**208**	191	165
3	**Phenyl alanine (5 mM)**	ND	17	134	150	173	**184**	166	140
4	**Veratryl alcohol (7 mM)**	6	38	67	77	93	**97**	90	88
5	**Benzyl alcohol (10 mM)**	ND	ND	36	59	73	81	**86**	74
6	**Anisyl alcohol (8 mM)**	ND	ND	14	39	57	**60**	51	22
7	**Tyr (5 mM)+Ver.alc (7 mM)**	12	49	107	202	226	**240**	212	200

Control cultures did not contain any added inducers. ND – not detectable.

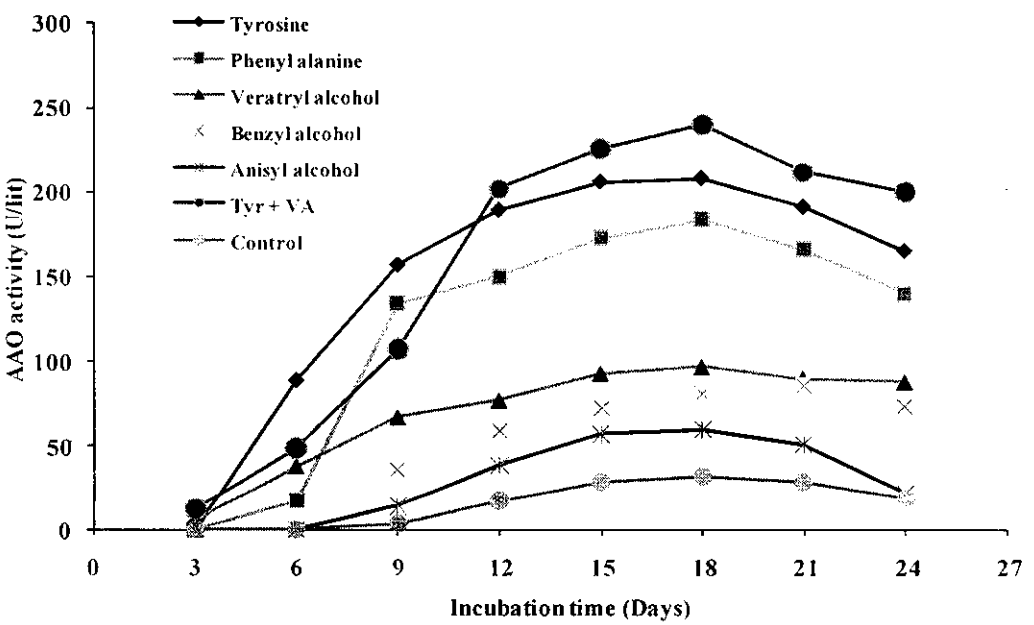


Fig.5.11. Effect of different inducers during growth on enzyme production in *P. platypus*.

Table.5.10: Comparative study of different inducers in production of *Pleurotus sp*

S.no	Inducer	*P.eous*	*P. ostreatus*	*P. platypus*
1	**Control**	18	40	32
2	**Tyrosine (5 mM)**	83	220	208
3	**Phenyl alanine (5 mM)**	39	76	184
4	**Veratryl alcohol (7 mM)**	80	156	97
5	**Benzyl alcohol (10 mM)**	62	113	86
6	**Anisyl alcohol (8 mM)**	20	79	60
7	**Tyr (5 mM)+Ver. Alc (7 mM)**	103	281	240

Control cultures did not contain any added inducers.

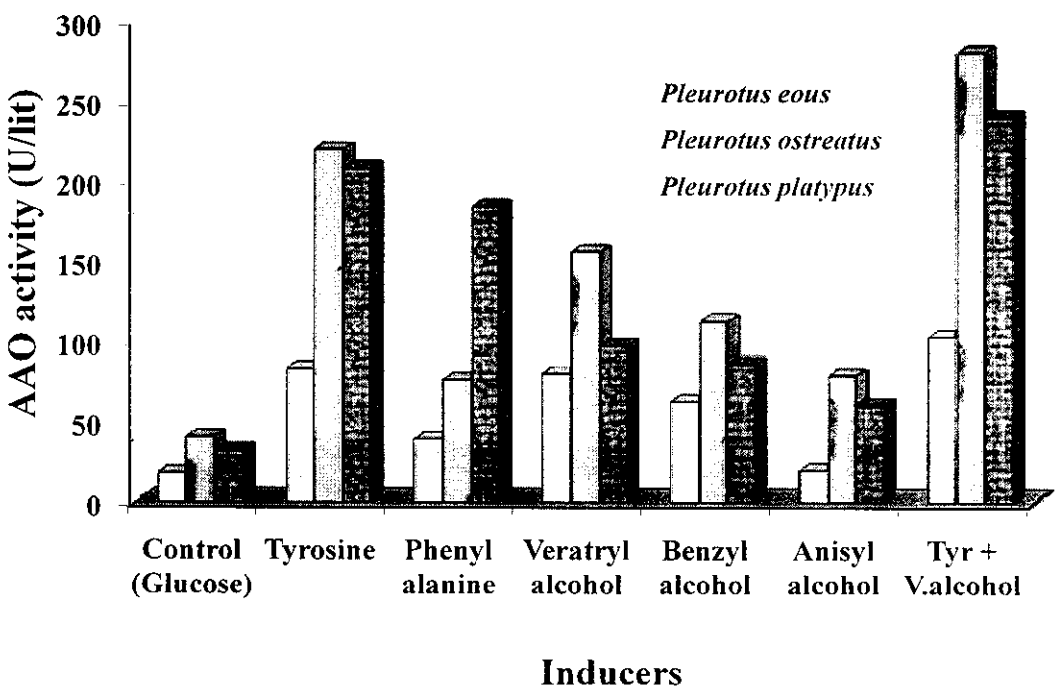


Fig.5.12: AAO enzyme activity in different *Pleurotus species*

In *P. pulmonaris*, nitrogen was the limiting nutrient in the glucose medium, and AAO activity occurred in the extracellular medium, attaining nearly 500 U/lit after 9 days (Gutiérrez *et al.*, 1994). Many lignolytic basidiomycetes generally require long incubation time, 15-20 days or even more to complete their growth and to reach their idiophase [Pal *et al.*, 1980]. Studies on the production of AAO by *Pleurotus sp* were conceded with the same culture conditions used for AAO by *Pleurotus ostreatus* K 16-2 [Okamoto and Yanase, 2002].

Elicitors reported so for AAO production are vanniIyl alcohol, veratryl alcohol [Asada *et al.*, 1995], benzyl alcohol [Asada *et al.*, 1995; Regaldo *et al.*, 1999; Lapadatescu and Bonnarme, 1999], tyrosine [Okamoto and Yanase, 2002], phenyl alanine [Lapadatescu and Bonnarme, 1999], kraft lignin [Regaldo *et al.*, 1999], straw alkali lignin and wheat straw [Gutiérrez *et al.*, 1994]. Veratryl alcohol could be used as inducer in the production of intracellular AAO from *P. chryosporium*; however they reported that vannilyl alcohol was better inducer than veratryl alcohol [Asada *et al.*, 1995]. The likely biosynthetic pathway of veratryl alcohol in *P. chryosporium* as follows: phenylalanine - cinnamate - benzaldehyde – veratryl alcohol.

5.3 Solid State Fermentation

5.3.1 Enzyme Production in SSF Using Various Substrates

Agricultural byproducts rich in cellulosic biomass can be exploited as cheap raw material for the production of industrially important enzymes and chemicals. In present study, various inexpensive agro-residues were tried as substrates for AAO production by *Pleurotus ostreatus* in SSF. The enzyme activity produced at several cultivation times using tyrosine as inducer. Using the solid/liquid ratio corresponding to the minimum amount of solid that retained nutrient solution without drainage, no appreciable liquid loss or gain was observed during the cultivations under water saturated air (Elinbaum *et al.*, 2002). In order to compare the results, the activity of AAO produced on different solid materials was expressed as units per gm of substrate (Figure.5.13). Among the inexpensive and abundantly available lignocellulosic substrates tested, grape seed resulted in maximum yield of AAO (1426 U/kg solid substrate) as compared to other

substrates viz. rice bran, wheat bran, groundnut shell, and sago hampus after 144 h of incubation at 28°C (Table.5.11; Figure.5.14). It can be seen that the titres obtained when using sago hampus were appreciably greater than those from wheat bran. Very low enzyme production was produced from rice bran and groundnut shell. Few reports are available on the production of AAO in SSF by fungi and the enzyme yield was lower in these fungi as compared to *Pleurotus ostreatus*.

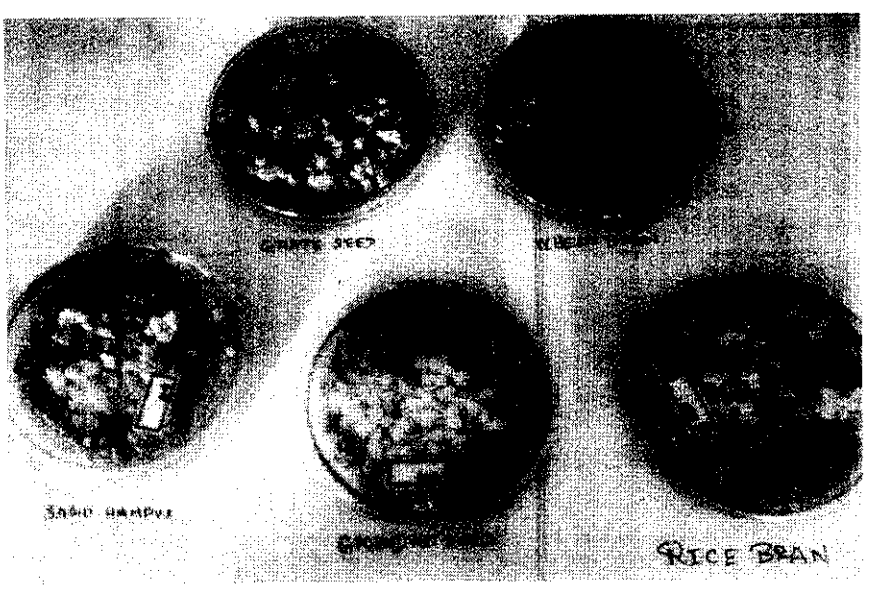


Fig.5.13: Solid state fermentation of AAO on various substrates

Apart from AAO, laccase and protease also produced in the substrates. Wheat bran, groundnut shell and rice bran supports high titer of protease, hence sago hampus does not support the enzyme production. Grape seed, a high AAO producing substrate was produce low titer of protease (110 U/kg) (Table.5.12; Figure.5.15). The enzyme production increased with time of incubation reaching a maximum after 144 h but decreased thereafter for grape seed. Laccase activity was present in the all substrates. maximum activity was produced in grape seed, followed by groundnut shell, wheat bran, sago hampus and rice bran.

Table.5.11: Effect of different solid substrates during growth on enzyme production in *P. ostreatus*

S.no	Solid substrates	Dry solid (g ml^{-1} nutrient solution)	AAO activity (U/kg)				
			3d	6d	9d	12d	15d
1	Rice bran	1.0	198	**440**	290	78	-
2	Wheat bran	1.0	15	**24**	-	-	-
3	Grape seed	1.0	0	**260**	1166	**1426**	1240
4	Groundnut shell	0.5	18	**76**	214	287	326
5	Sago hampus	0.5	224	**1200**	1028	668	542

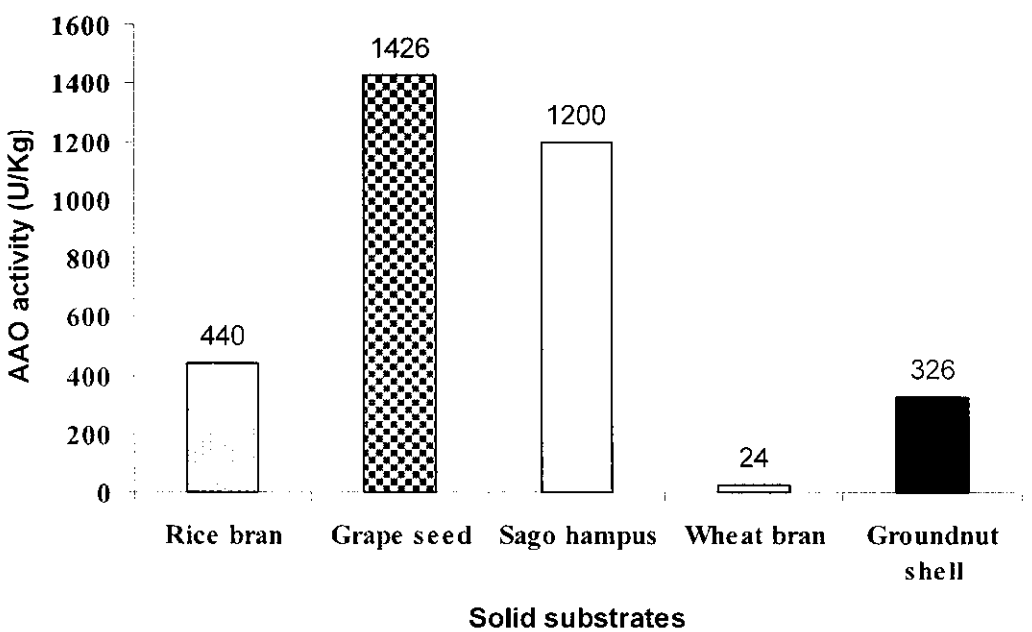


Fig.5.14: Screening of solid substrates for AAO production

Table.5.12: Screening of different parameters during growth on the enzyme production in *Pleurotus ostreatus*

S.no	Parameters	Incubation time (Days)					
		0	3	6	9	12	15
1	AAO (U/kg)	0	0	260	1166	1426	1240
2	Laccase (U/kg)	0	67	314	280	170	0
3	Protease (U/kg)	0	76	110	63	0	0
4	Reducing sugar (µg/ml)	1835	2314	1238	484	168	156
5	Protein (µg/ml)	29	70	463	792	1648	2408
6	pH	6.3	6	5.8	5.81	5.45	5.32

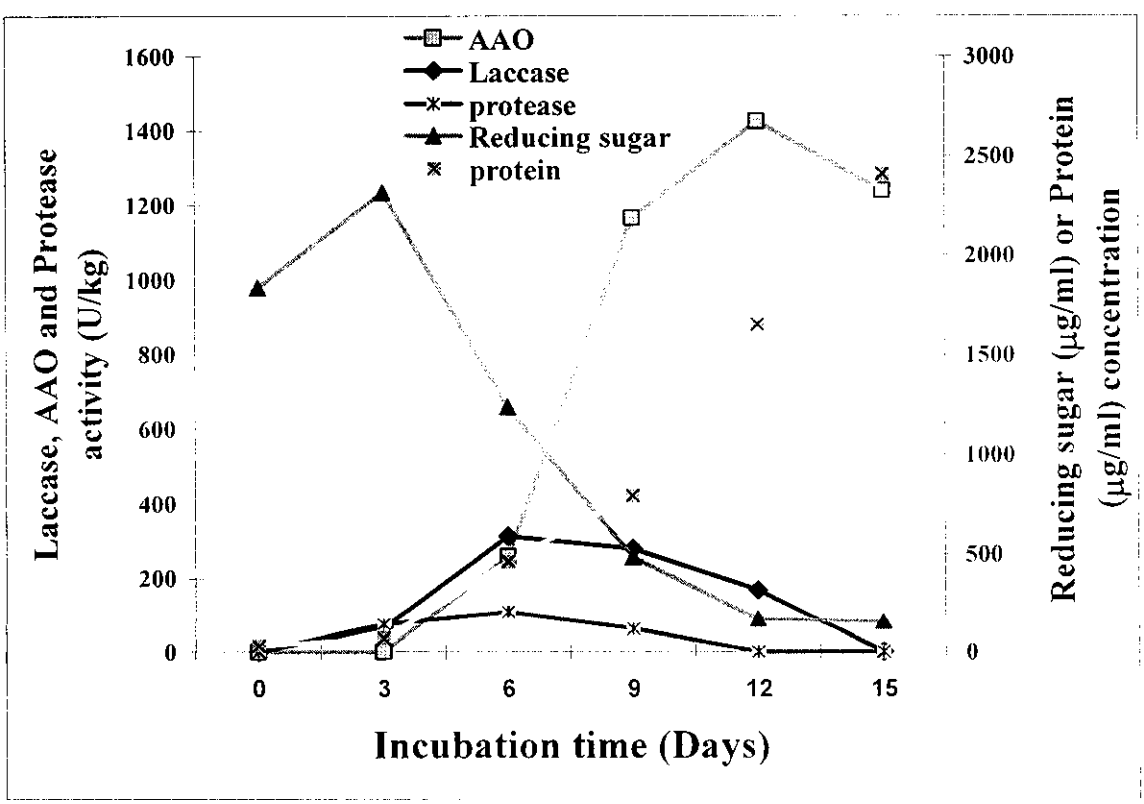


Fig.5.15: Screening of different parameters during growth on the enzyme production in *Pleurotus ostreatus*

5.3.2 AAO Production during Growth

AAO production was monitored up to 360 h of incubation using grape seed, wheat bran, rice bran, sago hampus and groundnut shell as the substrate. The enzyme production increased with time of incubation reaching a maximum after 144 h but decreased thereafter for all substrates except groundnut shell (360 h) and grape seed (288 h). Maximum AAO activity was obtained in sago hampus (1200 U/kg), followed by rice bran (440 U/kg) of substrate respectively after 144 hours. The enzyme yield was maximum (1426 U/kg) for grape seed are after 288 h and minimum (220 U/kg) after 72 h of incubation (Table.5.11). The difference in incubation period may be due to the substrates, difference of organisms, growth rate of microorganism and its enzyme production pattern (Sanghi *et al.*, 2008). An incubation period for maximum AAO production in SSF was 144 h reported for *Bjerkendra adusta* grown on wheat bran and polyurethane foam (Lapadatescu and Bonnarme, 1999). They also mentioned wheat bran as a substrate and benzyl alcohol as an inducer, but we failed to detect AAO production in this substrate. This may be due to the fact that the composition of the substrate and culture medium is known to substantially affect the production of this enzyme in the *Pleurotus ostreatus*. Due to high production of AAO in the substrate groundnut shell was used in further studies. In *Pleurotus eryngii*, an extracellular AAO activity was obtained at the end of the incubation period (40 days), when wheat straw as substrate. They also mentioned absence of straw supplementation: AAO attained its highest level at the end of the incubation period (Barassa *et al.*, 1998).

5.3.3 AAO Production as Influenced by Inducers

The AAO producing *Pleurotus ostreatus* were grown separately on solid substrates containing one of the following inducers: veratryl alcohol, benzyl alcohol, anisyl alcohol, tyrosine and phenyl alanine. In grape seed, compared with the control, inducers increased the AAO production in *Pleurotus ostreatus* in the following order: tyrosine > phenyl alanine > veratryl alcohol > benzyl alcohol (Figure.5.16). p-anisyl alcohol was failed to induce the AAO production in *Pleurotus ostreatus*. In the presence of tyrosine and phenyl alanine, maximum AAO activity was obtained 1460 U/Kg and 1308 U/Kg respectively. Similar to Smf, combination of both tyrosine and veratryl

alcohol induces the enzyme production. Hence the production was similar to tyrosine, so further SSF studies were carried out with tyrosine.

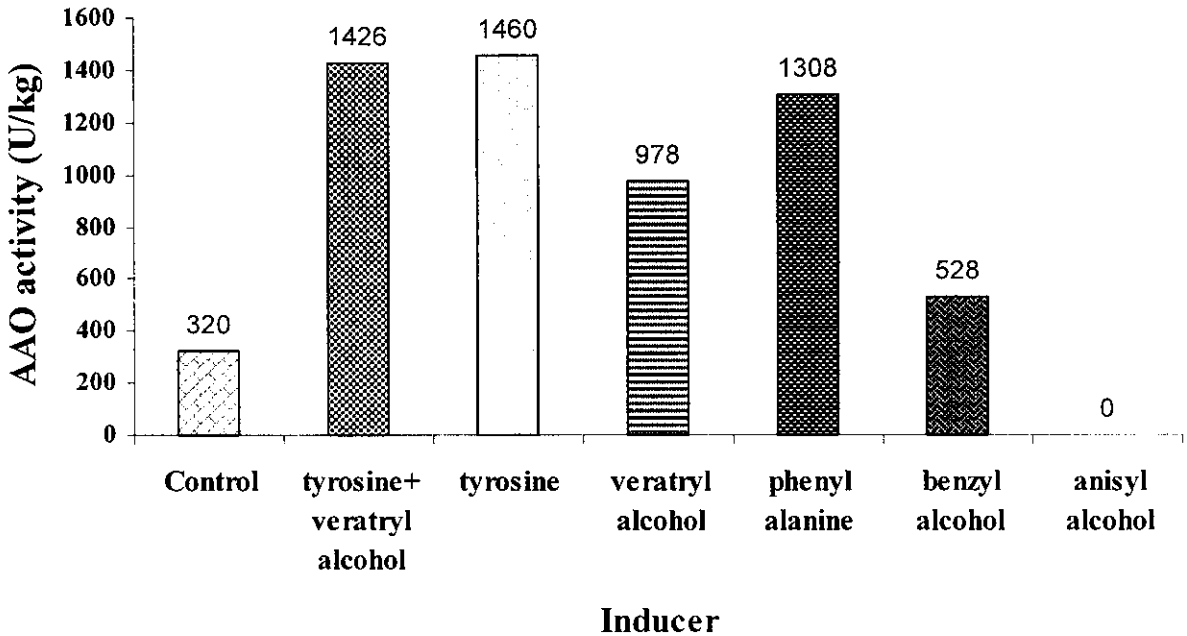
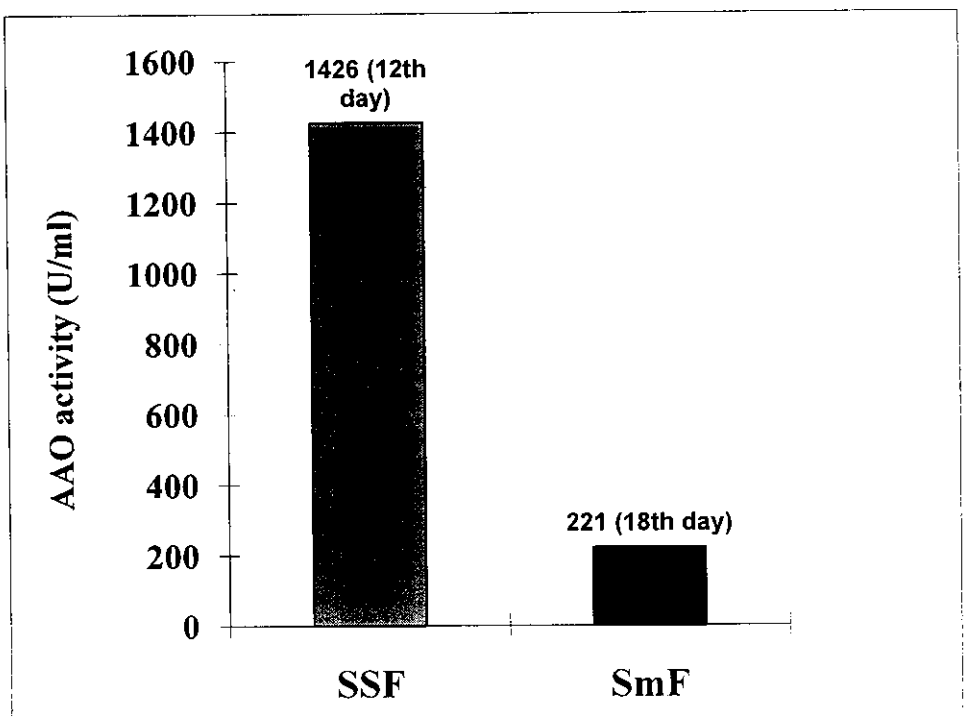


Fig.5.16: Effect of different inducers on SSF of AAO production in *Pleurotus ostreatus*

5.3.4 Comparison of Enzyme Production in SSF and SmF

These results show that the production of AAO is significantly increased and accelerated in SSF when an organic support (grape seed) is used as compared with submerged fermentation (SmF). The time to reach maximum enzyme production was considerably reduced in SSF (12 days of incubation) as compared to SmF (18 days). Moreover, AAO activity was 6.44-fold higher in SSF (1426 U/lit nutrient solution) than in SmF (221 U/lit) (Figure.5.17). This can partly explain the superiority of SSF over SmF at producing aryl alcohol oxidase. Table 1 compares the maximum AAO activity and the time required to attain the maximum activity obtained in this study to those reported for *P. ostreatus* by different authors in the literature. Under SSF conditions, the maximum AAO activity has been remarkably improved and the time required has been reduced to

12 days from 18 days (Figure.5.17). Because of the response to that phenolic compounds and lignin present in grape seed stimulated the AAO production in *P. ostreatus*.



*The value in the bracket indicate growth period at which maximal activity was obtained.

Fig.5.17: Comparison of enzyme production in SSF and SmF

The maximum AAO activity obtained in SSF was thrice that in SmF. It further shows that no AAO was produced in both SSF and SmF after the three days of biomass growth. At the later phase of growth (9 –18 days), AAO production was increased in both SSF and SmF. This is due to nutrient depletion, as is evident from the residual glucose concentration (Figure.5.15). AAO activity was increased at the later phase of growth in SSF, whereas in SmF AAO activity peaked on the 18th day, after which it decreased. The fall in AAO activity in SmF is possibly due to specific degradation of AAO by extracellular proteases present in the culture broth. Figure.5.15 shows that the sharp increase in protease activity on the 6th day resulted in a fall of the AAO activity in SmF. In contrast, although protease activity was present in SSF, it was more than three times lower than in SmF, and thus the AAO activity was found to rise steadily in SSF, possibly

due to lower proteolytic activity. The induction of tyrosine in the nutrient medium was developed in this investigation remarkably enhances the AAO production by *P. ostreatus*; possibly due to combined effects of the aromatic amino acids and aryl alcohols present in the grape seed and a lower proteolytic activity in SSF.

5.4 Effect of pH on Enzyme Activity

Pleurotus species were grown for 18 days in standard conditions in defined medium at pH 6.3, and 30 °C. The enzyme extracts were prepared and incubated in buffers of varying pH and relative AAO activity was determined. Results represent the average of three experiments. The effect of pH on the AAO activity in *Pleurotus sp* is presented in Figure.5.18. Optimum pH for *Pleurotus ostreatus, P. eous* and *P. platypus* was found to be around 5.5 to 6. As it can be observed, the AAO was active over a broad pH range, displaying over 81 % of its activity in the pH range of 4.0–7.0 (*Pleurotus eous*), and 5.0–7.0 (*Pleurotus ostreatus*). A further increasing these pH values decreased the relative activity down to 15%. This optimum temperature was in agreement with the studies conducted by several authors using different strains (Guillen *et al.*, 1990; Okamoto and Yanase, 2002; Ferreira *et al.*, 2005; Kim *et al.*, 2001). Therefore, AAO can be a potential candidate for different applications in the industries demanding acid pH.

5.5 Effect of Temperature on Enzyme activity

Pleurotus species were grown for 18 days in standard conditions in defined medium at pH 6.3, and 30°C. The enzyme extracts were prepared and incubated in buffers (pH 6.0) of varying temperature and relative AAO activity was determined. Results represent the average of three experiments. The *Pleurotus eous* AAO was active over a broad temperature range of 30–60 °C with an optimum temperature of 50 °C (Figure.5.19). It retained more than 70% of its activity at 60 °C. In *Pleurotus ostreatus*, optimum temperature for AAO was 40 °C. *Pleurotus platypus* AAO was active over a temperature range, displaying over 70% of its activity in the temperature range of 25–45 °C and its optimum was at 35 °C.

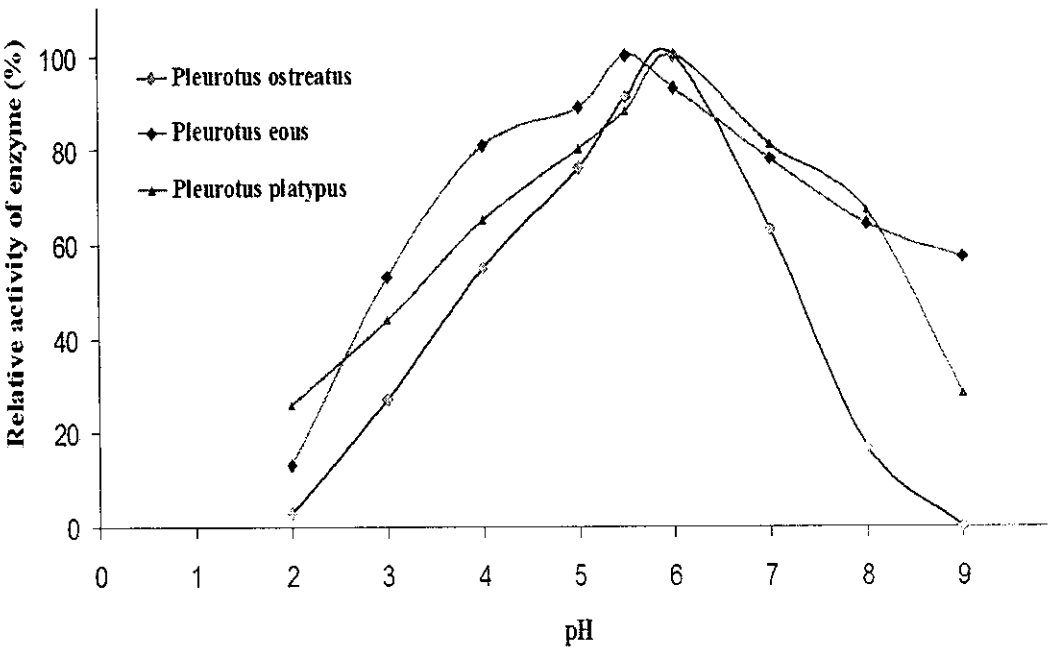


Fig. 5.18: Effect of pH on AAO activity

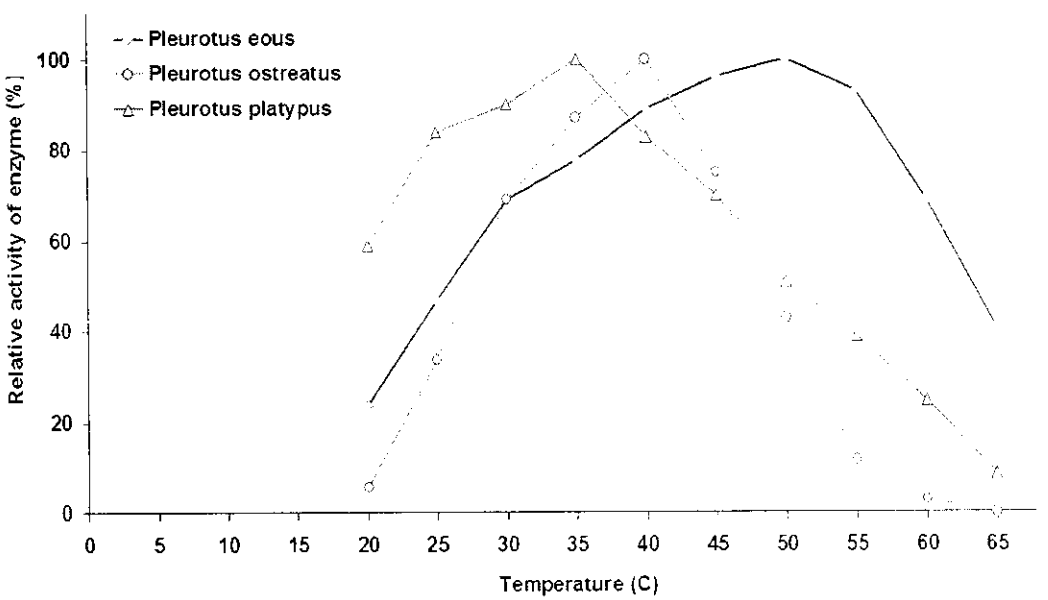


Fig.5.19: Effect of temperature on AAO activity

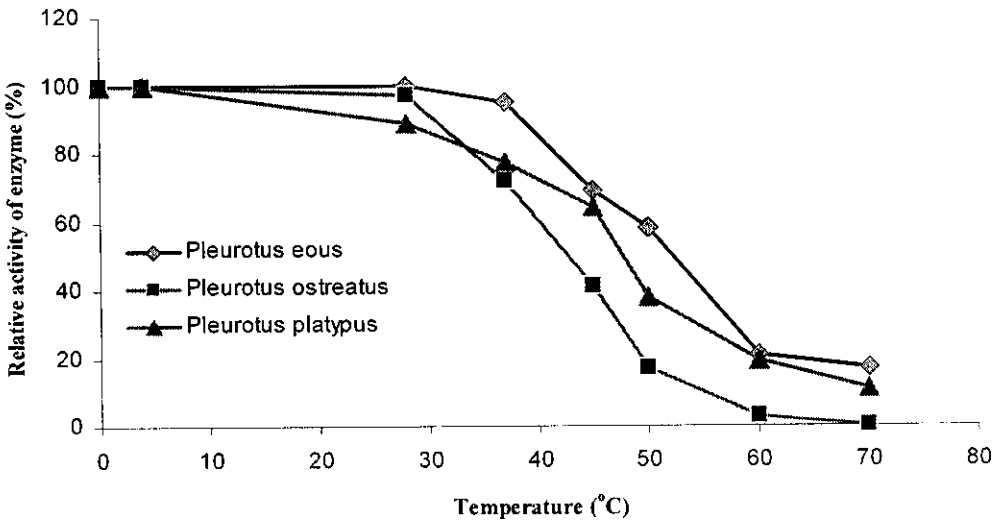


Fig.5.20: Thermostability of AAO

The optimum temperature of AAO, as reported for other species was between 35 and 60 °C (Okamoto and Yanase, 2002; Kim *et al.*, 2001; Guillen *et al.*, 1990). The thermostability of the enzyme was mentioned in Figure.5.20.

5.6 Kinetic Parameters for Enzyme Activity

The kinetic parameters of AAO for veratryl alcohol were obtained by a double reciprocal Lineweaver Burk plot.

Table.5.13: Kinetic parameter of AAO studied using veratryl alcohol in *Pleurotus ostreatus*

S (mM)	V (U/mg)	1/S (mM^{-1})	1/V (mg/U)
2	1.58	0.5	0.63
1	1.36	1	0.73
0.66	1.11	1.5	0.9
0.5	0.91	2	1.09
0.4	0.77	2.5	1.29
0.33	0.58	3	1.72

The K_m values for *Pleurotus ostreatus* oxidizing veratryl alcohol is 0.86 mM. The V_{max} values for oxidizing veratryl alcohol for *Pleurotus ostreatus* are 1.6 U/mg. A *Km* value of 1.4 was reported for *Pleurotus eryngii* (Guillen *et al.*, 1990), and it is evident that and *P. platypus* produces an AAO that has 2.5 fold higher affinity for veratryl alcohol. The kinetic parameter of AAO using veratryl alcohol of *Pleurotus ostreatus* were mentioned in Table.5.13 and Figure.5.21.

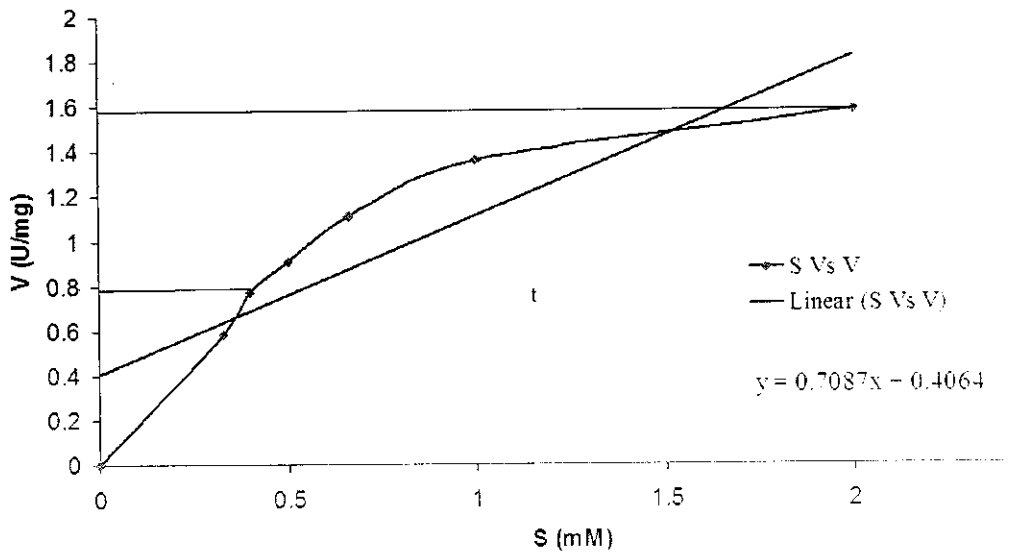


Fig.5.21: Michaelis menten plot of the kinetics of AAO activity.

5.7 Three Phase Partitioning of AAO

Efforts were also made to optimize the ammonium sulphate concentration, ratio of volume of t-butanol to fermented media and temperature for efficient yield and degree of purification. These three parameters have major influence on the purification of AAO. The optimum condition for TPP was carried out at 30°C, to use of 30% ammonium sulphate along with 1:1 ratio of *t*-butanol to aqueous crude extract. The optimum concentration determined here seems to be, in general the best concentration to operate TPP with various systems [Pike and Dennison 1989; Sharma and Gupta 2000]. The interfacial layer obtained after first TPP, was collection of high molecular weight contaminants, cell debris and it precipitates 2.61% of AAO activity (Figure.5.22).

Therefore aqueous layer was subjected to second stage of TPP under same conditions. When it was subjected to second round of TPP, 10.95% recovery of AAO activity and 10.19 fold purification was observed in middle layer. The overall purification of AAO by TPP is given in Table.5.14.

Table. 5.14: Overall purification of AAO by TPP

Steps	Total activity (U)	Protein concent (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	8.4	54	0.155	1	100
TPP – 1st precipitate*	0.22	1.74	0.126	0.8	2.61
TPP – 2nd precipitate+	0.92	0.58	1.58	10.19	10.95

*The crude extract was saturated with 30% (w/v) ammonium sulphate followed by addition of 1:1 tert-butanol.

+First step aqueous phase was saturated with 30% (w/v) ammonium sulphate followed by addition of 1:1 tert-butanol.

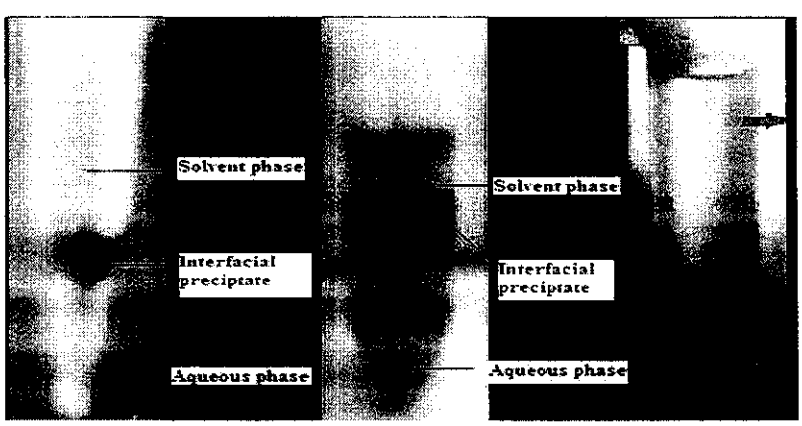


Fig.5.22: Three phase partitioning of AAO

CONCLUSION

6. CONCLUSION

In conclusion, Primary and secondary screening was carried out for the AAO producing fungi. Out of twenty fungal species, intense brown colour zone was formed around the colonies of six species was found to be lignolytic and the culture filtrates were assayed for AAO activity. Our results showed that *Pleurotus sp* (*P.ostreatus, P. platypus* and *P. eous*) is able to produce AAO efficiently, in the presence of the inducers tyrosine and veratryl alcohol. The highest activity was found with *Pleurotus ostreatus*. Among the inducers tested, we detected a quick induction (15^{th} day) of AAO activity by veratryl alcohol, while combination of tyrosine and veratryl alcohol shows resulted in maximal induction (289 U/lit).

Solid state fermentation was carried out to improve AAO production. Cheap agricultural byproducts rich in lignin content like grape seed was employed for the production of AAO. Results show that the production of AAO is significantly increased and accelerated in SSF when an organic support (grape seed) is used as compared with submerged fermentation (SmF). The time to reach maximum enzyme production was considerably reduced in SSF (12 days of incubation) as compared to SmF (18 days). Moreover, AAO activity was 6.44-fold higher in SSF than in SmF. To the best of our knowledge, this is the first report on the use of (TPP) to purify the AAO from *Pleurotus ostreatus*. Total purification fold was about 10.19 and 10.95 % recovery of AAO activity was obtained. So it was recommended that, this modified procedure could be recommended as simple purification step of AAO and holds a potential to be applicable to industrial scales.

The crude AAO from *Pleurotus ostreatus* was characterized, its optimum pH was found as 6.0, optimum temperature was 40°C and its Km value was obtained as 0.6 mM. The kinetic properties, broad pH and temperature stability displayed by *Pleurotus* AAO described here suggests its possible utility in biotechnological applications for paper pulp industries and decolourize of textile dyes. Results of the study indicate *P. platypus* and *P. eous* to be good producers of AAO, and they could be promising species for purification of AAO for biotechnological applications.

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