STUDIES ON TANNASE OF *Aspergillus niger* van Tieghem

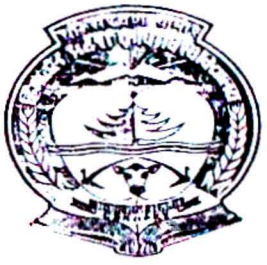
THESIS

By

RITA BHARDWAJ

(S-99-30-03)

Submitted to



Choudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur - 176 062 (H. P.) INDIA

in

Partial fulfillment of the requirements for the degree

of

MASTER OF SCIENCE IN BASIC SCIENCE

(BIOCHEMISTRY)

(2001)

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Palampur (H.P.), India.

CERTIFICATE - I

This is to certify that the thesis entitled: **"Studies On Tannase of *Aspergillus niger* van Tieghem"** submitted in partial fulfillment of the requirement for the **Master of Science (Basic Sciences)** in the subject of **Biochemistry** (Minor-Microbiology) of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Ms. Rita Bhardwaj** (S-99-03-03) daughter of Sh. C.S. Bhardwaj, under my supervision and that no part of the thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Dr. T. K. Bhat
Chairman, Advisory Committee

Place: Palampur
Date: October 5, 2001

CERTIFICATE - II

This is to certify that thesis entitled: **"Studies on Tannase of *Aspergillus niger* van Teighem"** submitted by **Ms. Rita Bhardwaj** daughter of Sh. C.S. Bhardwaj to Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfillment of the requirement for the degree of **Master of Science (Basic Sciences)** in the subject of **Biochemistry**, has been approved by the examining committee after an oral examination of the student in collaboration with the external examiner.

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Rita Bhardwaj

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LIST OF ABBREVIATIONS

A. niger	*Aspergillus niger*
CTAB	N- Cetyl - N, N, N, - trimethyl ammonium bromide
cm	Centimeter
$CdCl_2$	Cadmium chloride
$CaCl_2$	Calcium chloride
^0C	Degree Celsius
g/L	Grams per litre
g	Gram(s)
g	Gravity
GA	Gallic acid
h	Hour
KOH	Potassium Hydroxide
K_m	Michaelis-Menten constant
K_i	Inhibition constant
kDa	Kilodalton
l	Litre
MT	Mildew test
M	Molar
m	Milli
min	Minute(s)

$MgSO_4$	Magnesium sulphate
mBar	MilliBar
mA	Milliampere
$MnCl_2$	Manganese chloride
µ	Micro
nm	nanometer
N	Normal
OHP sheet	Overhead projector sheet
PDA	Potato-Dextrose-Agar
PEG-6000	Polyethylene glycol (M.W. 6000)
rpm	Revolutions per minute
SDS	Sodium dodecyl (lauryl) sulphate
TAA	Tannic acid agar
TCA	Trichloroacetic acid
TEMED	N, N, N', N'- Tetra methyl ethylenediamine
Tween-20	Polyethylene sorbitan monolaurate
Triton X-100	t-Octoylphenoxy polyethoxy ethanol
UV	Ultraviolet
V	Volts
v/v	Volume/Volume
$ZnCl_2$	Zinc chloride
%	Percent

INTRODUCTION

CHAPTER – 1

INTRODUCTION

Tannins are naturally occurring water-soluble polyphenols with a molecular weight of 500-3000 Da, which differ from most other natural phenolic compounds in their ability to precipitate proteins from solution (Spencer *et al.*, 1988). They are widely distributed in plant kingdom (pteridophytes, gymnosperms and angiosperms) and are present in almost every part or tissue of the plant – fruits, leaves, root, stem and bark (Haslam, 1989).

Tannins are mainly of three types: hydrolysable tannins, which have a sugar core linked to gallic acid (gallotannins) or ellagic acid (ellagitannins) by esterification; condensed tannins, formed from the monomer flavan-3-ol or a derivative thereof and are recalcitrant to hydrolysis, and catechin gallates which share the properties of hydrolysable and condensed tannins (Bhat *et al.*, 1998).

Tannins inhibit the growth of a number of microorganisms. Tannin containing plants resist microbial attack and are recalcitrant to biodegradation, and this gifts these plants a significant evolutionary advantage over their enemies. Tannins may deter herbivores from predation (Waterman and Mole, 1994). They may also deter microbes, either by increasing the resistance against pathogens or by protecting essential tissues such as wood against decay. Durable timbers have a higher tannin content (Scalbert, 1992). However, some microoganisms, particularly fungi, are quite resistant to tannins and can tolerate high concentrations of tannins (Deschamps, 1989).

A fungus *Aspergillus niger* van Tieghem having high tannin-tolerating capacity and tannin-degrading activity was isolated from faecal samples of hill cattle, fed largely on oak leaves (Bhat *et al.*, 1996). This strain can utilize tannins as sole carbon and

energy source and tolerate gallotannin concentration as high as 20 % without its growth being inhibited (Bhat *et al.*, 1997). The key enzyme responsible for hydrolyzable tannin degradation is tannin acyl hydrolase (EC 3.1.1.20) commonly called tannase, which catalyzes the hydrolysis of ester and depside bonds in these tannins (Dykerhoff and Ambruster, 1933). The enzyme tannase was first reported by Knudson, (1913) who found that it had a role in the degradation of tannic acid, which is the commercial name of Chinese gall tannin. I ater, the enzyme was described and purified from various fungal strains and was found to be induced by methyl gallate and tannic acid but not by other simple phenols such as gallic acid, salicylic acid or methyl salicylate (Haslam and Stangroom, 1966; Otuk and Deschamps, 1983). Among all known microbial producers of tannase, strains of some of the *Aspergillus* spp. are commercially the most efficient producers of this enzyme (Lekha and Lonsane, 1996). Tannase finds widespread application in food and beverage processing. It also finds extensive use in the manufacture of instant tea (Coggon *et al.*, 1975).

The optimal growth conditions for the production of mycelial mass rich in tannase from *A. niger* van Tieghem have been standardized and a simple and sensitive assay method has been developed for this enzyme (Sharma, 1998). Tannase from *A. niger* van Tieghem has been partially purified and its optimum pH, temperature and thermostability have been determined (Sharma *et al.*, 1999). In continuation with earlier work, the present study included the development of a better protocol for purification of tannase from this strain of *A. niger*, improving the yield of the enzyme, investigation on the effect of activity modulators on tannase activity, distinguishing its esterase and depsidase activity, and tannin biotransformation studies.

REVIEW OF LITERATURE

CHAPTER - 2

REVIEW OF LITERATURE

Tannase, discovered in 1786 by Scheele, is an enzyme that hydrolyzes the ester bonds of tannic acid to produce gallic acid and glucose. Tannase is known to be a ubiquitous enzyme of the microbial world (Deschamps, 1989; Field and Lettinga, 1992; Lekha and Lonsane, 1997) and has widespread occurrence in various fungi, bacteria and yeast. Tannase has potential applications in the clarification of beer and fruit juices, manufacture of coffee, flavoured soft drinks, improvement in the flavour of grape wine and as an analytical probe for determining the structure of naturally occuring gallic acid esters (Masschelein, 1981; Cantarelli, 1989).

1. Tannins

1.1 Types and structures

Tannins are defined as naturally occurring water soluble polyphenols of varying molecular weight, which differ from most other natural phenolic compounds in their ability to precipitate proteins from solutions (Spencer *et al.*, 1988). This property is the basis for their past and present use in tanning industry.

Based on their structures and properties, they are distributed into two major groups-hydrolysable and condensed tannins (Table 1). Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core which is usually glucose, and are readily hydrolysed by acids or enzymes into monomeric products (Fig 1). They are usually present in low amounts in plants. Tannic acid has been used as a typical hydrolysable tannin in various studies on polyphenol

Table 1: The major groups of tannins with their representative types and main sources.

Hydrolysable Tannins	**Catechin Tannins**	**Condensed Tannins**
1. Gallotannins e.g. tannic acid (commercial name of Chinese gall tannins); yield gallic acid & glucose on hydrolysis.	Catechin and epicatechin gallates; yield catechin/epicatechin and gallic acid on hydrolysis; have properties of hydrolysable & condensed tannins. from the wood of quebracho tree.	Polymeric proanthocyanidins; yield monomeric flavonoids such as flavan-3,4-diols & flavan-3-ols on hydrolysis e.g., quebracho tannins
Sources: Tara pods *(Caesalpina spinosa)*, gall nuts (pathological excrescences) from *Quercus infectoria* (Turkish gall) & *Rhus semialata* (Chinese gall), sumac leaves *(Rhus coriara)*	**Sources:** Tropical shrub legumes, tea leaves	**Sources**: Commonly found in fruits and seeds such as grapes, apple, olives, beans, sorghum grains, carob pods, cocoa & coffee, besides tree bark & heart wood.
2. Ellagitannins - yield ellagic acid & glucose on hydrolysis.		**Common types are** 1. Quebracho tannins from wood of *Schinopsis* spp., *Loxopterygium* spp. 2. Wattle tannins from *Acacia* spp. 3. Bark tannins from pine *(Pinus* spp.), oak *(Quercus* spp.) and gaboon wood *(Aucoumea kleneana)*.
Sources: Wood of oak *(Quercus* spp.), chestnut *(Castanea* spp.) and myrobalan *(Terminalia chebula)*		

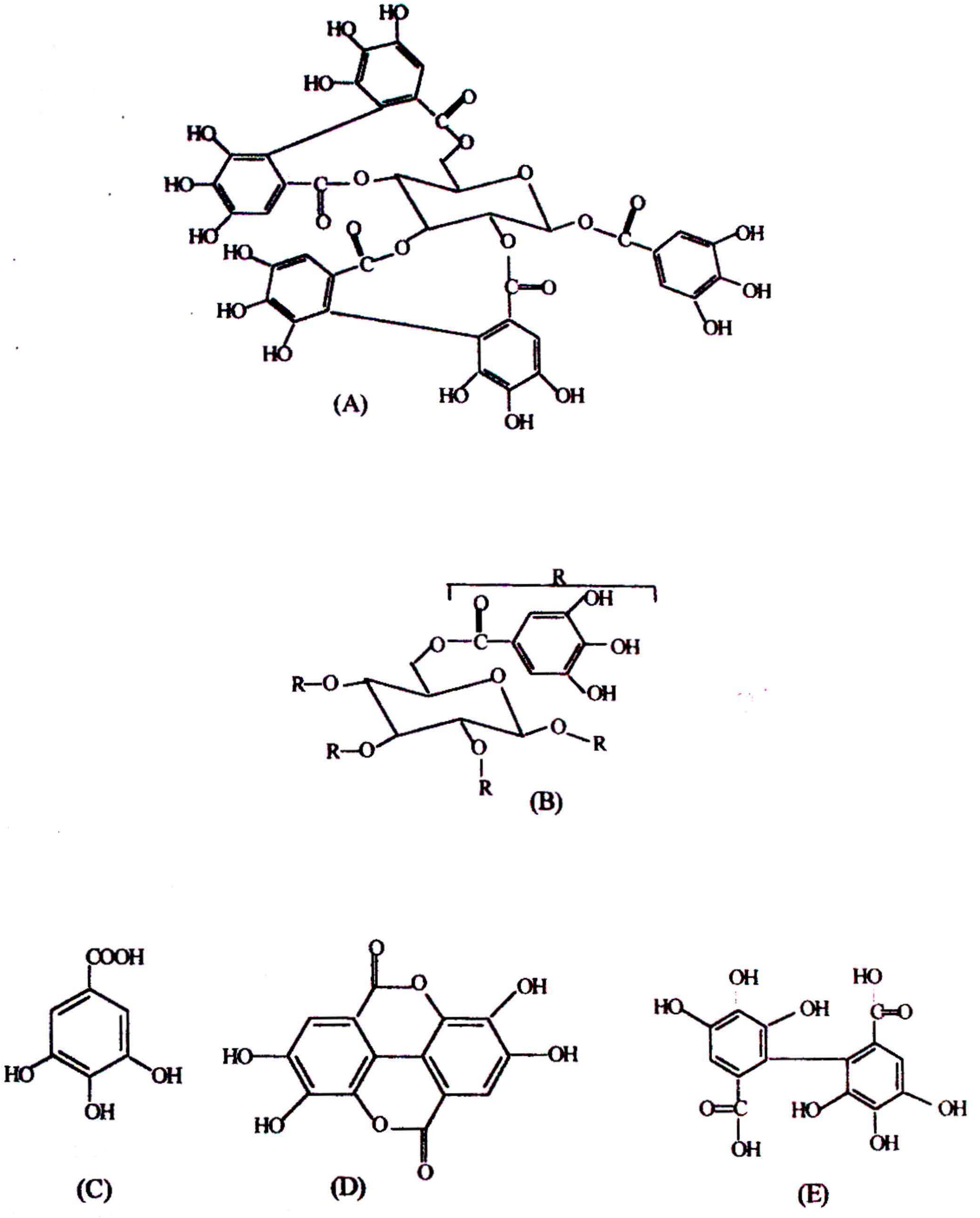


Fig 1: Structure of hydrolysable tannins and their monomers. (A) gallotannin, (B) ellagitannin, (C) gallic acid, (D) ellagic acid and (E) hexahydroxy diphenic acid.

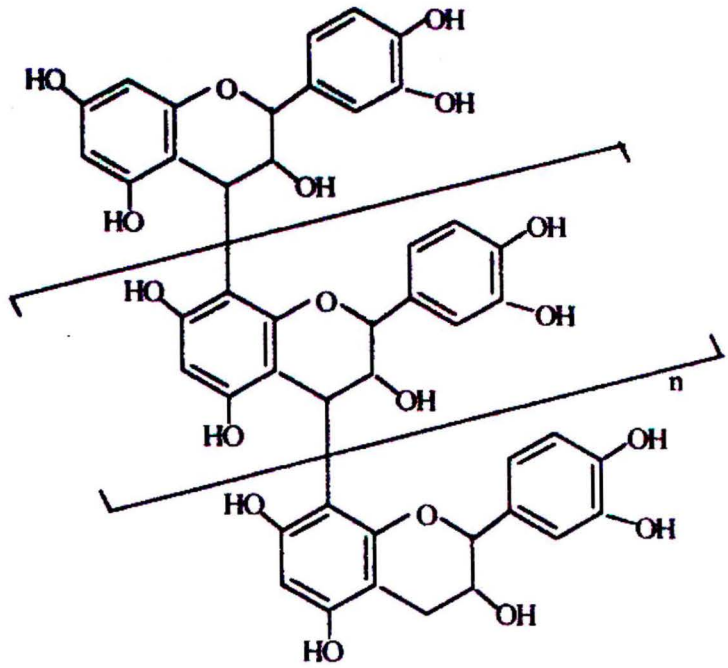
biotransformation (Lekha and Lonsane, 1997) and toxicity studies. Tannic acid is a mixture of polygalloylglucoses. Commercial tannic acid also contains small amount of gallic acid, digallic acid, trigallic acid and ellagic acid (Zhu and Filippich, 1995).

Condensed tannins, also known as polymeric proanthocyanidins, are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. They are usually more abundant in tree barks and woods than their hydrolysable counterparts. The most important monomers of condensed tannins are catechin and epicatechin (Fig 2).

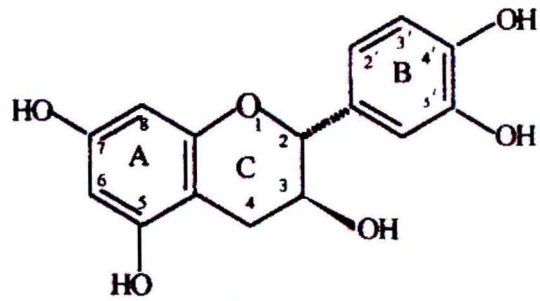
A group that occupies an intermediate position in the tannin hierarchy is the family of catechin tannins (Table 1) combining elements of hydrolysable and condensed tannins. These tannins are quite common in tropical shrub legumes (Mueller-Harvey *et al.*, 1987) and tea leaves (Graham, 1992).

1.2 Significance of tannins

Tannins are present in large number of feeds and forages and have capacity to form complexes with carbohydrates and proteins. Formation of complexes of tannins with biomolecules, especially proteins, has both negative and positive effects on their utilization (Reed, 1995) (Fig 3). Hydrolysable tannins have toxic effects in the animals that feed on forages rich in these tannins. High concentrations of dietary condensed tannins (6-20 % of dry matter) depress voluntary feed intake, digestive efficiency and animal productivity (Reed, 1995). In contrast, forages containing moderate concentrations of condensed tannins (2-4 % of dry matter) can exert beneficial effects on protein metabolism in ruminants especially sheep, by slowing degradation of dietary protein to ammonia by rumen microorganisms and increasing protein outflow from the rumen. This results in the increased absorption of amino acids in the small intestine and



(A)



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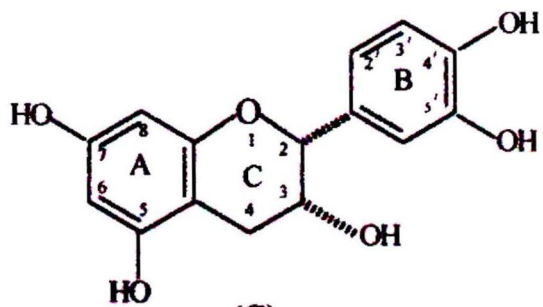


(C)

Fig 2: Structure of condensed tannins and their monomers. (A) 4,8 linked procyanadin (condensed tannin), (B) catechin and (C) epicatechin.

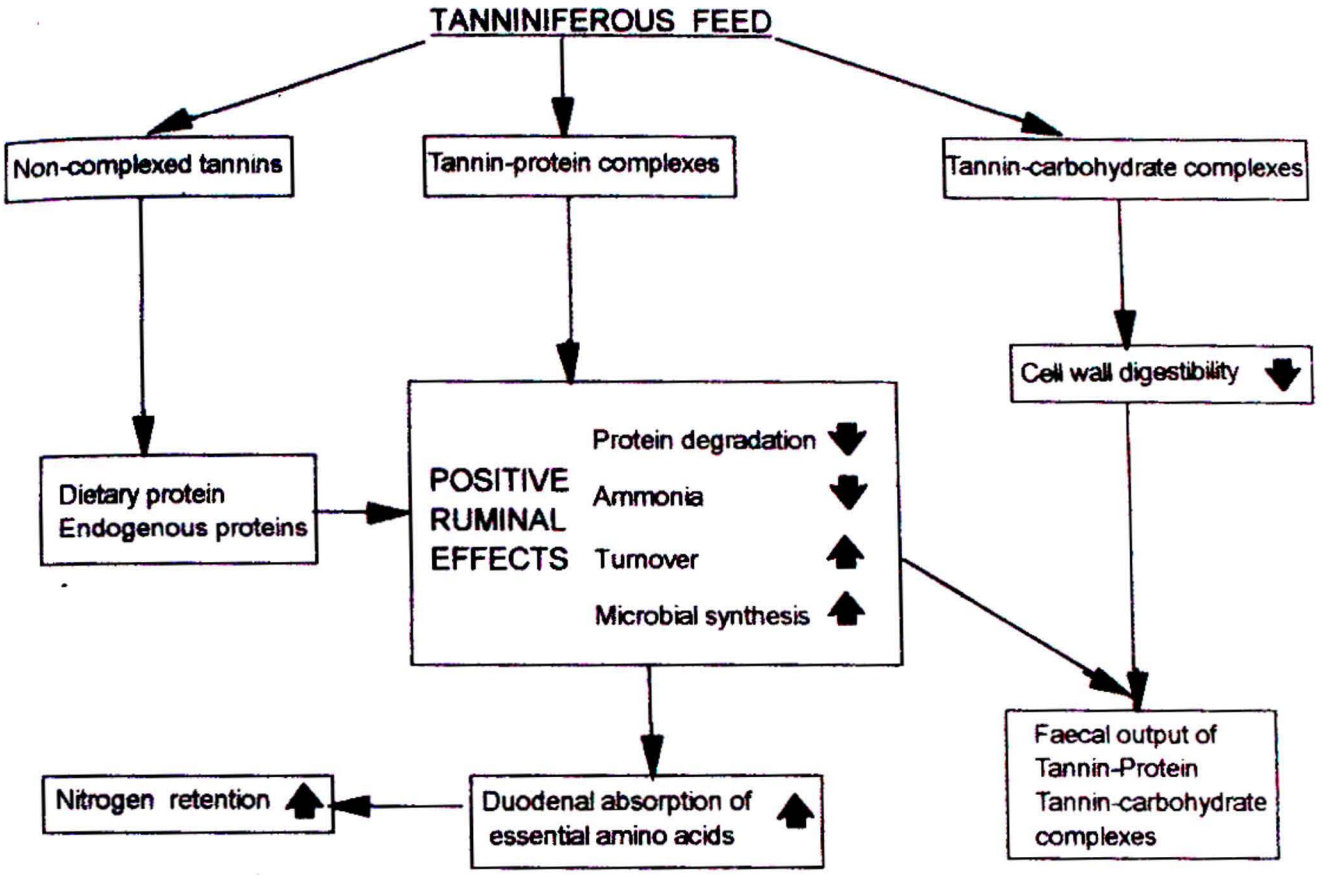


Fig 3: Positive and negative effects of tannin-nutrient interaction in ruminants.

ultimately leads to increase in lactation, wool growth and live weight gain, without changing voluntary feed intake of the animal (Singh and Bhat, 2001). Intake of condensed tannins at a moderate level can also contribute to improved animal health by reducing the detrimental effects of internal parasites in small ruminants and the risk of bloat in cattle (Singh and Bhat, 2001). Therefore, forages containing moderate concentrations of condensed tannins can increase sustainability and productivity in intensive grazing systems through increasing the efficiency of animal production, decreasing urinary nitrogen excretion and reducing chemical inputs for controlling internal parasites and rumen bloat in cattle (Singh and Bhat, 2001).

Tannins are believed to occur in the vacuoles of intact plant cells (Forsyth, 1964). The exact function of tannins in plants is not clear. At the intracellular level of metabolism, tannins are of little value to the plant, although they function as pigments and help in wound healing. Some plants accumulate tannins, particularly in the bark and heartwood. Such accumulated tannins protect the vulnerable parts of the plants from microbial attack by inactivating viruses and invasive extracellular enzymes of microbes by direct tanning action (White, 1957). Enzymes secreted by attacking micro-organisms are wholly or partially inactivated by complex formation with tannins (Goldstein and Swain, 1965), while such microbial substrates as polysaccharides and non-enzyme proteins present in the plants, become highly resistant to microbial attack after binding to a tannin molecule (Betnoit *et al.*, 1968). The inhibitory action of tannins on the growth of bacteria (Sivaswamy, 1982; Henis *et al.*, 1964), fungi (Mur, 1953; Scalbert, 1991), yeast (Scalbert, 1991) and viruses (Cadman, 1960) is well established.

2. Microbial Tannase

Despite the antimicrobial properties of tannins, many fungi, bacteria and yeast are quite resistant to tannins, and can grow and develop on them (Deschamps, 1989). Some of the fungi such as *Aspergillus* or *Penicillium* have evolved tannin-degrading systems to withstand high concentrations of tannins (Yamada *et al.*, 1968a, b). The key enyzme which plays a prominent role in the degradation of gallotannins is tannin acyl hydrolase (EC 3.1.1.20), commonly known as "tannase". Among all known microbial producers of tannase, strains of some of the *Aspergillus* spp. are commercially the most efficient producers of this enzyme (Deschamps, 1989; Field and Lettinga, 1992; Lekha and Lonsane, 1997). Tannase is now known to be a ubiquitous enzyme of the microbial world (Deschamps, 1989; Field and Lettinga, 1992; Lekha and Lonsane, 1997) and has widespread occurrence in various fungi, bacteria and yeasts (Table 2). It is produced both as membrane bound and extracellular forms. A number of investigations on microbial tannase have shown that this enzyme is not equally active against all hydrolysable tannins (Deschamps, 1989; Scalbert, 1991). Fungal tannases are quite versatile and efficiently degrade different types of hydrolysable tannins (Lewis and Starkey, 1969). However, yeast tannases are effective only in decomposing tannic acid and weakly degrade natural tannins (Deschamps, 1989). Bacterial tannases can degrade tannic acid as well as natural tannins like chestnut, tara, oak and myrobalan tannins (Lewis and Starkey, 1969; Deschamps *et al.*, 1980, 1983; Deschamps and Lebeault, 1984). Skene and Brooker, (1995) observed tannase activity for the first time in an anaerobic ruminal bacterium isolated from goats browsing on tannin-rich forage. These workers reported presence of this enzyme in *Selenomonas ruminantium* subsp. *ruminantium* and described its characteristics. Tannase activity has recently also been

Table 2: Microorganisms producing tannin acyl hydrolase (tannase)

BACTERIA

❖	*Achromobacter* sp.	(Lewis and Starkey 1969)
❑	*Bacillus pumilis*	(Deschamps *et al.*, 1983)
❑	*Bacillus polymyxa*	(Deschamps *et al.*, 1983)
❑	*Bacillus licheniformis*	(Mondal and Patil 2000)
❑	*Corynebacterium* sp.	(Deschamps *et al.*, 1983)
❑	*Klebsiella planticola*	(Deschamps *et al.*, 1983)
❖	*Pseudomonas solanacearum*	(Deschamps 1989)
❖	*Selenomonas ruminantium*	(Skene and Brooker 1995)

FUNGI

◆	*Aspergillus niger*	(Knudson 1913; Haslam and Stangroom 1966; Barthomeuf *et al.*, 1994; Lekha and Lonsane 1994; Bajpai and Patil 1996; Bradoo *et al.*, 1996; Sharma *et al.*, 1999)
◆	*Aspergillus oryzae*	(Iibuchi *et al.*, 1967; Doi *et al.*, 1973; Beverini and Metche 1990; Bajpai and Patil 1996; Bradoo *et al.*, 1996)
❖	*Aspergillus flavus*	(Yamada *et al.*, 1968a)
◆	*Aspergillus japonicus*	(Ganga *et al.*, 1977; Bradoo *et al.*, 1996)
❖	*Aspergillus aureus*	(Bajpai and Patil 1996)
◆	*Aspergillus awamori*	(Bradoo *et al.*, 1996; Seth and Chand 2000)
❖	*Aspergillus fischerii*	(Bajpai and Patil 1996)
❖	*Aspergillus rugulosus*	(Bradoo *et al.*, 1996)
❖	*Aspergillus parasiticus*	(Bajpai and Patil 1996)
❖	*Aspergillus terreus*	(Bajpai and Patil 1996)
❖	*Penicillium chrysogenum*	(Rajakumar and Nandy 1983; Bajpai and Patil 1996; Bradoo *et al.*, 1996)
❖	*Penicillium notatum*	(Ganga *et al.*, 1977)
❖	*Penicillium islandicum*	(Ganga *et al.*, 1977)
❖	*Penicillium digitatum*	(Bradoo *et al.*, 1996)
❖	*Penicillium acrellanum*	(Bradoo *et al.*, 1996)
❖	*Penicillium carylophilum*	(Bradoo *et al.*, 1996)
❖	*Penicillium charlesii*	(Bradoo *et al.*, 1996)
❖	*Penicillium citrinium*	(Bradoo *et al.*, 1996)
❑	*Cryphonectria parasitica*	(Farias *et al.*, 1992)
❑	*Fusarium solani*	(Bajpai and Patil 1996; Bradoo *et al.*, 1996)
❖	*Fusarium oxysporium*	(Bradoo *et al.*, 1996)
❑	*Rhizopus oryzae*	(Hadi *et al.*, 1994)
❑	*Trichoderma viride*	(Bajpai and Patil 1996; Bradoo *et al.*, 1996)
❖	*Trichoderma hamatum*	(Bradoo. *et al.*, 1996)
❖	*Trichoderma harzianum*	(Bradoo *et al.*, 1996)
❖	*Helicostylum* sp.	(Bradoo *et al.*, 1996)
❖	*Cunnighamella* sp.	(Bradoo *et al.*, 1996)
❖	*Syncephalastrum racemosum*	(Bradoo *et al.*, 1996)
●	*Neurospora crassa*	(Bradoo *et al.*, 1996)

YEASTS

❑	*Candida* sp.	(Aoki *et al.*, 1976a)
❑	*Pichia* spp.	(Deschamps 1989)
❖	*Debaryomyces hansenii*	(Deschamps 1989)

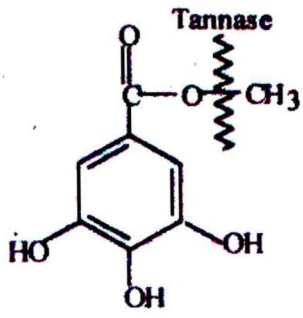
● **Poor producer** ❖ **Moderate producer** ❑ **Good producer** ◆ **Best producer**

reported in human lactobacilli (Osawa *et al.*, 2000) and tannin-tolerant ruminal bacteria (Odenyo *et al.*, 2001).

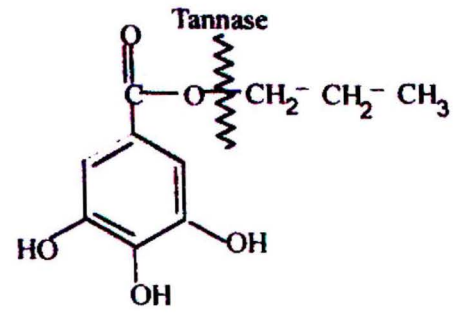
2.1 Properties of tannase

Tannase is active on galloyl residues of galloyl esters, as well as hexahydroxy diphenoyl and other residues of ellagitannins. Galloyl residues are usually more easily hydrolysed than the other groups (Scalbert, 1991). Tannase cleaves ester and depside linkage in hydrolysable tannins e.g. tannic acid. It also acts on the ester linkage in methyl gallate (Dykerhoff and Ambruster, 1933) (Fig 4). The pH optimum of the enzyme is 5.0 - 6.0 and it is usually unstable above pH 6.0 (Iibuchi *et al.*, 1968, 1972; Thomas and Murtagh, 1985). Recent work on *A. niger* tannase has indicated that the enzyme is a glycoprotein with a molecular weight of about 186 kDa, and contains 43 % sugar (Barthomeuf *et al.*, 1994).

O-phenanthroline, phenyl methyl sulfonyl fluoride, ethylene diamine tetraacetic acid (EDTA), 2-mercaptoethanol, and sodium thioglycolate are inactivators of tannase of *A. niger* (Barthomeuf *et al.*, 1994). It is also strongly inhibited by copper and to a lesser extent by ferric and zinc ions at 20 mM concentration. Pyrogallol, gallic acid, gallaldehyde and gallamide were reported to be competitive inhibitors of tannase. Several attempts have been made to produce or isolate tannase from the culture media of *Aspergillus*, and in particular to separate the esterase activity catalysing the hydrolysis of galloyl esters attached to glucose, from the depsidase activity catalysing the hydrolysis of depside linkages between two galloyl residues (Haslam and Stangroom, 1966; Beverini and Metche, 1990). These studies showed that this fungus produces several tannase isoenzymes, but failed to provide fractions with exclusive esterase or depsidase activity. The ratio of the two activities did vary in the different fractions but the relative



(a)



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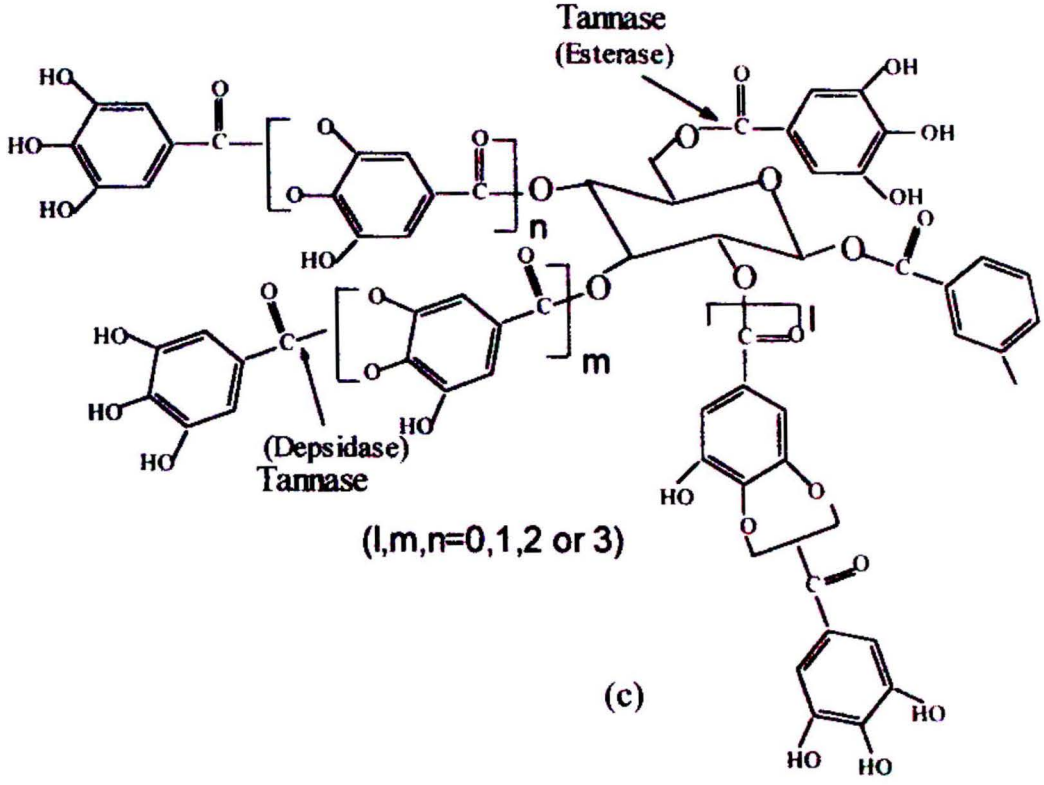

(c)

Fig 4: Structure of tannase substrates (a) methyl gallate, (b) propyl gallate, and (c) tannic acid

specificity of each enzyme was low. Barthomeuf *et al.*, (1994) could however, separately assay the activities of total tannase and its esterase component in *A. niger* using tannic acid and methyl gallate as substrates, respectively.

2.2 Tannase assay

Tannase catalyses the hydrolysis of ester and depside linkages in a hydrolysable tannin such as tannic acid, releasing glucose and gallic acid. However, it does not act on condensed tannins (Dykerhoff and Ambruster, 1933; George and Sen, 1960). Different studies have been carried out on the development of assay systems for estimation of microbial tannase activity. Most of the old methods for tannase assay are based on the titration of gallic acid released by the action of the enzyme on tannic acid, and do not give correct results due to the problem of accurately determining the end point (Madhavakrishna *et al.*, 1960; Nishira, 1961; Haslam, 1966; Farias, 1992). Madhavakrishna *et al.*, (1960) reported a method for tannase assay based on the estimation of glucose liberated by incubation with the enzyme for 24 h, which is not suitable for routine assays of the enzyme. Methods using decrease in absorbance of substrate tannic acid at 310 nm were also used but the procedure has a shortcoming that it is based on a small difference in UV absorption of the product (gallic acid) and the substrate (methyl gallate or tannic acid) (Haslam and Tanner, 1970). Jean *et al.*, (1981) developed a gas-chromatographic method for estimation of tannase activity by determining gallic acid released as a result of enzymatic hydrolysis of methyl gallate. This method is said to be rapid, specific, reliable, and reproducible but requires more sophisticated instrumentation and time, and thus is not suitable for routine assays. Inoue and Hagerman, (1988) developed a colorimetric estimation of gallic acid based on the formation of chromogen between gallic acid obtained by the acid hydrolysis of

gallotannins, and rhodanine. This method was later adapted by Skene and Brooker, (1995) for bacterial tannase assay which was modified further into a convenient assay system (Sharma *et al.*, 2000). Iacazio *et al.*, (2000) have reported another method for tannase assay using protocatechuic acid p-nitrophenyl ester 5 as substrate, which releases p-nitrophenol due to tannase action and is spectrophotometrically easily measured either at 350 nm for pH values greater than 6 or at 400 nm for pH values of 6 - 7 (yellow chromogen).

2.3 Production of tannase

Tannase is produced by submerged, liquid-surface and solid state fermentation. Submerged fermentation involves the growth of the microorganisms as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids (Frost and Moss, 1987). Liquid-surface fermentation involves the growth of culture on the surface of a liquid medium at a shallow depth and held in a suitable container (Mitchell and Lonsane, 1992). The essential feature of solid-state fermentation is the growth of microorganisms on an insoluble substrate without a free-liquid phase (Mitchell and Lonsane, 1992). Among these types, submerged fermentation is most extensively used for tannase production (Lekha and Lonsane, 1997).

A number of protocols have also been developed for production of tannase by various fermentation procedures (Yamada, 1967). These are the surface culture of *A. niger* (Doi *et al.*, 1973; Barthomeuf, *et al.*, 1994), solid-state process for economic production of tannase by *A. niger* (Lekha and Lonsane, 1994), solid-state fermentation of *Rhizopus oryzae* (Hadi *et al.*, 1994; Chatterjee *et al.*, 1996) and liquid-surface fermentation of *A. japonicus* (Bradoo *et al.*, 1997). Tannic acid concentration is the crucial factor in all these methods for tannase production and greatly influences the level

of enzyme (Yamada *et al.*, 1968; Nishira and Mugibayashi, 1956). This is due to the fact that tannase is an inducible enzyme produced only in the presence of tannic acid and methyl gallate (Knudson, 1913; Nishira and Mugibayashi, 1953). The minimum structural requirement for induction of tannase formation is gallic acid moiety. The minimum concentration of tannic acid that could stimulate formation of tannase was found to be 0.1 % (Knudson, 1913). No catabolite repression and/ or feed back inhibition was earlier observed (Knudson, 1913). However, Farias *et al.*, (1994) and Barthomeuf *et al.*, (1994) have reported feedback inhibition and end-product repression in different fungal tannases.

2.4 Immoblization of Tannase

The first report on the immbolization of tannase was by Weetal and Detar, (1974). Tannase was covalently attached to alkylamine porous silica activated with glutaraldehyde. The immobilization of tannase on glass beads by diazo-coupling was patented and used for green tea conversion (Sanderson *et al.*, 1974) and production of instant tea (Coggon *et al.*, 1975). Bajpai *et al.*, (1999) immobilized fungal mycelia on calcium alginate gel and studied gallotannin hydrolysis using the packed bed reactor. They standardized temperature, pH, residency period and substrate concentration for maximum hydrolysis of gallotannin. They also compared the operational stability and stability in terms of retention of enzyme activity after 150 days on storage at 4 ^{0}C .

Abdel-Naby *et al.*, (1999) immobilized tannase on various carriers by different methods and found that enzyme immobilized on chitosan with a bifunctional agent (glutaraldehyde) had the highest activity. The immobilized enzyme retained about 85 % of the initial catalytic activity, even after being used seventeen times. Guo and Yang, (2000) immobilized tannase by embedding it with calcium alginate carriers and studied

immobilization conditions and some properties of the immobilized enzyme. They used immobilized enzyme for gallic acid production and got 61 % yield. Boadi and Neufeld, (2000) have given a number of protocols for tannase immobilization by encapsulating the enzyme on various biomaterials. Tannase activity levels comparable to the free enzyme controls were observed with tannase entrapped within a chitosan coat, applied to an alginate bead core. After one month storage in an encapsulated form, activity levels of the enzyme comparable to freshly prepared bead preparations were observed.

2.5 Tannase gene cloning

The tannase gene from *A. oryzae* has been isolated, sequenced and cloned. It encodes a protein consisting of 588 amino acids including 18 amino acid signal peptide and lacks introns. The tannase gene product is translated as a single polypeptide and cleaved into two tannase subunits linked by disulphide bonds (Hatamoto *et al.*, 1996).

2.6 Application of tannase

Tannase has various industrial applications. It hydrolyses tannic acid to glucose and gallic acid (Iibuchi *et al.*, 1972). Gallic acid is mainly used as a synthetic intermediate for the production of pyrogallol and gallic acid esters, which are used in food and pharmaceutical industries (Deschamps and Lebeault, 1984). In pharmaceutical industries, gallic acid is used in the synthesis of trimethoprim. Gallic acid is also utilized in the enzymatic synthesis of propyl gallate, which is used as an antioxidant in fats, oils and beverages (Lekha and Lonsane, 1997).

Tannins are antinutritional agents and are present in a variety of plant materials that are used as feed (McLeod, 1974). The use of tannase in the pretreatment of tannin

containing feed may prove beneficial in removal of these undesirable compounds and also for improved digestibility (Lekha and Lonsane, 1997)

Instant tea, which is mostly used to prepare iced tea has an important requirement of being soluble in cold water (Coggon *et al.*, 1975). But when brewed tea beverages are allowed to stand for a few hours at or below 4 ^{0}C, they form a cold water insoluble precipitate referred to as "tea-cream" (Sanderson, 1972). Chemical methods for solubilizing tea cream reduce the consumer acceptance and are thus not preferred (Coggon *et al.*, 1975). Efforts are afoot to use tannase for instant tea preparation, and safety evaluation studies have shown that tannase can be regarded as safe for its intended use in processing tea (Lane *et al.*, 1997).

Tannase is also used in wine making. The enzyme hydrolyzes chlorogenic acid to caffeic acid and quinic acid, which favourably influence taste. Tannase has been used to remove phenolic substances from grape juice for stabilization of the beverage (Cantarelli, 1986; Cantarelli *et al.*, 1989) and also for preventing discoloration and haze development during beer chilling and storage. It has potential application in clarification of beer and fruit juices, manufacture of coffee, flavored soft drinks, and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Masschelein, 1981; Cantarelli, 1989).

Tannase may also find use in the leather industry to homogenize tannin preparation for production of high-grade leather tannins and in cosmetology to eliminate the turbidity of plant extracts used in biocosmetics (Barthomeuf *et al.*, 1994).

MATERIALS AND METHODS

CHAPTER - 3

MATERIALS AND METHODS

3.1 CHEMICALS

Tannic acid (analytical grade) was purchased from Qualigens Fine Chemicals, Mumbai. Tannic acid (ACS grade), gallic acid methyl ester (methyl gallate), gallic acid and molecular weight markers were obtained from Sigma Chemicals Co. USA. The molecular weight markers were: glyceraldehyde-3-phosphate dehydrogenase: 36 kDa, ovalbumin: 45 kDa, glutamic dehydrogenase: 55 kDa, albumin: 66 kDa, fructose-6-phosphate kinase: 84 kDa, phosphorylase b: 97 kDa, β- galactosidase: 116 kDa and myosin: 205 kDa. Folin-Ciocalteu's reagent and chemicals for electrophoresis (Molecular Biology Grade) were obtained from Sisco Research Laboratories, Mumbai. N, N, N', N' – tetra methyl ethylenediamine (TEMED) was purchased from Fluka Biochemicals, Switzerland. Sephadex G-150 and DEAE-Sephadex A-50 were procured from Pharmacia Fine Chemicals, Sweden. Dialysis membrane (12-14 kDa cut off; pore size 2.4 nm) was purchased from Hi-Media, Mumbai. Rhodanine was procured from Merck-Schuchardt, Germany. Lyophilized fungal tannase prepared from *A. oryzae* was a kind gift from Dr. J. Yamakoshi (Kikkoman Corp., Japan) and was used as a standard enzyme (Kikkoman tannase). All other chemicals were of analytical grade.

3.2 Inoculum preparation

Aspergillus niger van Tieghem, earlier isolated at IVRI Palampur (Bhat *et al.*, 1996) and now maintained at Microbial Type Culture Collection and Gene Bank Centre, Institute of Microbial Technology, Chandigarh as strain no. MTCC 2425, was used in the present studies. It was maintained on potato-dextrose agar (PDA) slants at 4 ^{0}C and

subcultured every alternate month. For inoculum preparation, the culture was grown on tannic acid agar (TAA) slants at 30 ^{0}C for 7 days.

3.3 Culture medium preparation and inoculation with the fungus

Mildew test (MT) medium was used for growing the fungus in the presence of tannic acid as the sole source of carbon and energy. The composition (g/L) of MT basal medium was as follows:

Sodium nitrate	3.0
Dipotassium hydrogen orthophosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5

The pH of the basal medium was adjusted to 5.0 and 40 ml of the medium was taken in 250 ml flasks. The medium was autoclaved at 121 ^{0}C for 20 min and allowed to cool to room temperature. The filter-sterilized (Cellulose nitrate membrane of 25 mm diameter and 0.45 µm pore size, Whatman Ltd. Maidston, England), tannic acid (Analytical grade) solution in the MT- basal medium (10 ml) was added to each of the flasks which contained the autoclaved basal medium. The final concentration of tannic acid was 2 %. The medium was inoculated with the fungal spores (3- 4 x 10^7) maintained on TAA. The inoculated culture medium was incubated at 30 ^{0}C in an orbital shaker (Orbitek, Scigenics India, Chennai) at 120 rpm for 120 h.

3.4 Harvesting of fungal biomass

The mycelial suspension was filtered through Whatman No.1 filter paper, washed thrice with glass distilled water and finally with 0.05 M citrate buffer, pH 5.0 (extraction buffer), pressed against filter paper and wet weight was noted. The mycelial

mass was processed for enzymatic studies. If the mycelial mass was not to be immediately processed, then the same was stored at –20 ^{0}C.

3.5 Preparation of mycelial extract

The procedure described by Bridge, (1996) was followed with slight modifications. The mycelial mass was harvested at the scheduled time. A 10 % mycelial suspension was made in extraction buffer and frozen overnight at –20 ^{0}C. Acid-washed sand, four times the weight of the mycelium was added, and the mixture was ground in a chilled pestle-mortar kept in an ice bath. The homogenate was centrifuged at 12,000 x *g* for 30 min at 4 ^{0}C using K-24 (Janetzki) centrifuge. The supernatant (mycelial extract) was used for tannase assay (Sharma, 1998). The mycelial extract was stored at 4 ^{0}C to preserve the enzymatic activity. The pellet was discarded.

3.6 Tannase assay

Tannase (tannin acyl hydrolase; EC 3.1.1.20) was assayed by the method of Sharma *et al.*, (2000), using gallic acid as standard.

Reagents

1. Citrate buffer	0.05 M, pH 5.0
2. Substrate solution	Dissolved 18.4 mg of gallic acid methyl ester (methyl gallate) in 10 ml of extraction buffer.
3. Rhodanine solution	0.667 % in methanol.
4. 0.5 M KOH	Dissolved 28.05 g of potassium hydroxide pellets in one l of distilled water.

Mycelial extract (0.25 ml) and substrate solution (0.25 ml) were pre-incubated at 30 ^{0}C for 10 min.

3.6.1 Protocol for the assay of tannase activity

	Blank	Test	Control
Citrate buffer (ml)	0.25	---	---
Mycelial extract (ml)	---	0.25	---
Substrate solution (ml)	0.25	0.25	0.25
Incubated at 30 ^{0}C for 5 min			
Rhodanine solution (ml)	0.3	0.3	0.3
Incubated at 30 ^{0}C for 5 min			
KOH solution (ml)	0.2	0.2	0.2
Incubated at 30 ^{0}C for 2.5 min			
Mycelial extract (ml)	---	---	0.25
H_2O (ml)	4.0	4.0	4.0
Kept the tubes at 30 ^{0}C for 10 min			

Absorbance was recorded against water at 520 nm. The enzyme activity was calculated from the change in absorbance.

$$\Delta A_{520} = (A_{test} - A_{blank}) - (A_{control} - A_{blank})$$

One unit of enzyme activity was defined as the number of micromoles of gallic acid formed per min.

3.7 Gallic acid estimation

3.7.1 Gallic acid produced during tannin biotransformation studies using purified tannase was estimated by the protocol given at 3.6.1. For estimation of gallic acid (GA) standard curve, aliquots of GA solution containing 1-10 μg GA were taken.

3.7.2 Gallic acid produced during tannin biotransformation studies using whole fungus was estimated by the method of Inoue *et al.*, (1988) using GA as standard.

Reagents

GA solution (1mg/10ml) for use as standard was prepared fresh in extraction buffer. Other reagents were as given above for tannase assay.

3.7.3 Protocol for GA estimation

(a) For estimation of GA standard curve, aliquots of gallic acid solution containing 10-100 µg gallic acid were taken and the volume was made to 1.0 ml with extraction buffer. The blank contained 1.0 ml extraction buffer.

(b) For estimation of GA yield in culture filtrate, 1.0 ml of the culture filtrate was taken.

(c) Added 1.5 ml 0.667 % rhodanine (in methanol) solution to the tubes, and incubated at 30 ^{0}C for 5 min.

(d) Added 1.0 ml of 0.5 M KOH solution to the tubes and incubated at 30 ^{0}C for 2.5 min.

(e) Added 21.5 ml distilled water to the tubes and incubated at 30 ^{0}C for 10 min.

Absorbance was recorded against water at 520 nm. GA concentration was calculated from the standard curve. The increase in absorbance over blank was used for calculations.

3.8 Protein Estimation

Protein was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin (BSA) as a standard.

Reagents

1. Bovine Serum Albumin (BSA) standard solution: 1 mg/ml.
2. Sodium hydroxide (0.1 N); Prepared 0.1 N NaOH solution by dissolving 4 g NaOH pellets in 1000 ml water.
3. Reagent A: Anhydrous sodium carbonate solution (8 %); dissolved 80 g of anhydrous sodiun. carbonate in one litre of distilled water.
4. Reagent B: Sodium potassium tartarate and copper sulphate solution; dissolved 0.6 g of copper sulphate and 21.2 g of sodium potassium tartarate in one litre distilled water.
5. Reagent C: Mixed reagent A and reagent B in the ratio of 1:1 just before use.
6. Folin-Coicalteau's reagent: Prepared 1 N Folin reagent by diluting 2 N Folin reagent with distilled water.
7. 20 % TCA solution in distilled water.

3.8.1 Protocol for protein estimation

The sample was precipitated by addition of 0.5 ml of 20 % TCA to 0.5 ml of sample. The tubes were left overnight at 4 ^{0}C and then centrifuged at 3000 rpm for 15 min to obtain the protein pellet. The supernatant was discarded and the pellet was dissolved in 1.0 ml of 1 N NaOH. Aliquots (0.1-0.3 ml) were taken and volume was made to 0.5 ml with 1 N NaOH. The blank contained 0.5 ml of 1 N NaOH.

Reagent C (5 ml) was added to all the tubes, mixed immediately and incubated at 37 ^{0}C for 10 min. Folin & Ciocalteu's reagent (1 N, 0.5 ml) was added, mixed thoroughly and incubated at 37 ^{0}C for 30 min.

Absorbance was measured at 725 nm. The increase in absorbance over the blank was used for calculations.

3.9 Improving the yield of tannase

3.9.1 Using Polytron homogenizer

A 10 % suspension of mycelial mass was made in extraction buffer and kept overnight for freezing (congelation). The frozen mass was extracted using Polytron PT3000 Kinematica AG homogenizer at 20,000 rpm for 8-10 min, and the homogenate was further processed in the usual manner. For comparing Polytron homogenization with pestle and mortar extraction, a control sample was extracted by using pestle and mortar. To minimize the batch to batch variation, mycelial mass of the same batch and equal weight were taken for both the processes.

3.9.2 Using detergents

Four detergents, t–Octylphenoxypoly-ethoxyethanol (Triton X-100), polyoxy-ethylene sorbitanmono-laurate (Tween 20), N- Cetyl- N, N, N- trimethyl ammonium bromide (CTAB) and sodium dodecyl (lauryl) sulphate (SDS) were tried. The detergent (1 mM) was added to 10 % mycelial suspension made in extraction buffer and frozen overnight at –20 ^{0}C. Simultaneously, a control of the same mycelial batch and equal weight was extracted, without pretreating it with any detergent. Extraction was done using pestle and mortar and processing was done in usual manner. After extraction, the extract was dialyzed against extraction buffer. Protein content and enzyme activity were estimated after dialysis. To minimize the variation, mycelial mass of equal weight and the same batch, were taken for all treatments.

3.9.3 Using extraction buffers of different pH values

During harvesting of fungal biomass when extraction buffer was added for final rinsing, it was observed that filtrate obtained was almost colourless in comparison to the filtrate obtained when distilled water was used for washing. The effect of buffer pH on pigment and enzyme release, was therefore investigated for finding out the correlation between them. Citrate buffer 0.05 M (pH 4.5. 5.0, 5.5, and 6.0), and phosphate buffer 0.05 M (pH 6.5, 7.0, 7.5, and 8.0), were used. A 10 % suspension of mycelial mass was made in buffer of each pH value. Extraction was done in the usual manner. Protein content and enzyme activity were estimated. To minimize variation, mycelial mass of the same batch and equal weight were taken for all the treatments.

3.10 Enzyme purification

3.10.1 Enzyme extraction

The mycelial mass was harvested after 120 h of incubation by filtering it on Whatman No.1 filter paper, and was washed thoroughly with distilled water till most of the pigments appeared in the filtrate. The final washing was with extraction buffer using 10 ml buffer per g mycelial mass, and was kept overnight at –20 ^{0}C. This step was needed for disrupting the fungal cell wall structure for facilitating release of the enzyme. Crude mycelial extract was prepared as described in section 3.5.

3.10.2 Concentration of the crude extract (Reverse dialysis).

A measured volume of the crude extract was taken in a dialysis membrane bag kept in a beaker at 4 ^{0}C. The dialysis bag was completely covered with sucrose powder. PEG-6000 was avoided as it is known to bind proteins. The extract was periodically

monitored for decrease in volume of its contents, to nearly one-tenth of the original volume. The concentrated sample was recovered from the bag. The bag was rinsed with extraction buffer for ensuring complete recovery.

3.10.3 Dialysis

The concentrated crude extract was exhaustively dialysed at 4 ^{0}C against extraction buffer, on a magnetic stirrer using dialysis membrane. The dialysed extract was recovered from the bag. The bag was rinsed with extraction buffer to ensure complete recovery of the enzyme.

3.10.4 Lyophilization

Dialysed crude extract was frozen and lyophilized at –40 ^{0}C in a freeze dryer (Edwards EF4 Modulyo) till a minimum volume or powder was obtained.

3.10.5 Sample reconstitution

Lyophilized sample was reconstituted with minimum amount of water. Reconstitution of the sample was done with water to avoid increase of salt concentration due to addition of extraction buffer, and its effect on chromogen formation. An aliquot was kept for protein estimation and enzyme assay, and the remaining portion was loaded on the column.

310.6 Sephadex G-150 Chromatography

The details of gel preparation and chromatography were as given in Gel Filtration: Theory and Practice (Pharmacia Fine Chemicals, Sweden). The swollen gel was degassed and was packed in column to a bed size of 2.5 x 35 cm (bed volume

171 ml). The column was equilibrated with extraction buffer. The concentrated sample from the preceding step was applied on the column. Elution was done with extraction buffer. Forty fractions of 5 ml each were collected and monitored for elution profile of proteins by measuring absorbance at 280 nm, and for tannase activity. Fractions with high tannase activity were pooled and lyophilized for next purification step.

3.10.7 DEAE Sephadex A-50 Chromatography

DEAE Sephadex A-50 gel preparation was according to the protocol given in Manual on Ion Exchange Chromatography, Principles and Methods (Pharmacia Fine Chemicals, Sweden). Column was packed to a bed size of 1.5 x 25 cm (bed volume 44 ml) and was equilibrated with extraction buffer. The concentrated sample was applied on the column. Elution was done with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 M citrate buffer, pH 5.0 (5 fractions of 5 ml each for buffer of each molarity). All the fractions were monitored at 280 nm for the elution profile of proteins and assayed for tannase activity. Fractions with high tannase activity were pooled for further processing.

3.10.8 Dialysis of fractions

The pooled fractions with tannase activity from the preceding step were subjected to exhaustive dialysis against water at 4 ^{0}C, on magnetic stirrer using dialysis membrane.

3.10.9 Lyophilization of fractions

Dialysed fractions were frozen and lyophilized at –40 ^{0}C in freeze drier (Edwards EF4 Modulyo) till powder or minimum volume was obtained and was used for further investigations.

3.11 Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the number of polypeptides in enzyme protein and their molecular weight. SDS-PAGE was done according the method of Takdes, (1979) and it was run in a vertical slab gel electrophoresis apparatus (Mini Model type 0501; Bangalore Genei Pvt ltd). Acrylamide concentration was 4 % for stacking gel and 10 % for separation gel. TEMED and ammonium persulphate were used as reaction catalysts. Sample was hydrolysed by adding equal amount of 2 X sample buffer to tannase I (refer section 4.1.1) and Kikkoman tannase, and then heating it in a boiling water bath for 2 minutes. Molecular weight markers (20 µl), tannase I (20 µl; 1.7 mg/ml) and Kikkoman tannase (20 µl; 2 mg/ml) were loaded and the gel was run at 150 V, 25 mA for 1 h or till the tracking dye bromophenol blue reached the other end of the gel.

Staining of the gel was done by keeping the gel overnight at room temperature in 0.25 % solution of Coomassie blue in methanol: acetic acid: distilled water (5:1:5). Excess dye was removed by keeping in a destaining solution containing 10 % acetic acid and 35 % ethanol for 2 h, or till the gel became transparent.

Drying of the PAGE gel was according to the method of Vasiliki *et al.*, (2000) by soaking gel twice in 95 % ethanol. The gel was placed in a Petri dish or an evaporating tray containing 10 gel volumes of ethanol (95 % v/v) and stirred for 15 min. After that, the ethanol solution was replaced with fresh 95 % ethanol, and soaking was continued for 5 min. In the final step, the gel was removed from the ethanol solution and placed on a OHP sheet, and then it was covered with a glass plate to avoid curling during the final stages of ethanol evaporation. The gel was ready for storage in 12 h.

3.12 Michaelis - Menten Constant (K_m) for different substrates

K_m was calculated for three substrates *viz.* tannic acid (ACS grade), methyl gallate and propyl gallate, by monitoring tannase I activity at different concentrations (0.1 mM – 1.0 mM) of each substrate. The reaction product in each case was gallic acid and was measured in usual manner. K_m was calculated by method given by Segel, (1968).

3.13.1 Effect of Enzyme Activity Modulators

Six different compounds *viz.* $ZnCl_2$, EDTA, $MgSO_4$, $CdCl_2$, $MnCl_2$, and $CaCl_2$ were used as modulators. Inhibition and activation studies were done at 20 mM concentration. Tannase I and an individual salt were incubated for 30 min prior to tannase assay.

3.13.2 Enzyme inhibition

Two different concentrations, (20 and 40 mM) of inhibitor $CaCl_2$ were used. Enzyme activity was monitored at different substrate methyl gallate concentrations *viz.* 0.2, 0.4, 0.6, 0.8 and 1.0 mM. Inhibitor, substrate and enzyme (tannase I) were incubated for five min, and the assay was done by the standard procedure. K_i was calculated by method given by Segel, (1968).

3.14 Determination of Esterase and Depsidase activity

Total enzyme activity of two forms of tannase (I and II; refer section 4.1.1) was estimated using tannic acid (ACS grade; 1 mM) as substrate, and esterase activity was determined by using methyl gallate (1mM) as substrate. Depsidase activity was calculated from the difference between the total tannase and esterase activities.

3.15 Biotransformation of Tannic acid

3.15.1 By *A. niger* van Teighem

Biotransformation studies were carried out by culturing *A. niger* using 0.2 % tannic acid (ACS grade). The production of gallic acid was monitored by harvesting inoculated flasks at 0, 24, 48, 72, 96 and 120 h of incubation, and estimating the gallic acid in the culture filtrate. An uninoculated control was kept for each stage of harvesting.

3.15.2 Using Tannase

Biotransformation studies were conducted by monitoring the amount of gallic acid produced from 0.1 % tannic acid (ACS grade), by the action of tannase I. Incubation was done for 0, 6, 12, 18, 24, 30, 36, 42, 54, 60, 66, 72, 78, 84, 96, 102 and 120 h. A control was kept for each hour of incubation.

3.16 Statistical Analysis

The analysis of data was done by using the statistical software (Sigma Stat for Windows, version 1.0), developed by Jandel Scientific Corporation, USA. Each experiment was repeated three times and the data are means of triplicate determinations. Results with probability levels greater than 5 % were regarded as non-significant.

RESULTS

CHAPTER - 4

RESULTS

The present study had four aims and the results from the same have been presented under the following sections:-

1. Studies on improving the yield and purity of tannase.
2. Substrate affinity studies.
3. Investigations on the effect of activators and inhibitors on tannase activity.
4. Esterase and depsidase activity of tannase.
5. Biotransformation of tannic acid.

4.1 Improving the yield

Homogenization using Polytron homogenizer was used for extraction of tannase from mycelial mass. For comparison, pestle and mortar extraction was kept as control. Polytron extraction gave tannase yield which was significantly lower ($p < 0.05$) than the yield obtained through pestle and mortar method (Fig 5). However, protein extraction obtained by Polytron homogenizer, and pestle and mortar were comparable.

Four detergents *viz.* CTAB, SDS, Tween-20, and Triton X-100 were used for pretreatment of mycelial mass prior to congelation (Fig 6). Comparison of tannase activity among them and with control showed that pretreatment with Triton X-100 gave highest mean enzyme activity, followed by CTAB and then control. SDS and Tween-20 gave tannase activity which was lower than the control. Protein yield was maximum for SDS followed by Tween-20, Triton X-100, control and CTAB. The values observed for the various treatments and control for tannase activity and protein yield were statistically nonsignificant.

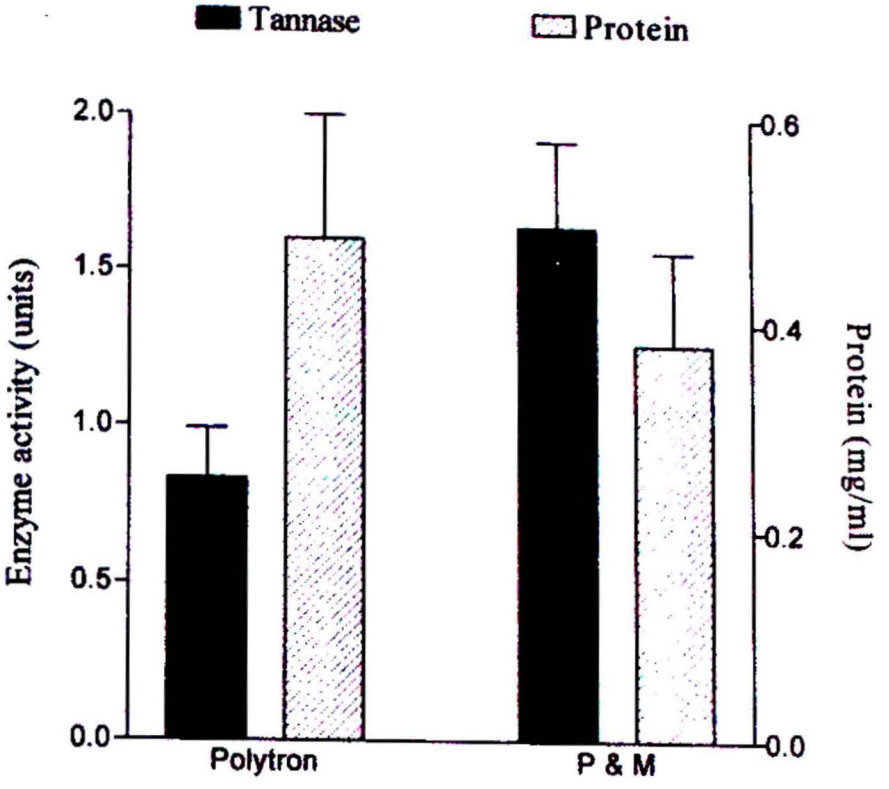

Fig 5 : Effect of physical methods (Polytron - polytron homogenizer; P&M - pestle and mortar) on protein extraction and tannase activity of *A. niger* (Mean ± S. D.).

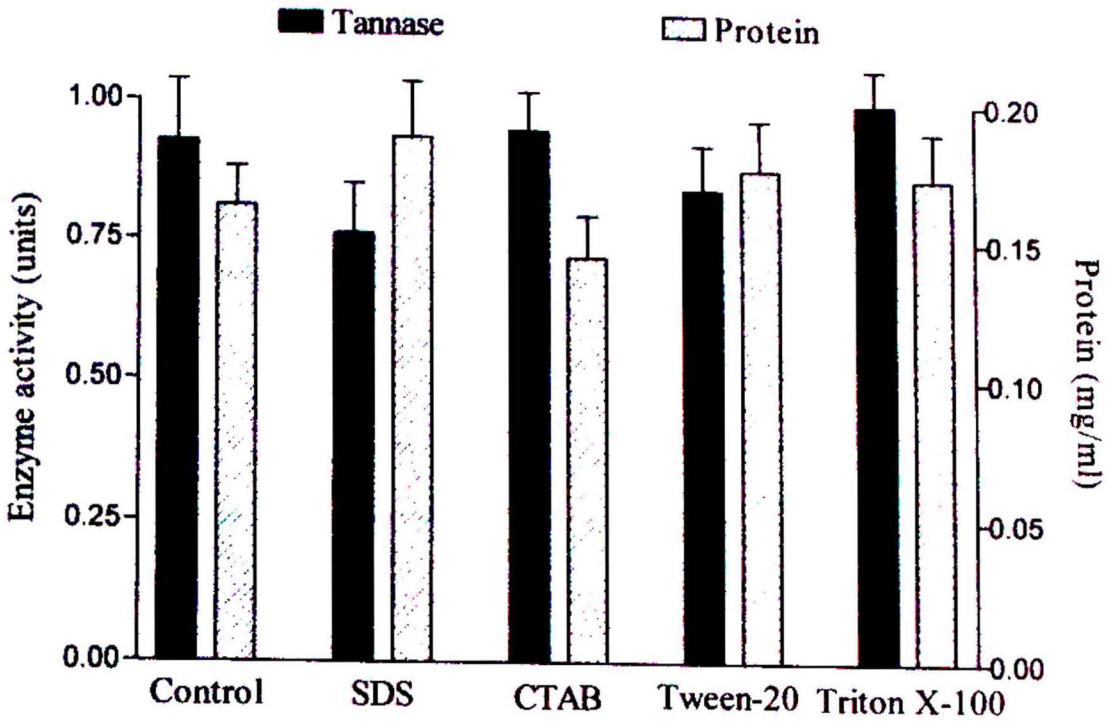

Fig 6: Effect of detergents on protein extraction and tannase activity of *A. niger* (Mean + S. D.).

During the routine harvesting of fungal mass, it was observed that the release of melanin, the fungal pigment, was pH dependent. For verifying the pH dependence of protein and enzyme release, and its correlation with melanin release, mycelial mass was extracted using buffers of eight different pH *viz.* 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The set of pH 5.0 was kept as control. It was observed that enzyme and protein release were highly pH dependent (Fig 7). The highest tannase activity was in the extracts prepared at pH 6.0 and the lowest was in the samples extracted at pH 6.5. The maximum protein extraction was at pH 7.5 and the minimum was at pH 4.5. The highest enzyme activity vis-a-vis protein concentration was maximum at pH 5.5. The tannase activity of the extracts prepared at pH 4.5, 5.5, 6.0, 6.5 and 7.0 was significantly different ($p < 0.05 - 0.01$) from the control. The differences between protein yield in the mycelial extracts prepared at pH 6.5, 7.0, 7.5 and 8.0 were significant ($p < 0.01 - 0.001$), when compared to control value. A visual observation of the colour intensity of mycelial extract indicated that the fungal pigment was released in the following order: pH 7.5 > 8.0 > 7.0 > 6.5 > 6.0 > 5.5 > 5.0 > 4.5.

4.1.1 Enzyme purification

The elution profile of Sephadex G-150 column chromatography for protein and tannase activity is shown in Fig 8. Protein was distributed in one major and one minor peak. A major peak towards the end of the elution was contributed by the fungal pigments. This chromatographic step successfully removed the pigments from the crude mycelial extract. Fractions from Sephadex G-150 column chromatography containing high tannase activity were pooled and lyophilized to a minimum volume and applied on DEAE-Sephadex A-50 column. The elution profile of proteins and tannase eluted from DEAE- Sephadex A-50 column is given in Fig 9. Absorbance at 280 nm for protein

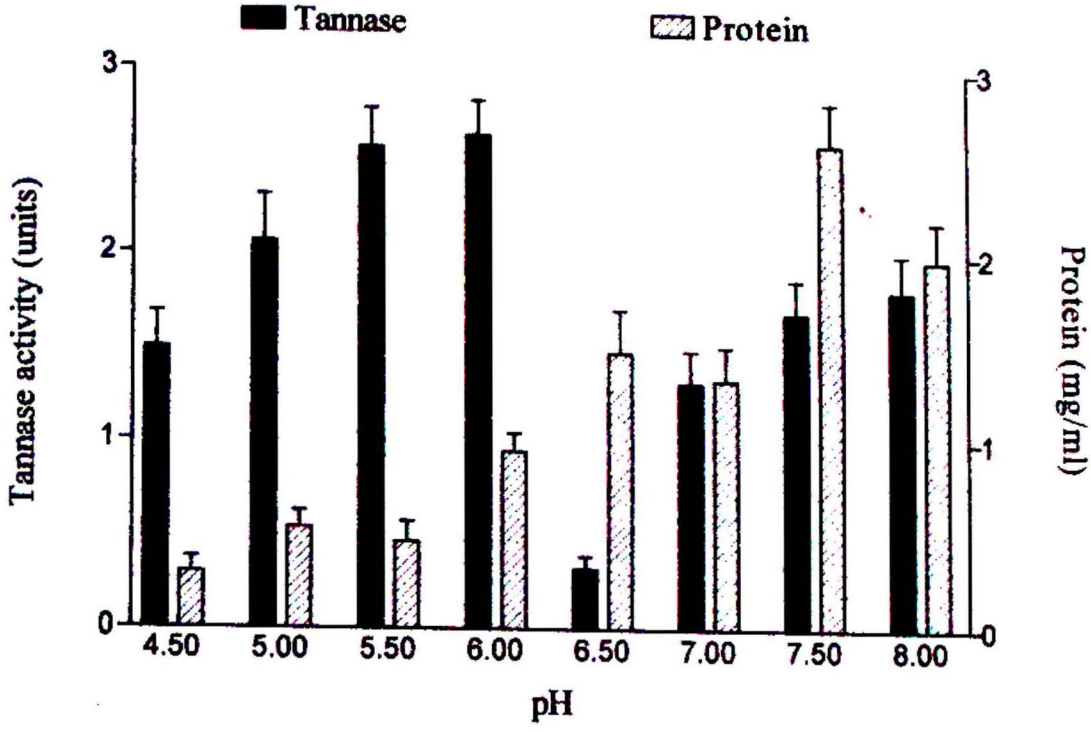

Fig 7: Effect of pH of buffer on protein extraction and tannase activity of *A. niger* (Mean ± S. D.).

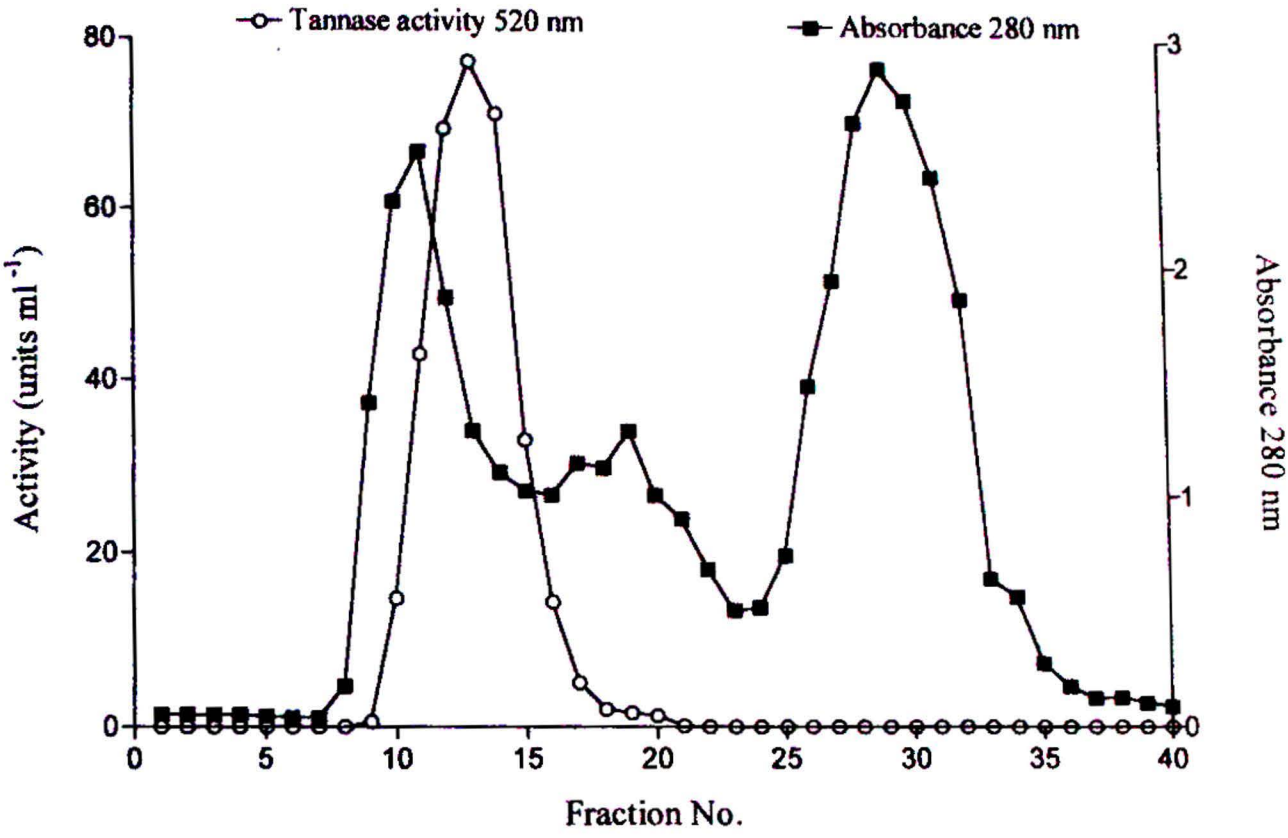

Fig 8 : Elution profile of *A. niger* tannase on Sephadex G-150 column. Each fraction was 5 ml.

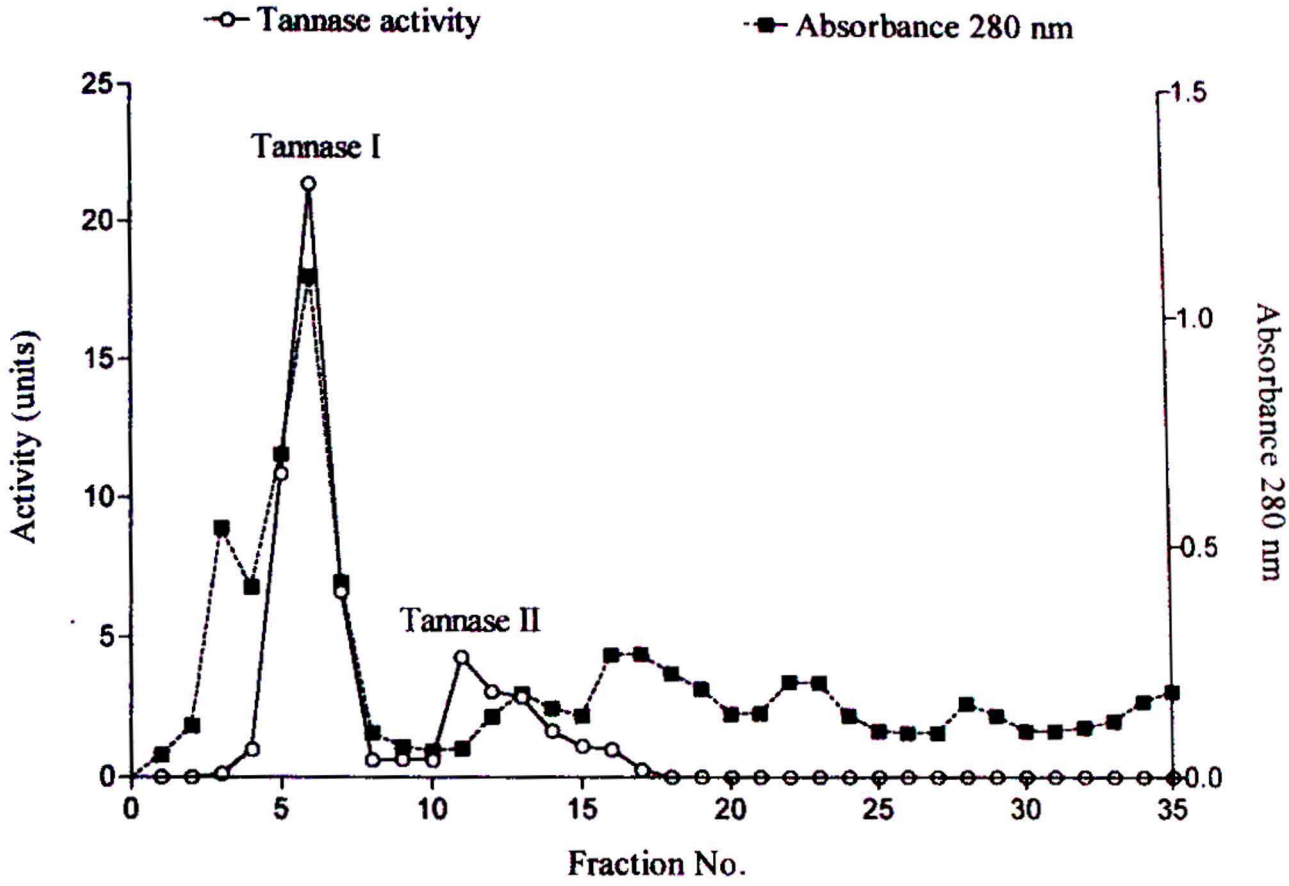

Fig 9: Elution profile of *A. niger* tannase on DEAE-Sephadex A-50 column. Each fraction was 5 ml.

content showed one major peak (fraction 5 to 7) which coincided with the major peak for tannase activity. A minor tannase peak was obtained between fraction 11 to 16. So fractions 5 to 7 and 11 to 16 were pooled separately and termed as tannase I and tannase II, respectively. They were dialysed against water for removal of salts, lyophilized for storage and used in various studies. Enzyme preparation obtained after DEAE-Sephadex A-50 column chromatography had a recovery of 20 %, and overall purification was 51-fold. The details of data obtained after different stages of purification are given in Table 3.

4.1.2 Electrophoresis

SDS-PAGE electrophoresis of tannase I gave two major bands indicating that the enzyme is composed of two polypeptides of molecular weight 102 kDa and 83 kDa. Kikkoman tannase also gave two bands of molecular weight 87 kDa and 56 kDa (Fig 10).

4.2 Michaelis-Menten constant (K_m) for different substrates

The Lineweaver-Burke plots of purified tannase I with tannic acid, methyl gallate, and propyl gallate as substrates are shown in Fig 11, 12, and 13, respectively. The Michaelis-Menten constant (K_m) for tannic acid, methyl gallate and propyl gallate were 2.8×10^{-4} M, 9.5×10^{-4} M and 20.5×10^{-4} M, respectively.

4.3 Effect of enzyme activity modulators

Different chemicals were tested for their modulatory effect on tannase activity (Fig 14). None of them had an activating effect on tannase and EDTA neither activated nor inhibited the enzyme. $CaCl_2$ showed maximum inhibition (58 %), followed by $CdCl_2$

Table 3: Summary of purification of tannase from *A. niger* van Tieghem.

Purification step	Volume of extract (ml)	Protein (μg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Crude extract	1000	23	23. 0	0.16	160	7.0	100	1
Sephadex G-150	35	83	2.9	2.4	83	28.6	52	4
DEAE Sephadex A-50	15	6	0.09	2.1	32	355.6	20	51

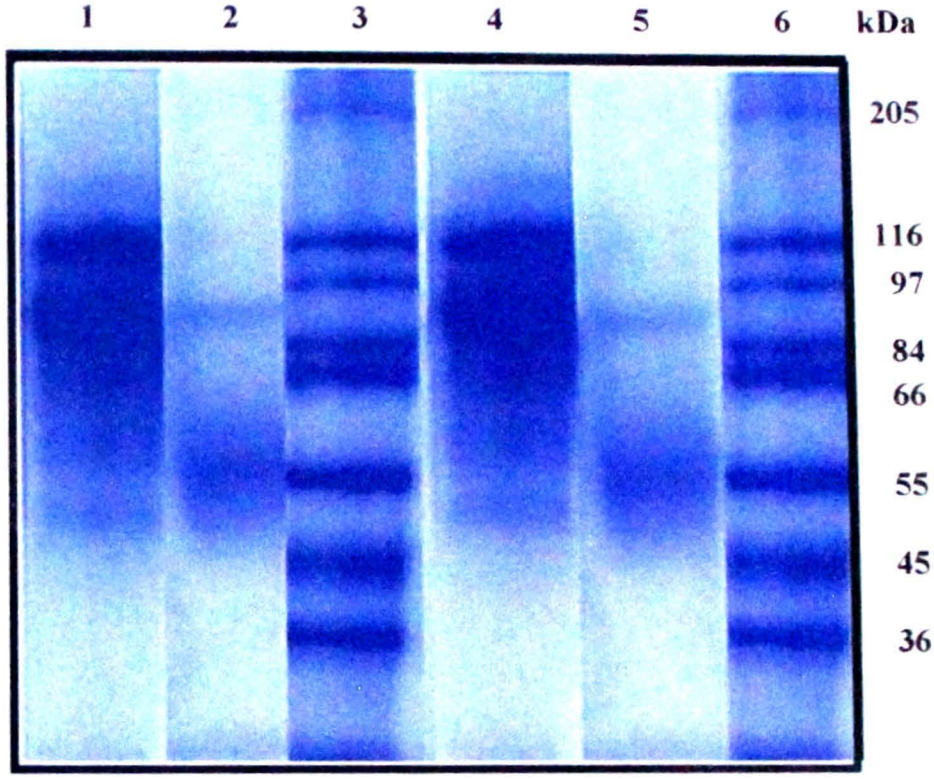

Fig 10: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of tannase from *Aspergillus niger* van Tieghem. The lanes contain (1) & (4) *A. niger* tannase, (2) & (5) Kikkoman tannase, (3) & (6) molecular weight markers.

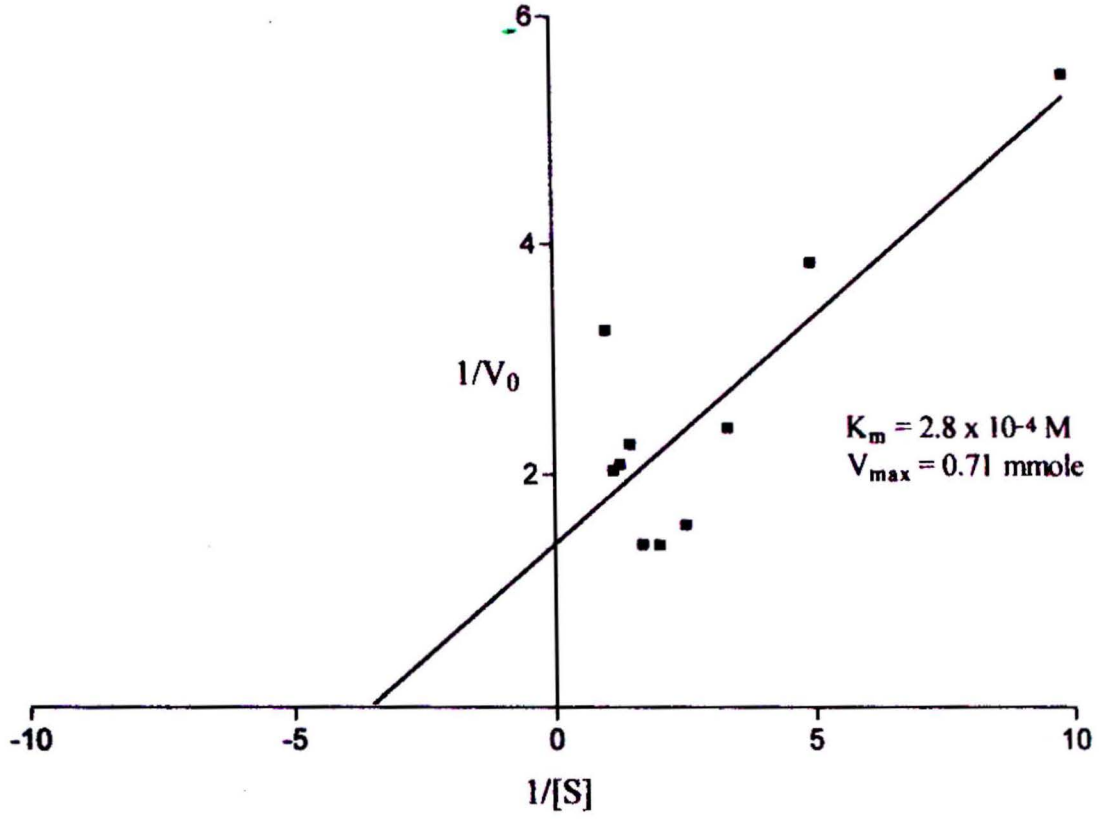

Fig 11: Lineweaver-Burke plot of *A. niger* tannase with tannic acid as substrate

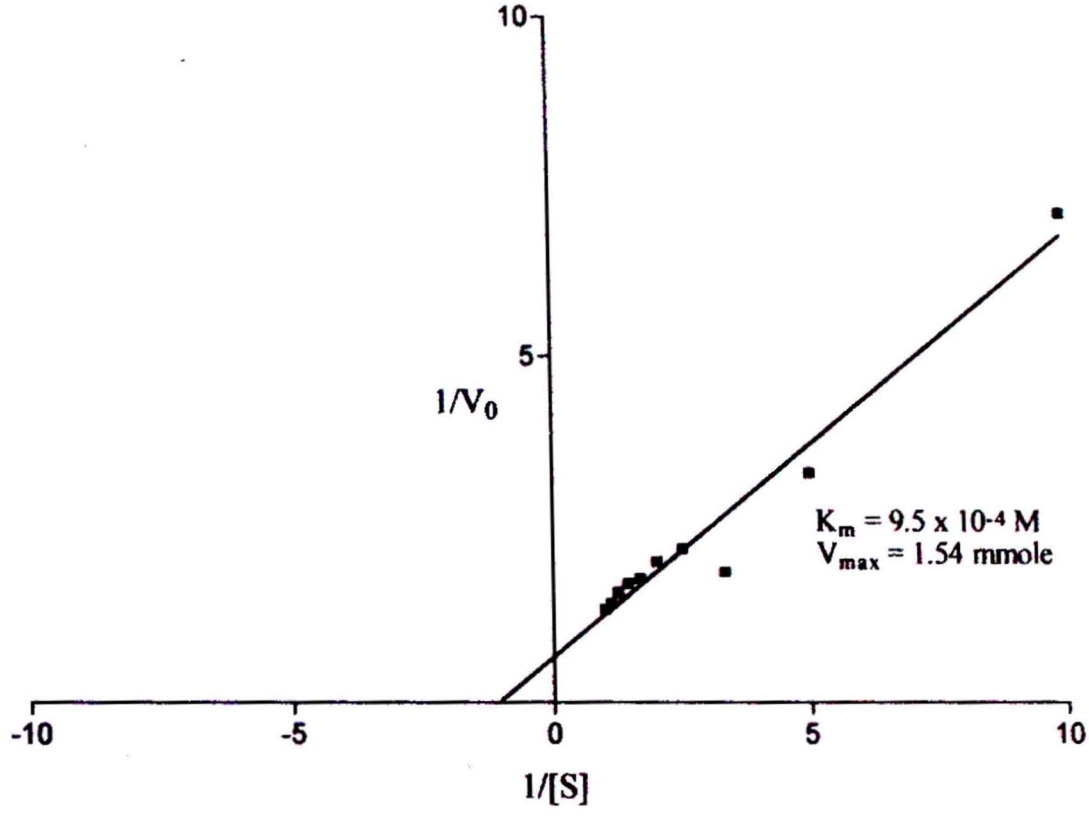

Fig 12 : Lineweaver-Burke plot of *A. niger* tannase with methyl gallate as substrate

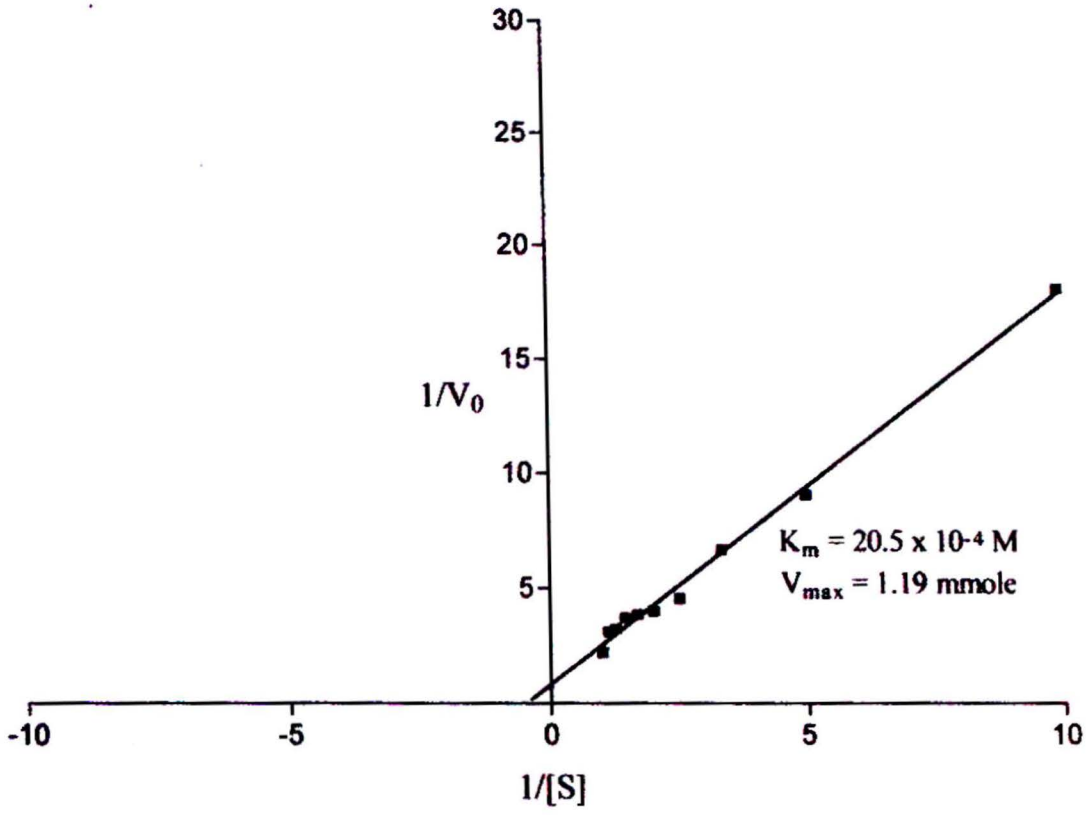

Fig 13: Lineweaver-Burke plot of *A. niger* tannase with propyl gallate as substrate

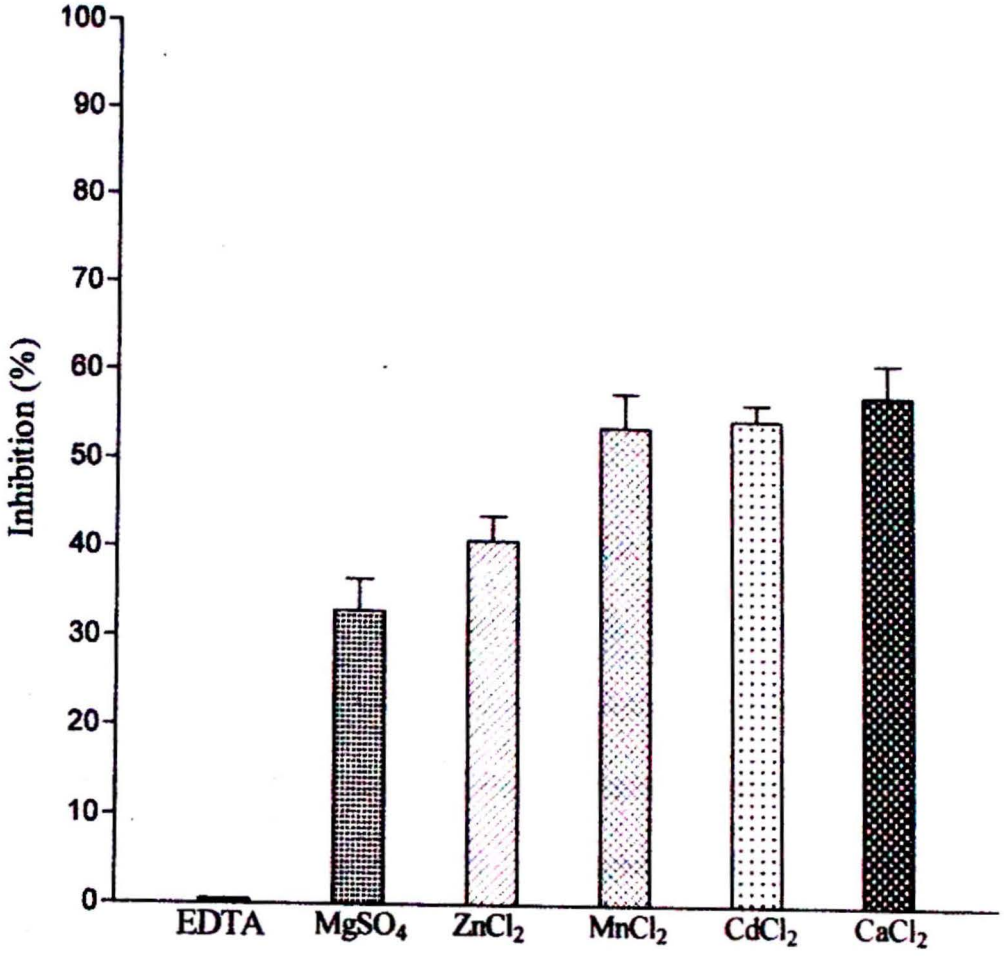

Fig 14: Effect of activity modulators on *A. niger* tannase activity (Mean ± S. D.).

(55 %), $MnCl_2$ (54 %), $ZnCl_2$ (41%) and $MgSO_4$ (33 %). K_i of $CaCl_2$ the salt that had maximum inhibitory effect, was found to be 5.9×10^{-4} M and the inhibition was of non-competitive type (Fig 15).

4.4 Esterase and depsidase activity

Tannase I and tannase II were investigated for esterase and depsidase activities (Fig 16). Results show that tannase I which appeared as the major peak had 52 % esterase and 48 % depsidase activity, and tannase II which appeared as a minor peak had 46 % esterase and 54 % depsidase activity.

4.5 Biotransformation of tannic acid

The per cent transformation of tannic acid into gallic acid at, different intervals of incubation with whole fungus *A. niger* van Teighem and purified enzyme (tannase I), is shown in Fig 17. Tannase I was used for biotransformation of tannic acid as it was the major form of the enzyme and constituted its predominant component.

With the whole fungus, tannic acid conversion could not be observed up to 18 h. Between 18 to 72 h there was an increase, and from 72 to 120 h there was a decrease in the tannic acid conversion. The maximum conversion of tannic acid to gallic acid (25.4 % yield of gallic acid) was observed at 72 h of incubation.

With purified tannase, the tannic acid conversion sharply increased up to 12 h of incubation. After that, between 12 to 84 h, the conversion was more or less constant, giving maximum conversion (36.4 % yield of gallic acid) at 54 h of incubation. After 84 h incubation there was a slow down in the production of gallic acid.

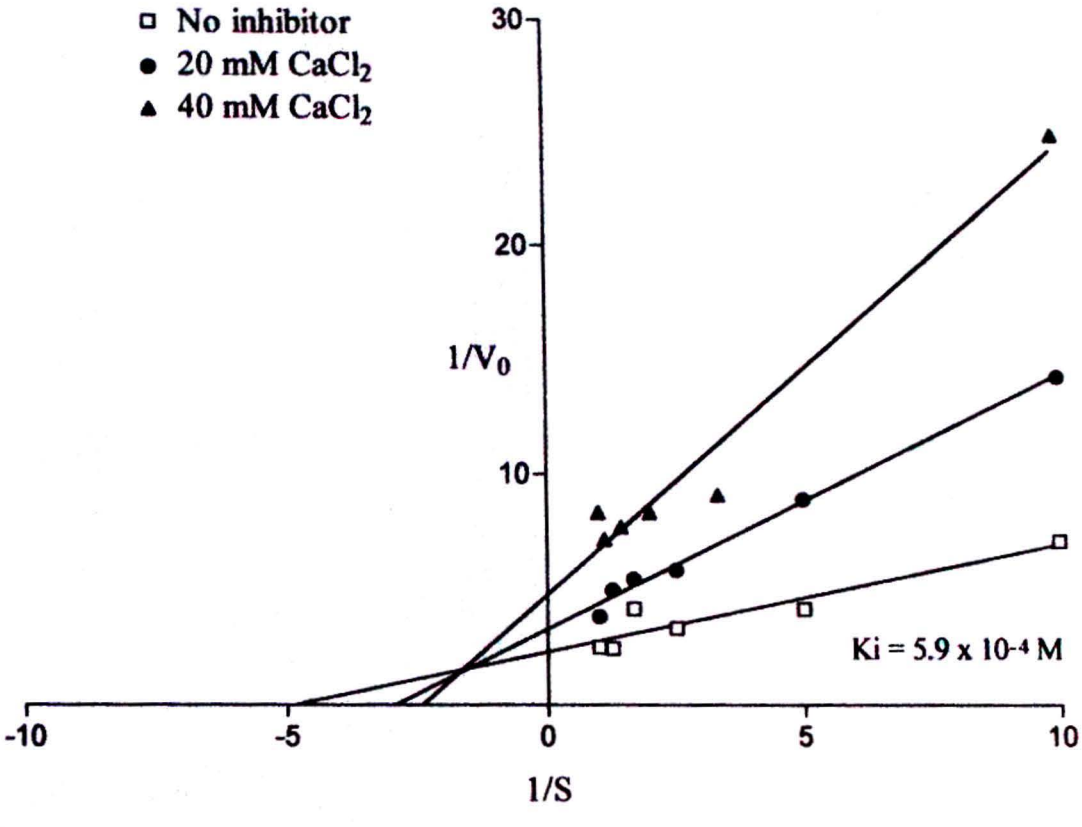

Fig 15 : Lineweaver-Burke plot of *A. niger* tannase in presence of calcium chloride as inhibitor and methyl gallate as substrate.

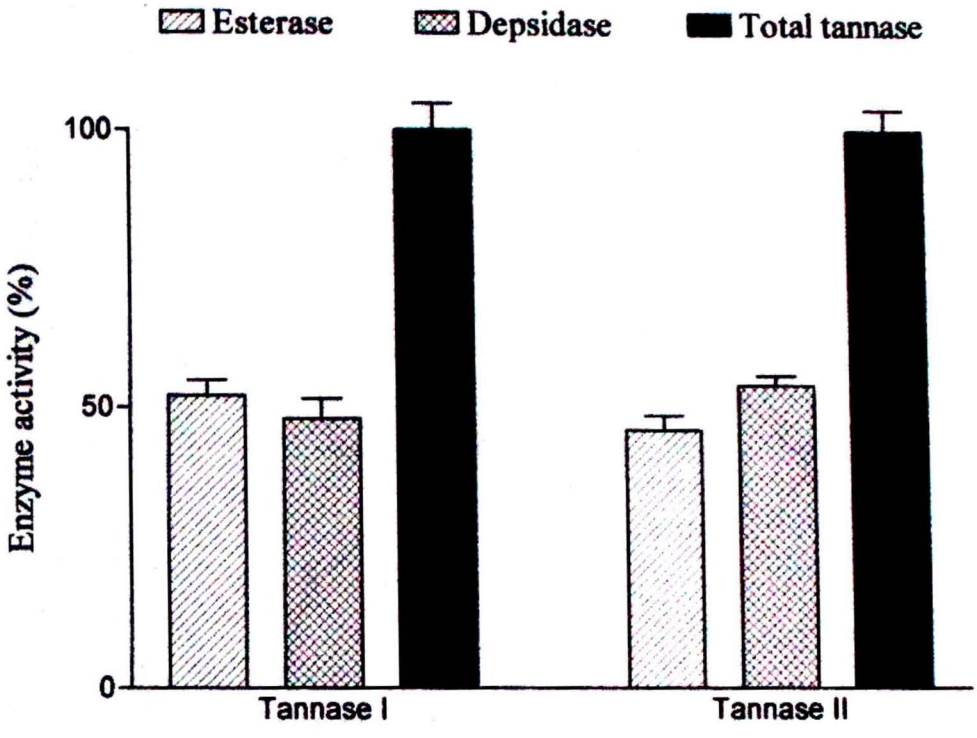

Fig 16: The esterase, depsidase and total tannase activities of tannase I and tannase II forms of *A. niger* (Mean ± S. D.).

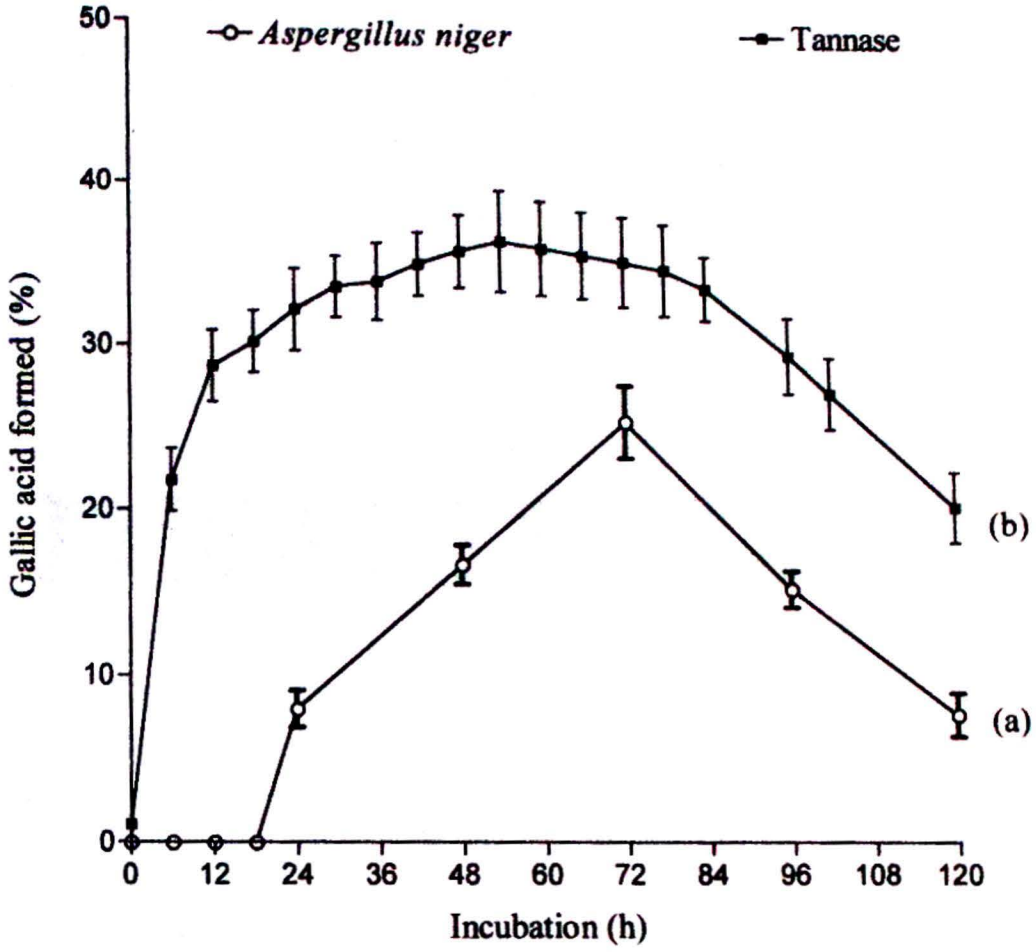

Fig 17: Effect of time of incubation on the yield of gallic acid from tannic acid when (a) incubated with *A. niger*, and (b) reacted with *A. niger* tannase (Mean ± S. D.)

DISCUSSION

CHAPTER - 5

DISCUSSION

Tannase is produced by a number of microorganisms (Lekha and Lonsane, 1996). In the present investigations, the enzyme was produced by submerged fermentation process and the type of tannase studied was of the intracellular type. Earlier studies have shown that *Aspergillus niger* van Teighem mostly produces intracellular tannase and has very low extracellular tannase activity (Sharma, 1998). However, the problem with intracellular tannase is that it is strongly bound with the mycelium (Barthomeuf *et al.*, 1994). Hence, we attempted to maximize the extractability of tannase from mycelial mass by using physical and chemical methods, separately or in combination.

Extraction of the mycelial mass by the conventional pestle and mortar method gave a higher extractability of the enzyme as compared to that obtained when the mycelial mass was disintegrated in a Polytron homogenizer. The latter method gave a higher protein yield than the pestle and mortar method and may be suitable for those conditions where higher yield of total protein is required. However, for tannase extraction pestle and mortar method seemed to be better suited as it gave higher yield of the enzyme protein which was indicated by its increased activity. A number of detergents were tried for increasing the protein and tannase yield. The mycelial mass was pretreated with a particular detergent and disintegrated using pestle and mortar. Treatment with Triton X-100 gave highest enzyme activity whereas treatment with SDS gave the highest protein yield. However, in both the cases a statistical comparison of the data indicated that pretreatment with detergent did not have a significant effect on the extraction of total protein including tannase protein.

During the course of extraction, it was frequently observed that pestle-mortar grinding of mycelial mass congelated in extraction buffer, yields mycelial extract which contains pigments. It was also observed that extraction of the mycelial mass using distilled water as the extraction medium gave higher yield of fungal pigments. This led to the feeling that the extraction of the pigment, and may be fungal protein, was pH dependent. When the mycelial mass was extracted using buffers of different pH values, a clear pattern emerged from the results of this experiment. At pH 6.0 and below, tannase activity was significantly higher than protein yield. The extractability of melanin also followed a decreasing trend in acidic pH. Thus, it seems that due to low release of melanin in the acidic pH range, less of tannase protein becomes bound to the pigment and the greater proportion of the enzyme is in the free form for expressing its activity. It appears that tannase protein has greater binding affinity to fungal pigments. It is known that interactions of fungal melanins with cytosolic proteins are dependent on the pH of the solution (Doering *et al.*, 1999). Mani *et al.*, (2001) have demonstrated the importance of an acidic pH of 6.0 for the binding of melanin with proteins in human melanosomes. In the acidic pH range of 3.0-6.0 and in neutral and alkaline conditions, the binding of melanin with protein decreased (Mani *et al.*, 2001). Depending on the pH of solution, the melanin-protein interaction resulted in the formation of different states of melanin *viz.* insoluble protein bound (pH 2.0 - 2.8) and soluble protein-bound form (pH 6.0 - 6.5). This phenomenon may also explain the presence of low amount of detectable protein and enzyme activity in *A. niger* mycelial extract prepared at pH 6.5.

During earlier studies on *A. niger* tannase purification, a very low amount of purified enzyme protein was available after two-stage purification (Sharma *et al.*, 1999). The present purification protocol also yielded a low amount of protein after two-stage purification. However, the recovery (yield) and specific activity of the purified enzyme

were higher at 20 % and 356, respectively, and 51-fold purification was also more than that achieved in earlier study. Barthomeuf *et al.*, (1996) also obtained a similar type of yield (15 %) but specific activity of the enzyme was higher at 1980. Another notable feature of the present study was that the problem of binding of fungal pigment to matrix of ion exchanger (DEAE-Sephadex A-50) was overcome by interchanging the steps of ion-exchange chromatography with molecular exclusion chromatography. The fungal pigment did not bind to Sephadex G-150 and was eluted after the proteins. A higher specific activity, recovery and purification of *A. niger* tannase in this study indicates that the present protocol for purification of the enzyme is an improvement on the purification procedure reported earlier by Sharma *et al.*, (1999).

In the present investigation, two peaks of tannase activity were observed in fractions collected from DEAE-Sephadex A-50 column. The fractions of the first peak which were eluted in the beginning, represented the major component (85 %) of the enzyme protein and activity. As it was interesting to know whether the minor component of tannase was similar or dissimilar to the major component, it was important to determine the nature of this component. This was done by checking their esterase and depsidase activities, and the ratio in which these activities were present in the total tannase activity. The results show that these two forms of enzyme have both esterase activity (E) and depsidase activity (D) with an E/D ratio of 1.08 for tannase I and 0.85 for tannase II, and none of them is of pure esterase or depsidase type. Haslam and Stangroom, (1966) have found a comparable ratio for these two activities of tannase from *A. niger* grown in tannic acid-containing medium. This ratio has been found to change with the type of tannins used for culture of the microorganisms (Barthomeuf *et al.*, 1996). As tannase I was found to be the predominant component having major

portion of tannase activity, this form of the enzyme was used for studying the physico-chemical characteristics of the enzyme.

SDS-PAGE electrophoresis of tannase I revealed it to be a protein composed of two polypeptide chains having molecular weights of 102 and 83 kDa. Thus, the molecular weight of the tannase used in the study was about 185 kDa. Tannase is known to be a high molecular weight protein whose molecular weight varies from 186 to 300 kDa, depending on the strain of the microorganism (Lekha and Lonsane, 1996). The native enzyme is reported to consist of two subunits of similar molecular size (Farias *et al.*, 1994; Barthomeuf *et al.*, 1994; Hatamoto *et al.*, 1996;). In the present case, however, two polypeptides of different molecular size were seen. The same was the case with the standard enzyme (Kikkoman tannase) which separated into two different polypeptides of dissimilar molecular size (87 and 56 kDa). The molecular weight of Kikkoman tannase (143 kDa) is, therefore, somewhat lower than reported so far for microbial tannases.

The inhibition pattern of the enzyme slightly varied from the observations of earlier workers. As was observed in the present case, Iibuchi *et al.*, (1968) also found no modulatory effect of EDTA. Barthomeuf *et al.*, (1994) found no effect of $MgSO_4$, $CaCl_2$, and $MnCl_2$ on the activity of tannase from *A. niger*. However, Rajakumar and Nandy (1983), reported partial inhibition of tannase by manganese (22 %) and magnesium (17 %) salts. In the present investigation, $MnCl_2$ and $MgSO_4$ gave a greater inhibitory effect of 54 % and 33 %, respectively. Iibuchi *et al.*, (1968) observed a strong inhibition (70 %) of *A. oryzae* tannase activity with $CdCl_2$, whereas only 41 % inhibition of tannase activity by $CdCl_2$ was seen in the present case. Inhibition of microbial tannase activity by $ZnCl_2$ has not been reported by earlier workers. In the present study, 55% inhibition of tannase activity was observed. Calcium chloride exhibited maximum inhibitory effect of 58 % on *A. niger* van Teighem tannase, whereas Barthomeuf *et al.*, (1994) have reported

no effect of this salt on tannase of their strain of *A. niger*. The noncompetitive type of inhibition caused by $CaCl_2$ in the present case is different from the competitive type of inhibition reported earlier for microbial tannases with various types of inhibitors (Iibuchi *et al.*, 1972; Farias *et al.*, 1994).

Rajakumar and Nandy, (1983) have reported a K_m value of 0.48×10^{-4} M for *Penicillium chrysogenum* tannase using tannic acid as a substrate. Yamada *et al.*, (1968) observed K_m values of tannase produced by *A. flavus* as 0.5×10^{-4} M for tannic acid and 8.6×10^{-4} M for methyl gallate. The activity pattern for *A. niger* van Teighem tannase with tannic acid, methyl gallate and propyl gallate as substrates, followed a pattern similar to one seen by Iibuchi *et al.*, (1972) for relative activity of these substrates with their preparation of *A. oryzae* tannase. The K_m values for the three substrates revealed that tannic acid had the highest affinity, methyl gallate was less reactive than tannic acid, and propyl gallate had the lowest affinity for the *A. niger* van Teighem tannase. It is known that the substrate with the lowest K_m value has the highest apparent affinity for the enzyme, and the best substrate is one which has highest V_{max}/K_m value (Segel, 1976). In the present study, tannic acid gave the highest V_{max}/K_m value of 2.53 followed by 1.62 for methyl gallate and 0.58 for propyl gallate. Thus, it is evident that tannic acid was the best substrate for tannase in the present case also.

Biotransformation of tannins by whole microorganisms has been studied by earlier workers (Deschamps and Lebeault, 1984; Pourrat *et al.*, 1985). The yield of 25.4 % gallic acid obtained due to conversion of tannic acid by *A. niger* van Teighem was similar to the yield of 30 % gallic acid observed by Pourrat *et al.*, (1985) when they used gallotannins with their strain of *A. niger*, and 27.5 % yield reported by Kar and Banerjee, (2000) with their fungal strain in submerged fermentation condition. The yield of 36 % gallic acid by the action of *A. niger* van Tieghem on tannic acid was reasonably good for

a non-immobilized enzyme system. Bajpai *et al.*, (1999) using an immobilized fungal mycelial system, could obtain gallic acid yields of 27.8 %, 43.9 %, 50.4 % and 50.8 % for entrapped mycelia of *A. niger, A. fischerii, Fusarium solani and Trichoderma viride*, respectively, under optimum conditions. Guo and Yang, (2000) could obtain a production of 61 % gallic acid in an immobilized tannase system. Thus, it is evident that *A. niger* van Tieghem strain has good potential for enhanced production of gallic acid, either as an immobilized mycelial system or an immobilized tannase system.

SUMMARY

CHAPTER – 6

SUMMARY

Tannase (tannin acyl hydrolase EC 3.1.20) is an enzyme that hydrolyzes the ester bonds of hydrolyzable tannins, such as tannic acid. It is known to be a ubiquitous enzyme of the microbial world and has a widespread occurrence in various fungi, bacteria and yeast. Tannase is extensively used in feed, food, beverage, brewing, pharmaceutical and chemical industries.

Various attempts have been made to purify tannase from different microorganisms. The major hurdle in its purification from *A. niger* van Tieghem is that it is very tightly bound to the mycelia. During the present study various chemical and physical methods were tried to get increased yields. Polytron homogenization did not give any enhancement of tannase extraction. Similar was the case with detergent pretreatments, and they also did not have any remarkable effect on the extraction of enzyme protein. However, extraction of fungal pigments and proteins showed high pH dependence and maximum enzyme extraction was obtained at pH 5.5.

For purification, crude enzyme extract from *A. niger* (1000 ml) was concentrated against sucrose, dialysed against extraction buffer and lyophilized. The powder obtained was reconstitiuted with water and subjected to molecular sieve chromatography. This purification step gave complete separation of enzyme from mycelial pigments. Fractions having high enzyme activity were pooled, lyophilized and subjected to ion-exchange chromatography. The active fractions were exhaustively dialysed against water and then lyophilized. The two step purification protocol gave 51-fold purified enzyme with an yield of 20 %. SDS-PAGE electrophoresis of two-step purified enzyme protein revealed

that tannase form this particular strain is composed of two polypeptides of molecular weight 102 and 83 kDa.

Based on the Michaelis-Menten constant (K_m) of tannase for three substrates tested, tannic acid was the best substrate with K_m of 2.8×10^{-4} M, methyl gallate a good substrate with K_m of 9.5×10^{-4} M and propyl gallate was a poor substrate with a K_m of 20.5×10^{-4} M.

The inhibition percent observed for different modulators were $CaCl_2$ with a maximum of 58 %, followed by $CdCl_2$ 55 %, $MnCl_2$ 54 %, $ZnCl_2$ 41 % and $MgSO_4$ 33 %. EDTA had no modulatory effect on tannase activity. K_i of $CaCl_2$ the salt that had maximum inhibitory effect, was found to be 5.9×10^{-4} M and the inhibition was of noncompetitive type.

Biotransformation of tannic acid to gallic acid with whole fungus *A. niger* van Teighem, gave a maximum conversion of 25.4 % at 72 h of incubation, and with purified *A. niger* tannase a maximum yield of 36.4 % gallic acid was obtained at 54 h of incubation.

FUTURE PROJECTIONS

1. Studies on tannase production by processes other than submerged fermentation
2. Studies on extracellular form of *A. niger* tannase.
3. Effect of type of tannins in culture medium on the induction of esterase and depsidase activities of the enzyme.
4. Substrate affinity of tannase with other types of gallotannins, and ellagitannins and catechin gallates.
5. Improvement in the thermostability of *A. niger* van Teighem tannase.
6. Ultrapurification of both forms of tannase for X-ray crystallographic studies
7. Investigations on the immobilization of the enzyme for its optimal use in various applications.
8. Investigations on the synthetic capability of tannase in non-aqueous media.

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ABSTRACT

Title of Thesis	Studies on Tannase of *Aspergillus niger* van Tieghem
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Tannase, a hydrolysing enzyme, widely occurs in microorganisms and has various industrial applications. Tannase from *A. niger* van Teighem was isolated from mycelial mass and purified 51-fold with a yield of 20 % by gel filtration and anion-exchange chromatography. The estimated molecular weight is 185 kDa and the molecule is a dimer composed of two polypeptides with molecular weight of 102 and 83 kDa.

Use of Polytron homogenizer and detergents –Triton X-100, CTAB, SDS and Tween-20, did not give any enhancement of tannase extraction. However, enzyme extraction, fungal pigment release and protein extraction showed a high pH dependence with maximum enzyme extraction at pH 5.5.

Based on the Michalis-Menten constant (K_m) and V_{max}/K_m values of the enzyme for the substrates tested, tannic acid was the best substrate ($K_m = 2.8 \times 10^{-4}$ M) followed by methyl gallate ($K_m = 9.5 \times 10^{-4}$ M). However, propyl gallate with a ($K_m = 20.5 \times 10^{-4}$ M) was a poor substrate.

The percent inhibition observed for different modulators was 58 % for $CaCl_2$, followed by $CdCl_2$ (55 %), $MnCl_2$ (54 %), $ZnCl_2$ (41 %) and $MgSO_4$ (33 %). K_i of $CaCl_2$, the salt that had

maximum inhibitory effect, was found to be 5.9 x 10^{-4} M and the inhibition was of noncompetitive type.

Biotransformation of tannic acid to gallic acid using purified tannase of *A. niger* gave higher conversion, as compared to the yield of gallic acid when whole *A. niger* was used.

(Signature of Student with date)

(Signature of Major Advisor)

Countersigned

Head of Department