BIOCONVERSION OF TANNIC ACID TO GALLIC ACID BY USING FUNGAL TANNASE

A THESIS SUBMITTED TO

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

BY

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UNDER THE GUIDANCE OF

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PUNE – 411008

(INDIA)

DECEMBER 2010

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled, **"Bioconversion of tannic acid to gallic acid by using fungal tannase"** submitted by me for the degree of doctor of philosophy is the record of work carried out by me under the guidance of Dr. M. V. Deshpande and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

Ms. Shilpa Wagh

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ACKNOWLEDGEMENT

It is an honor for me to have Dr. M.V. Deshpande as my guide. I would like to take this opportunity to express my heartfelt gratitude to him for his valuable guidance, untiring efforts and constructive criticism.

I owe my deepest gratitude to Mr. R.P. Gaikaiwari, CMD, Hi Tech Bio Sciences India Ltd. who has made available his enthusiastic support for carrying out my research work. I am thankful to him for his help in fermentation studies.

I am indeed grateful to Dr. S.M. Gadgil, Director, Hi Tech Bio Sciences India Ltd. for his valuable help during the course of this work.

I am grateful to (late) Dr. V. Shankar, (Rtd) Senior Scientist, NCL for his valuable guidance in enzyme purification work. I thank Prof P. Wangikar, Scientist IIT Mumbai and Dr. Vandana Ghormade, Scientist, Agharkar Research Institute for their help, discussions and suggestions during the present work.

It is a pleasure to thank all my colleagues from NCL and Sci Molecules India especially Dr. Santosh Tupe, Fazal, Snehal, Priti, Ejaj, Manisha, Sarika, Aseem, Supriya, Pallavi and friends for making my journey delightful and for being there always. I am indebted to my parents and brother for their patience and unconditional support.

I thank Director, National Chemical Laboratory to permit me to carry out my research work in the Biochemical Sciences Division. This thesis would not have been possible without the financial support from Sci Molecules India Pvt. Ltd.

I thank Lord Ganesha for his blessing throughout my work.

Shilpa Wagh

LIST OF ABBREVIATIONS

EDTA	Ethylene diamine tetraacetic acid
h	Hour (s)
Mg	Microgram
Min	Minute (s)
mM	Milimole (s)
μM	Micromole (s)
PAGE	Polyacryl amide gel electrophoresis
SDS	Sodium dodecyl sulphate
w/w	Weight by weight
w/v	Weight by volume
v/v	Volume by volume

ABSTRACT

The advantages of bioconversion process such as high yields, environmental safety and process reproducibility using biocatalyst (enzyme or whole cells of microorganisms) have been well established over chemical technologies. Tannin acyl hydrolase, commonly known as tannase (EC 3.1.1.20) plays a central role in bioconversion of hydrolysable tannins to produce gallic acid, a well known precursor for manufacturing of broad spectrum antibiotic, trimethoprim. In the last decade, gallic acid has been found to have therapeutic value as antimicrobial, antiviral, antitumor and radio protective agent (Agarwal *et al*., 2006; Gandhi and Nair, 2005; Hsu *et al*., 2007; Kaur *et al*., 2005; Li *et al*., 2005; Shinno *et al*., 2005; Veluri *et al*., 2006). As a potential drug and recent application in agriculture for crop protection make gallic acid a molecule of huge market potential (Mahoney and Molyneux, 2004). The chemical technology of gallic acid production is disadvantageous because of environmental hazards and uneconomical due to poor yield.

Tannins are polyphenolic secondary metabolites of higher plants (such as chestnut, Oak wood, Trillo, Valonea, Myrobalan, Divi-Divi), classified as hydrolysable and non hydrolysable tannins (condensed tannins) (Khanbabee and Van Ree, 2001). They are considered as the classic defense compounds of plants against herbivores and have a broad spectrum of distribution in the plant kingdom which includes pteridophytes, gymnosperms and angiosperms and generally accumulate in large amounts in roots, bark, leaves and fruits (Frutos *et al*., 2004; Scalbert, 1991). The biosynthesis of hydrolysable tannins in higher plants was reported by coupling of a polyphenolic building block – gallic acid with diverse polyols such as D-glucopyranose (Khanbabee and Van Ree, 2001).

Microorganisms, especially filamentous fungi are the more potent producers of tannase. More than 60 fungal strains have been documented for tannase production. The commercial tannase production is also carried out by *Aspergillus* strains (Van De Lagemaat and Pyle, 2005b). However the bioconversion of tannin by fermentation has not been exploited fully. Little work has been done on the use of higher tannic acid concentrations at fermenter scale. Because of abundant natural tannin availability, huge market potential of the gallic acid, bioremediation requirement for the tannin waste, uneconomical processes for gallic acid and tannase production it is indeed useful to identify additional novel tannase producers as an alternative for gallic acid production.

The extensive literature survey on tannins, their types, bioconversion details by tannin acyl hydrolase (tannase) by microorganisms, production of gallic acid and applications of gallic acid and tannase was presented in the **Introduction (Chapter 1)**. Production optimization studies in solid state and submerged fermentation, tannase purification and characterization as well as its immobilization and use of immobilized enzyme was also reviewed.

Materials and Methods (Chapter 2) used during the investigation were described. The microbiological techniques used for isolation of tannase producing fungi from different sources (forest litter, pods/seeds, leaves, bark and fruits), media employed for isolation and screening of tannase production and tannin utilization by isolates as well as reference cultures viz. *A. niger*, *A. oryzae*, methods for extraction of tannins from different plant materials were given. The studies on mycelial morphology of *Aspergillus* were carried out by visual observation and light microscopy. Biochemical techniques used for purification and characterization *viz.* ammonium sulphate precipitation, cross flow filtration and ion exchange chromatography, electrophoresis as well as immobilization of extracellular enzyme and mycelia by alginate entrapment were described. The assays for estimation of tannase, gallic acid and tannic acid were also discussed.

The isolation and screening of fungal strains for tannase production (Chapter 3) able to withstand high tannin concentrations was carried out. Tannase producers were reported from majorly soil and also from surfaces of plant parts (Cruz-Hernandez *et al*., 2005; Mondal *et al*., 2001). In the present study, soil samples from tannin rich surroundings and tannin containing plant parts (bark, leaves, seeds, pods and fruits) were collected to isolate tannase producing fungi on tannic acid – glucose agar. The 106 fungal isolates were primarily screened for extracellular tannase production on agar medium as well as in liquid medium containing tannic acid as sole source of carbon. Further selection was carried out by secondary screening of high tannase producers in liquid medium containing varying concentrations of tannic acid (50 g/L – 500 g/L) as sole source of carbon. Thus it was possible to obtain two fungal isolates producing high tannase capable of withstanding 400 g/L tannic acid in 60 h under submerged fermentation conditions. The reference strains viz. *A. niger* NCIM 620 and *A. oryzae* NCIM 643 were found to tolerate low tannin concentrations, 100 g/L and 70 g /L respectively. The cultures were identified by colony morphology and partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene sequencing. The soil isolate SM 88

was identified as *A. parvulus* and *C. spinosa* pod isolate was identified as *A. allahabadi*. Both the fungal isolates were further screened for their ability to utilize tannic acid (20 – 400 g/L) under submerged conditions. Based on the tannase production (11.56 U/ml) and conversion efficiency (79.8 %) of high tannic acid (200 g/L) concentration, *A. allahabadi* was better strain than *A. parvulus* and therefore used for further studies of tannase production by submerged fermentation.

Chapter 4 (Studies and optimization of tannase and gallic acid production by fermentation) describes the effect of various nutritional parameters (carbon, nitrogen and minerals) and process parameters (agitation and aeration) on tannase and gallic acid production by *A. allahabadi* at shake flask level and scale up to 100 L stirred tank reactor (STR). The chapter further describes the work carried out on study of tannase production by using coculture and mutants of *A. allahabadi*. The initial time profile of *A. allahabadi* in an un optimized medium showed maximum tannase activity in 48 h while tannin conversion efficiency was 92 % when tannic acid concentration was 20 g/L. *A. allahabadi* tannase was found to be growth associated enzyme which peaked at late exponential growth phase. Basal levels of tannase were determined when *A. allahabadi* was grown in glucose containing medium in absence of tannic acid. The tannase levels increased when the culture was grown in tannic acid as sole source of carbon.

Naturally occurring plant tannins are major source of raw material for production of gallic acid. Tannases were reported to show different responses to different tannin substrates. Moreover the complex nature of plant tannin extracts may not be a good medium to support the growth of microorganism. In view of this, effect of different hydrolysable tannin containing plants viz. *C. spinosa, C. dignya, Q. infectoria, T. belerica* and *T. chebula* were studied on *A. allahabadi* tannase. The plant parts were extracted in water to obtain high tannic acid content. The results of extraction optimization of plant tannins have been discussed in Annexure I. The *A. allahabadi* tannase showed high tannase activity (8.10 U/ml) and high specific growth rate (0.437) when grown in medium containing *C. spinosa* tannin (20 g/L) as sole source of carbon. *A. allahabadi* was found to utilize high *C. spinosa* (pentagalloyl quinic acid) tannin (200 g/ L) concentration similar to that of tannic acid (pentagalloyl glucose). Therefore *C. spinosa* extract was used further for fermentation optimization studies.

The tannase production was studied at shake flask level and different nutritional and process parameters were optimized. Most of the studies on tannase production have been

reported on 20 – 50 g/L of tannic acid and concentration of tannic acid was reported to be significantly affecting the tannase production (Srivastava and Kar, 2009). Hence the effect of various parameters was optimized in two media containing different tannin sources *viz*. tannic acid (pentagalloyl glucose) and *C. spinosa* extract (pentagalloyl quinic acid) at 20 g/L tannic acid equivalence. Similarly effect of carbon, nitrogen and minerals was studied using high (200 g/L) tannic acid concentration to compare the findings at lower and higher tannic acid concentrations. The *A. allahabadi* tannase was found to be unaffected by the additional carbon source in the medium containing low tannin (20 g/L) concentration as primary carbon source except gallic acid. Gallic acid was found to increase the activity, whereas in presence of high tannin (200 g/L) no additional carbon was found to affect the tannase production. The optimization of nitrogen source revealed increase in tannase activity in presence of ammonium nitrate than that in other nitrogen sources. Zinc significantly increased tannase activity of *A. allahabadi* while effect of Ca on the activity was marginal. The effect of media volume was found to affect the growth of *A. allahabadi* and tannase production. Medium of 100 ml in 500 ml Erlenmeyer flask showed high tannase production. Agitation speed of shaker decides mixing efficiency and makes nutrients and oxygen available to the cells. Agitation speed of 200 rpm was found to be optimum for high tannase production by *A. allahabadi*. The profile of tannase production in an optimized medium under optimized process conditions at shake flask level showed the productivity of tannase production in medium with tannic acid as 433 U/L/h and that in medium with *C. spinosa* tannin as 503 U/L/h at 48 h.

The data obtained at shake flask level was considered for **optimization of tannase production at 35 L STR**. The parameters under consideration were agitation in terms of tip speed and aeration in terms of vvm (volume/volume/min). Tip speed at 2 m/s and aeration at 0.6 vvm was found to be optimum for high tannase production. Viscosity analyses demonstrated non Newtonian pseudoplastic behavior of fermentation broth. The time profile study of tannase production by *A. allahabadi* at 35 L STR using *C. spinosa* tannin showed increase in tannase production and in productivity than at shake flask level. High tannase production was achieved at 40 h while it was at 48 h under optimized fermentation conditions at shake flask level. In 35 L STR, the productivity of tannase (652 U/L/h) was increased by 1.29 fold as compared with the results of shake flask studies (502 U/L/h).The scale up of the process at 100 L STR was demonstrated using same agitation and aeration conditions optimized at 35 L STR. The productivity of tannase production (655 U/L/h) and that of gallic acid production (3.52 g/L/h) was

comparable with that in 35 L STR. The results of 100 STR indicated a reasonable degree of success in the scale up of tannase and gallic acid production from 35 L to 100 L.

Morphology of filamentous fungi and its effect on product formation under submerged conditions has been reported by many researchers. Four different type of morphology forms *viz.* small, fluffy pellets and /or free dispersed mycelia, big fluffy pellets, medium size, compact pellets and mycelial clumps were observed when *A. allahabadi* was grown in tannic acid / *C. spinosa* extract medium under submerged conditions. Dispersed mycelial form of morphology was observed to show high tannase activity while mixture of loosely packed pellets and free dispersed mycelia also showed comparable tannase activity. Shear rate (agitation), aeration, tannin concentration and initial pH were observed to the most important and deciding factor to define the morphology of *A. allahabadi* and tannase production under SmF.

Purification, characterization and immobilization of *A.allahabadi* tannase was carried out and **its application in bioconversion of tannic acid (Chapter 5)** was investigated. The *A. allahabadi* tannase was purified to 20 fold from the culture filtrate. It appeared as single band on SDS PAGE. The optimum temperature and pH for tannase activity were 35^0C and 5.0 respectively. Tannase activity was observed to be stable in pH range 4.5 – 6.0 and in the temperature range 25 – 45^0C. The effect of different additives was checked on tannase activity. No significant inhibition of enzyme activity was observed with Ca^{2+}, K^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Mn^{2+} except Hg^{2+} at 2 mM concentration. Tannase was partially inhibited by 1mM EDTA and completely inhibited by 2 mM concentration of EDTA. Anionic (Tween 20, Tween 60 and Tween 80) and non ionic surfactants (Triton X-100) were observed to inhibit tannase at 0.5 % concentration. The kinetic properties of *A. allahabadi* tannase were determined using tannic acid and methyl gallate substrates. The enzyme showed higher affinity towards tannic acid with K_m value of 2.96 $x10^{-3}$ mM while that of methyl gallate was 1.21 mM. The ratio of K_{cat}/K_m 4.08 $x10^7$ min/mM for tannic acid and 4.46 $x10^2$ for methyl gallate showed tannic acid as preferred substrate over methyl gallate. In case of tannic acid, catalytic time (time required for hydrolysis reaction of one acyl bond in substrate) was calculated as 8.26 $x10^{-6}$ min and that in case of methyl gallate was 1.8 $x10^{-3}$ min.

Purified tannase was immobilized by entrapment and covalent binding. Ca alginate entrapped tannase showed highest immobilization efficiency (63.12 %). Immobilized tannase showed optimum pH at 5.0 which was same as that of free tannase. The stability

of immobilized enzyme was observed over a slightly broader pH range (4.0 – 6.5). The optimum temperature of immobilized tannase was shifted to 40^0C from that of free enzyme at 35^0C. The temperature stability of immobilized tannase was observed to be over a broad temperature range from 20^0C – 60^0C. Efficiency of bioconversion of immobilized tannase was carried out in 200 g/L tannic acid. The efficiency of bioconversion (93.70 %) was maintained till 5^{th} cycle after which it reduced to 60 % by 9^{th} cycle. The tannase retained 86 % of its initial activity at the end of 9^{th} cycle. *A. allahabadi* mycelia were also immobilized in Ca alginate beads. The immobilized mycelia showed comparable results (92 % tannin conversion efficiency when 200 g/L tannin was used) as that with immobilized tannase.

The tannic acid extraction from plant parts in water was optimized at various conditions viz. different temperatures and time, stirring and non stirring conditions. The plant water extracts were also characterized for organic nitrogen, reducing sugar and mineral content **(Annexure I)**.

In the **conclusions (Chapter 6)** *A. allahabadi* for the first time was shown to produce tannase and efficient utilization of high tannic acid concentration (200 g/L). The effective utilization of *C. spinosa* plant tannin by *A. allahabadi* for tannase and gallic acid production will be useful for development of economical fermentation process due to cheap and easily available raw material. The purified tannase by *A. allahabadi* was found to have high turnover number (i.e. high K_{cat} value) suggesting the utilization of high tannin concentration in shorter period (50 % of tannin (100 g/L) was utilized in first 16 h). The increase in productivity at 100 L STR scale up suggested the possibility of developing an industrial scale process at larger fermenter level. Also this is the first report on the operational stability of immobilized tannase in high tannin (> 50 g/L) concentrations.

The references have been given in **Chapter 7.**

Shilpa Wagh **Dr. M.V.Deshpande**

(Research student) (Research Guide)

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Bioconversion process essentially involves the use of biocatalyst in the form of whole cells or enzyme (s) for production of bioproducts. The process was largely exploited to produce fine and bulk chemicals (amino acids, organic acids), fuels (ethanol, oil), carbohydrates (cellulose, starch, glycerol) and value added products (single cell protein, vitamins). The raw materials used for bioconversion are mainly renewable sources such as abundantly available biomass in nature (wood, plant parts – leaves, stems, seeds and seed husk) and agro-industrial wastes (beet pulp, wheat bran, apple pomace and olive mill residue) (Akinyele *et al*., 2011; Howard *et al*., 2003; Sampaio *et al*., 2003; Sampedro *et al*., 2005; Willke and Worlop, 2004). Industrially the bioconversion process has gained importance over chemical technologies because of process controls, manipulations of microorganisms, safety and reproducibility. While the chemical processes generally involve high temperature, high pressure and in some cases organic solvents. Number of bioconversion processes has employed biocatalysts such as lipases, amylases, cellulases, proteases and xylanses for commercial production of bioproducts (Punnapayak *et al*., 1999; Willke and Worlop, 2004).

Tannins are also abundant plant constituents, similar to cellulose and lignin. Tannins (commonly referred to as tannic acid) are water-soluble polyphenols (Chung *et al*., 1998). The connotation of the term tannins includes tannic acid as well as hydrolysable gallotannins, i.e., those which yield gallic acid on hydrolysis. Hydrolysable gallotannins may be obtained from many sources including *Caesalpinia spinosa* (Tara), *Caesalpinia digyna* (Teri) and many others (FAO JECFA Monographs 7, 2009).

Tannin acyl hydrolase (EC 3.1.1.20), commonly known as tannase was widely used for bioconversion of hydrolysable tannins to simple phenolics such as gallic acid.

Though the production of tannases using various microorganisms, their characterization, immobilization and application in bioconversion to gallic acid were studied extensively, the commercial sources of tannase were very limited (Van De Lagemaat and Pyle, 2005).

Filamentous fungi were reported to dominate among all tannase producing microorganisms and to withstand tannin concentration more than unicellular yeasts and bacteria. In view of this, the study was designed to isolate and screen for the fungal tannase producer (s) which can tolerate high tannin concentration. Further the isolate (s) was studied for tannase production and bioconversion by fermentation using various tannin sources. This study is also an attempt to check the effect of various parameters (carbon and nitrogen sources, operation conditions) on the fungal morphology and subsequently on tannase and gallic acid.

1.1 Tannins – natural substrate for bioconversion to gallic acid

Of the major classes of secondary metabolites, phenolics are most commonly synthesized in plants in response to herbivore attack, pathogens, UV light, cold or nutrient deficiencies. Plant phenolics comprise a diverse group of defense molecules ranging from low molecular weight phenolics such as coumarins, stilbenes, flavonoids to complex polymers like lignins and tannins (Constabel, 1999). As reported tannins are widespread in the plant kingdom which includes pteridophytes, gymnosperms and angiosperms and generally accumulate in large amounts in roots, bark, leaves and fruits (Scalbert, 1991). But they were found to be most abundant in the parts such as leaves or flowers which are more likely to get damaged or to be eaten by herbivores (Frutos *et al*., 2004).

1.1.1 Significance of tannins in plants

Bhat *et al* (1998) reported that tannins were the second most abundant group of polyphenols after lignins. They are considered as classic defense compounds of plants against herbivores. It was observed that the long lived trees have higher tannin content than short lived trees. The factors like high temperature, water stress, extreme light intensity and poor soil quality were reported to increase tannin contents in plants (Frutos *et al.*, 2004). Increased levels of (hydrolysable) tannins in leaves of plants such as oak, birch, willow and *Acacia* due to insects (e.g. Gypsy moth larvae) or by herbivore damage were also documented (Constabel, 1999). The chemical basis of tannin defense was attributed to their ability to precipitate proteins. The protein tannin complex was reported to inhibit the invasion of host tissues by microbes and also inhibit microbial growth by reducing the availability of metallic ions necessary for the metabolism of microbes (Beart *et al*., 1985; Scalbert, 1991). Tannin complex formation with proteins and carbohydrates

was documented to protect plants from herbivores due to unpalatability, and/ or astringent (strong) sensation. The antinutritional effect exerted by the tannin protein complex by reducing availability of food nitrogen or inhibiting digestive enzymes was also reported (Beart *et al*., 1985; Frutos *et al*., 2004).

1.1.2 Types of tannins

Based on their properties and structural characteristics, tannins are divided into three major groups and two subgroups (Fig. 1.1).

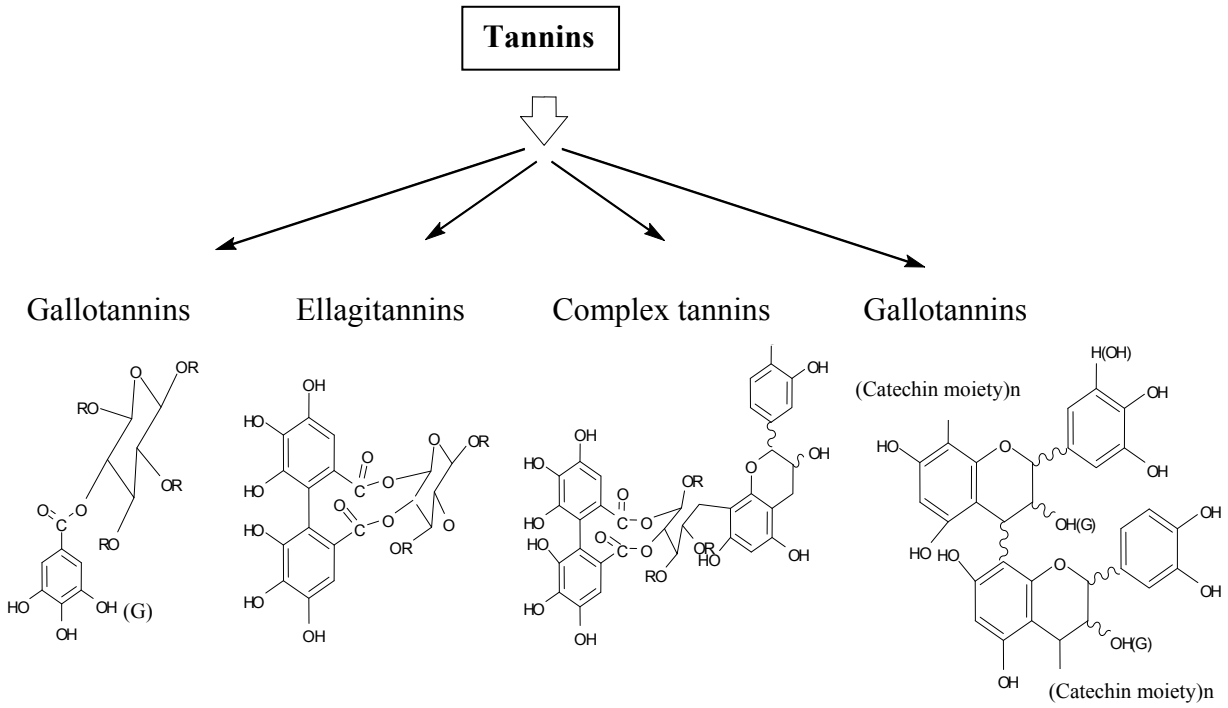


Fig. 1.1 Types of tannins (Source: Khanbabee and Van Ree, 2001)

Hydrolysable tannins commonly referred to as 'tannic acid' (Chung *et al*., 1998; Haslam, 2007; Niho *et al.*, 2001) are further classified as gallotannins and ellagitannins.

The plants *Rhus semialata* (Chinese galls), *Quercus infectoria* (Turkish galls), *Caesalpinia spinosa* (Tara pods), *Aver ginnala* (Korean maple, Acer tannin leaves), *Rhus coriaria* (Sumac leaves), *Hammelis virginiana* (Hamammelis –hazel nuts) to name a few were documented to be rich in gallotannins (Bhat *et al*., 1998). Gallotannins are the simplest hydrolysable tannins, containing polyphenolic and a polyol residue, whereas galloyl units or their meta-depsidic derivatives are bound to diverse, polyol-catechin-, or

triterpenoid units (Khanbabee and Van Ree, 2001). Gallotannins yield gallic acid and glucose or quinic acid on hydrolysis.

Plants such as *Caesalpinia coriaria* (Divi-Divi), *Caesalpinia brevifolia* (Algarobilla tannin) *Terminalia chebula* (myrobalan seeds), *Castanea sativa* (Chestnut), *Eucalyptous sieberiana*, *Schinopsis* sp., *Quercus velonia*, *Q. aegilops* (Valonea tannin), *Quercus coccifera* (Garouille) were reported to contain ellagitannins in their bark, nuts or leaves (Bhat *et al*., 1998; Mukherjee and Banerjee, 2003). Khanbabee and Van Ree (2001) reported the formation of ellagitannins from gallotannins by oxidative coupling of at least two galloyl units which are C-C coupled to each other and absence of a glycosidically linked catechin unit. Ellagitannins yield ellagic acid, gallic acid and glucose on hydrolysis.

Complex tannins are formed when gallotannin or ellagitannin unit forms acyl bond with pro anthocyanadines such as catechin e.g. tea polyphenols (Khanbabee and Van Ree, 2001).

Condensed tannins are referred to proanthocyanidins. These are generally found in plants which possess a woody growth and also in plant gums and exudates (Haslam, 1996). Common plants containing condensed tannins are *Acacia* sp. (Wattle tannins), *Schinopsis* spp. and *Loxopterygium* sp. (Querbacho wood), *Pinus* sp (Pine bark), *Quercus* sp. (Oak wood) and *Aucoumea kleneana* (Gaboon wood) (Bhat *et al*, 1998). The fundamental structural unit of condensed tannins is the phenolic flavan-3-ol (catechin). These oligomeric and polymeric proanthocyanidins contain two to ten or more catechin units. The flavan-3-ol units are linked principally through the 4 and 8 positions (Haslam, 2007; Khanbabee and Van Ree, 2001). Condensed proanthocyanidins degrade in strong acid to give corresponding anthocyanidin.

The words tannin/tannic acid referred hereinafter, are synonyms to hydrolysable tannins i.e. gallotannins or ellagitannins.

1.2 Gallic acid (3,4,5-trihydroxy benzoic acid)

Most of plant secondary metabolites are derived from phenylpropanoid, alkaloid, fatty acid (e.g. polyketide, terpenes) and shikimic acid pathways. Shikimate pathway gives rise to tannins, coumarins, quinines and hydroxy benzoic acid (Fig. 1.2). Amongst the hydroxybenzoic acids (such as salicyclic acid, caffeic acid, syringic acid and vanillic

acid) that are found in plants, majority of them are relatively rarely encountered because their occurrence is limited to particular plant family e.g. salicylic acid in Salicaeae.

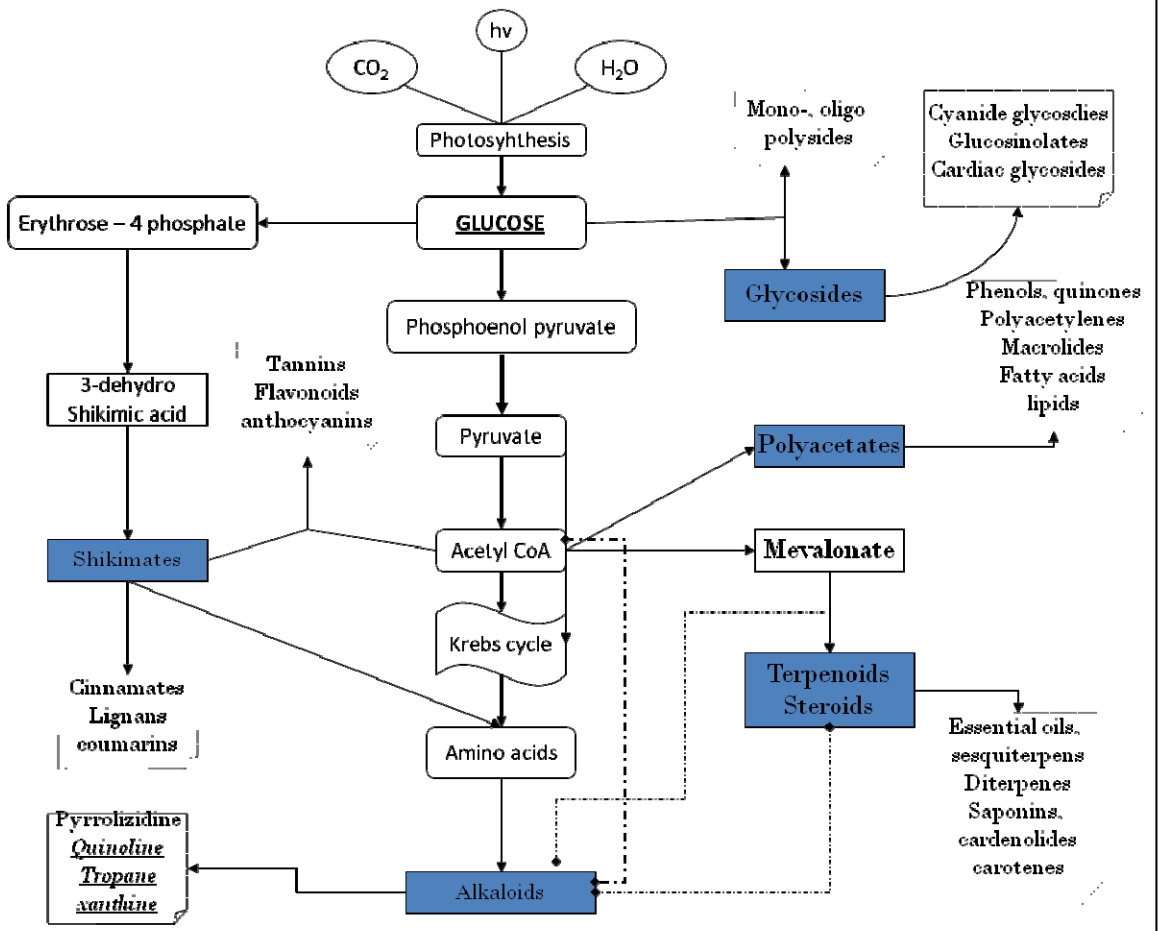


Fig. 1.2 Synthesis of natural products (Source: Bruneton, 1995)

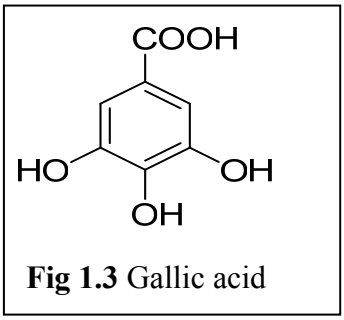


Fig 1.3 Gallic acid

While gallic acid (3,4,5 – trihydroxy benzoic acid) (Fig. 1.3) was unique amongst them because of its wide distribution in herbaceous dicotyledons, angiosperms and in some green algae (Haslam, 1986; Haslam and Cai, 1994). It is almost invariably located in plant tissues in the form of esters – with sugars, polyols, glycosides and other phenols (Haddock *et al*., 1982; Haslam, 1986). Almost 200 years ago, gallic acid was first isolated from gall nuts by Swedish chemist Scheele (Haslam, 1986). Various routes of gallic acid biosynthesis were reported. For example Werner *et al*. (2004) suggested a synthesis of gallic acid from phenylalanine via caffeic acid or trihydrocinnamic acid. One of the pathways could be direct dehydrogenation of 3 –

dehydroshikimic acid (Fig. 1.4), unlike other benzoic acid derivatives which were synthesized from side chain degradation of a C6-C3 unit (Bruneton, 1995).

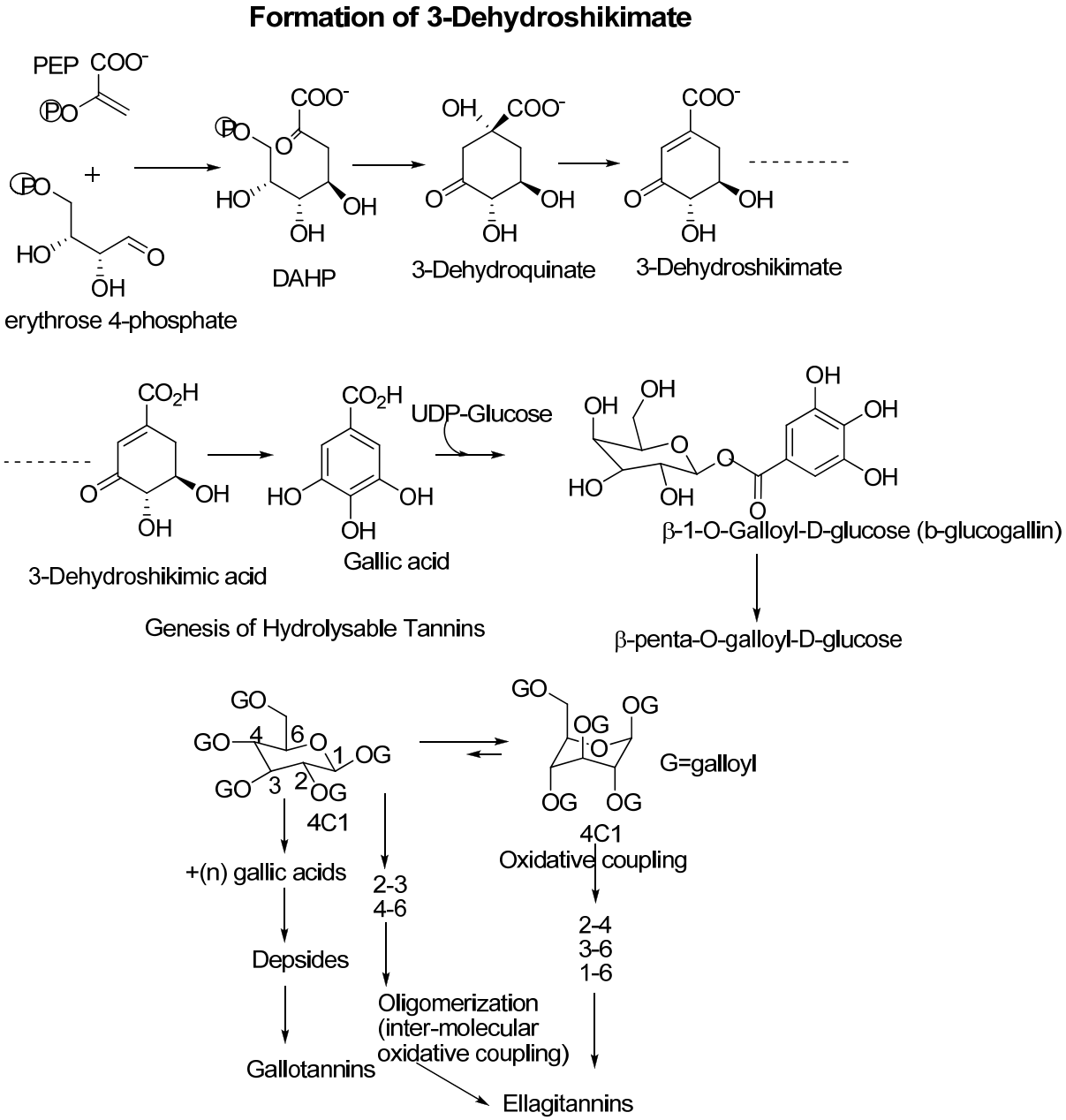


Fig 1.4 Synthesis of gallic acid and hydrolysable tannins (Source: Bruneton, 1995)

1.2.1 Distribution and occurrence of gallic acid in nature

Gallic acid is richly present in roots, bark (*Quercus* sp.), leaves (*Syzygium cumini*, *Phyllanthus emblica*), fruits (*Mangifera indica*), seeds, pods (*C. spinosa*) and galls (*Quercus*) of higher plants and also in vegetables (black radish, onion), fruits

(pomogranate) and beverages (tea, wine, fruit juices) (Table 1.1). Tea is one of the richest sources of gallic acid among daily dietary food.

Table 1.1 Dietary sources of gallic acid

Example	Content
Fruits and Vegetables	
Black berry	8-67 mg /kg
Black current	30-62 mg/kg
Raspberry	19.38 mg/kg
Strawberry	11-44 mg/kg
White current	3-38 mg/kg
Cashew nut	8.62 mg/kg
Cardamom	6.41 mg/kg
Buchanania lanzan	16 mg/kg
Black raisin	14.97 mg/kg
Fox nut	3.92 mg/kg
Groundnut	14.05 mg/kg
Coconut	11.64 mg/kg
Clove buds	175 mg/kg
Beverages	
Black tea#	3200-3600 mg/kg
Chinese green tea	5200 mg/kg
Japanese green tea*	2300 mg/kg
Semi fermented tea	4500 mg/kg
French wine and spirit	31-38 mg/L
Red wine	95 mg/L
White grape wine	1 mg/L
White wine (no Vinifera grapes)	0.67 – 2.05 mg/L

#: fresh wt; *: dry wt. Compiled from: Rice-Evans *et al*., 1997; Tiwari *et al*., 2009; Tomas-Barberan and Clifford, 2000.

1.2.2 Applications of gallic acid

Gallic acid has always been a molecule of industrial importance because of its applications in different sectors from healthcare and food to dyes, inks, paints and photography. It was used in preparation of high grade leather tannins, in the manufacture of inks, paints and colour developers and as a photosensitive resin in the semiconductor

production. It is also used in testing of free mineral acids, dihydroxy-acetone and alkaloids. The major application of gallic acid was a precursor for synthesis of broad spectrum antibacterial agent, trimethoprim (Anderson *et al*., 1980; Misro *et al*., 1997; Mukherjee and Banerjee, 2003; Ow and Stupans, 2003).

In the past decade, gallic acid has emerged as a potential drug molecule of therapeutic value as antimicrobial, antioxidant; anticancer, antiviral, antitumor and as a radio protective agent. (Agarwal *et al*., 2006; Gandhi and Nair, 2005; Hsu *et al*., 2007; Kaur *et al*., 2005; Li *et al*., 2005; Prasad *et al*., 2006; Shinno *et al*., 2005; Veluri *et al*., 2006). Researchers documented the potential health applications of this molecule against anaphylaxis (Kim *et al*., 2006), LT (heat labile enterotoxin by *E. coli*) induced diarrhea (Chen *et al*., 2006), Leishmaniasis (Kolodziej and Kiderlen, 2005), neurodegenerative disease (Bastianetto, 2006) and peptic ulcers (Bhattacharya *et al*., 2007). Liu *et al*. (2006) reported antiangiogenic activity of gallic acid by dose dependant inhibition of human angiogenic initiation and neovessel growth in human placental vein angiogenesis model. The cell line studies using HSV and HRV viruses showed the possible use of gallic acid as antiviral agent (Choi *et al*., 2010; Kratz *et al*., 2008). Appledoorn *et al*. (2006) reported cardioprotective activity of gallic acid. While Chuang *et al*. (2010) demonstrated use of gallic acid in the treatment of idiopathic pulmonary fibrosis.

The recent finding of inhibition of aflatoxin synthesis by gallic acid suggested its application in grain and food storage also (Mahoney and Molyneux, 2004). Recently Dandekar *et al.* (2007) described transgenic plants comprising a shikimate dehydrogenase gene which produced gallic acid precursor. These plants with elevated levels of gallic acid were claimed to be useful for protection of crops such as walnut, peanut, cotton, corn, alfa alfa and other forage crops that otherwise were susceptible to aflatoxin contamination. External application of gallic acid alone or in combination with other compounds such as pesticides or fertilizers was shown to control aflatoxin synthesis in fungus.

Gallic acid is used as a starting material for production of food additives like gallic acid esters and derivatives such as propyl gallate, octyl gallate, lauryl gallate and dodecyl gallate which reduce rancidity and work as preservatives. Propyl gallate, an antioxidant and antimicrobial agent is being used in hair products, adhesives and lubricants. Pyrogallol, a decarboxylated gallic acid, was used as a developer in photography, in

staining of fur, leather and hair, in manufacturing of dyes and for the determination of oxygen in gas analysis. The application of alkyl gallate as a modulator of β lactam antibiotics was also documented. Ethyl gallate and methyl gallate being a collagenase inhibitor showed potential in the treatment of periodontosis. Gallic acid and its esters especially methyl gallate exhibited antimicrobial activity against number of microorganisms. The various applications details are documented in Chaubal *et al*., 2005; Chung *et al*., 1993; Kang *et al*., 2008; Niho *et al*., 2001; Shibata *et al*., 2005; Yoshida *et al*., 1982; Yu and Li, 2008.

1.3 Production of gallic acid from tannins

Chemical synthesis of plant derived compounds is often uneconomical, because of the complex structures and stereo specificity (Namdeo, 2007). Simultaneously, extraction of the plant secondary metabolite is also disadvantageous because of interference of unknown impurities. Biotechnological route was an economical option in such cases. Commercially production of gallic acid was carried out by hydrolyzing tannins either by chemical hydrolysis or enzymatic hydrolysis.

The chemical route of gallic acid production involves mainly acid hydrolysis (hot mineral acid) of natural gallotannins (Mukherjee and Banerjee, 2003). This conventional method is economically non viable because of poor yield and less purity of gallic acid (Bajpai and Patil, 2008). It also involves high concentrations of acid or alkali which lead to corrosion of the process vessels and demands more safety precaution. Another disadvantage is the release of the large amount of toxic effluent which causes environmental hazards (Banerjee *et al*., 2001). Alternatively gallic acid is produced by microbial fermentation in which the microbial enzyme tannase cleaves the ester bond in (hydrolysable) tannins to release gallic acid molecules. The source of enzyme in most of the cases is either fungus or bacterium. The fermented cell free broth is precipitated at lower temperature to isolate gallic acid. The process is economical, non hazardous and eco friendly.

1.4 Tannase – an enzyme involved in bioconversion of tannin to gallic acid

Tannin acyl hydrolase (EC 3.1.1.20) plays an important role as biocatalyst in the bioconversion of hydrolysable tannins to gallic acid. A number of production processes using microbial tannases are available in the literature (Table 1.2). The tannase from

Aspergillus has been used widely for production of gallic acid from tannins. Apart from its use in gallic acid production, this enzyme was also found to be industrially important because of its wide applications in tannery, pharmaecuticals, alcohol and beverage industries (Purohit *et al*., 2006).

Table 1.2 Patents issued for production of tannase and gallic acid (Source: Aguilar *et al*., 2007)

Patent title	**Patent No.**
Production of tannase by *Aspergillus*	JP7225786 (1975), JP62272973 (1987), JP63304981 (1988)
Antioxidant catechin and gallic acid preparation	JP01268683 (1989)
Tannase production process by *Aspergillus* and its application to obtain gallic acid	EP339011, WO/1989/010400 (1989)
Process for the preparation of gallic acid by co-culture	US7118882 (2006)

1.4.1 Tannase applications

Most of the commercial applications of tannase are in the manufacture of instant tea to remove water insoluble precipitates called 'tea cream'. Tannase were documented to be used in deesterification of tea polyphenols in non converted green tea leaves which enhances the natural levels of gallic acid and epigallochatechin and favors the formation of epitheaflavic acids which were responsible for bright reddish color and good cold water solubility (Aguilar and Gutierrez-Sanchez, 2001; Lekha and Lonsane, 1997).

Tannase was employed to prevent discoloration and haze development during beer storage. It was also used to make acorn wine from Korean acorns (*Quercus* spp.) and to treat grape must and juice along with lactase to remove phenolic substances for stabilizing and increasing the quality of wine (Lekha and Lonsane, 1997). Tannase was reported for its use in enzymatic debittering of fruit juices. The mechanism of action involved reduction in the tannin contents responsible for haze, sediment, color, bitterness and astringency of the juice upon storage (Aguilar *et al.*, 2007; Rout and Banerjee, 2006). Use of tannase as a clarifying agent in the preparation of coffee flavoured soft drinks and

in the stabilization of malt polyphenols was also documented (Das Mohapatra *et al*., 2006; Sabu *et al*., 2005).

Pretreatment of tannin containing food and feed with tannase was reported to reduce the antinutritional effects by improving digestibility and nutrient availability of food and feed (Lekha and Lonsane, 1997). Application of tannase to lentil flours has also been documented for production of bioactive polyphenolic compounds (Duenas *et al*., 2009). The use of tannase proved to be an effective treatment for the removal of high quantities of tannins in tannery effluent which posed serious pollution problems (Aguilar and Guieterrez-Sanchez, 2001). Tannase was speculated to play an important role in plant cell wall degradation by cleaving some of the cross-links existing between cell wall polymers (Aguilar *et al*., 2007). They were also applied in preparation of high grade leather tannin (Das Mohapatra *et al*., 2006).

1.4.2 Sources of Tannase

Tannase was reported to be present in plants containing hydrolysable tannins such as myrobalan (*Terminalia chebula*) fruits, divi divi (*Caesalpinia coriaria*) pods, and dhawa (*Anogeissus latifolia*) leaves, English oak (*Quercus robur*), Pendunculate oak (*Quercus rubra*) and in the leaves of Karee (*Rhus typhina*) tree as well as in plants containing condensed tannins like avaram (*Cassia auriculata*), babul (*Acacia arabica*), and konnam (*Cassia fistula*) (Lekha and Lonsane, 1997; Niehaus and Gross, 1997). The physiological significance of tannase in plants was suggested in formation of tannins. Along with large quantities of sugar, plants synthesize chebulinic acid, gallic acid, and hexahydroxyphenic acid during growth. In the course of ripening, these acids might become esterified with glucose, with the help of tannase to form tannins (Lekha and Lonsane, 1997).

The gall larva was reported to produce tannase to hydrolyze tannic acid in plant galls. Low levels of tannase were reported in the mucosa membrane of rumen and small intestine of cattle (Lekha and Lonsane, 1997).

Number of bacteria and fungi were reported to produce tannase. The main genera among bacteria are: *Bacillus* (Das Mohapatra *et al*., 2006; Mondal, 2001a), *Citrobacter* (Ajaykumar *et al*., 1999), *Corynebacterium, Klebsiella* (Deschamps *et al*., 1983), *Lactobacillus* (Rodriguez *et al*., 2008a, b; Sasaki *et al.*, 2005), *Lonepinella* (Goel *et al*., 2007), *Pantonea* (Pepi *et al*., 2010), *Pesudomonas* (Selwal *et al*., 2010), *Serratia* (Belur

et al., 2010), *Selenomonas* (Skene and Brooker, 1995) and *Streptococcus* (Curiel *et al*., 2009).

A number of species belonging to fungal genera *Aspergillus, Penicillium, Paecilomyces* and *Trichoderma* were reported to be efficient tannase producers. Few genera such as *Arxula* (Boer *et al*., 2009), *Candida* (Aoki *et al*., 1976 a, b), *Cryphonectria* (Farias *et al*., 1994), *Debaryomyces* (Deschamps *et al*., 1983), *Fusarium sp*. (Bradoo *et al*., 1996), *Hyalopus sp*. (Mahapatra and Banerjee, 2009), *Mycotorula* (Belmares *et al*., 2004), *Myrothecium* (Bradoo *et al*., 1996), *Pichia* (Deschamps *et al*., 1983), *Saccharomyces* (Zhong *et al*., 2004), V*ertcillium sp*. (Kasieczka-Burnecka *et al*., 2007), *Aureobasidium, Cunnighamella, Heleicostylum, Syncephalastrum sp.* (Bradoo *et al*., 1996) and *Rhizopus* (Hadi *et al*., 1994) were also reported for tannase production. Most of the microorganisms were reported to produce intra and extracellular tannase and sometimes the activity was membrane bound also (Barthomeuf *et al*., 1994a; Pourrat *et al*., 1987).

1.4.3 Mechanism of action

Tannase acts on ester and depside linkages (acyl bond i.e. C-O) in hydrolysable tannins, mainly gallotannins (Fig. 1.5a). A typical reaction catalyzed by tannase is shown in Fig. 1.5 b wherein Tannic acid (I) was found to be hydrolysed to gallic acid and glucose through 2,3,4,6 tetragalloyl glucose (III) and monogalloyl glucose (IV) (Lekha and Lonsane, 1997). It also acts on (ester bond of) methyl gallate and (depside bond of) m-digallic acid. Few reports are available on the tannase catalyzed bioconversion of ellagitannins, however, biochemical mechanism was not clearly explained. (Aguilera-Carbo *et al.*, 2008). Tannase was also reported to act on complex tannins such as (-)-epicatechin gallate and epigallocatechin gallate but not on condensed tannins (Lekha and Lonsane, 1997).

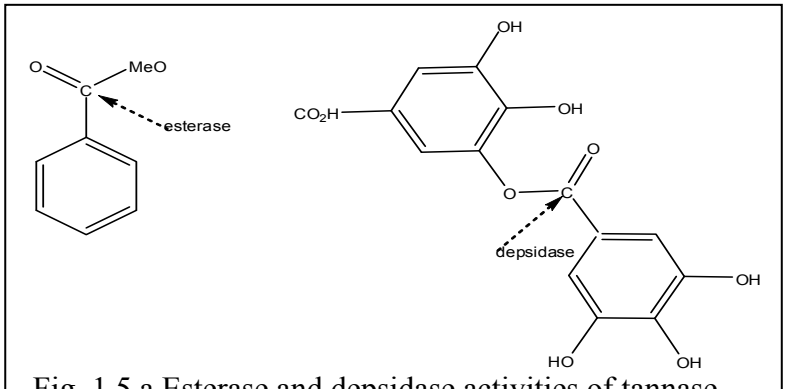


Fig. 1.5 a Esterase and depsidase activities of tannase

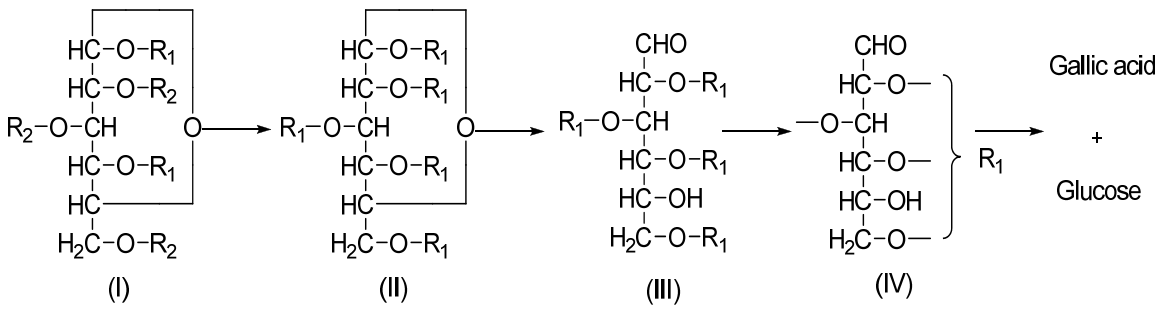


Fig. 1.5 b Typical reaction catalyzed by tannase (R_1- gallic acid; R_2 – m-di gallic acid) (Source: Lekha and Lonsane, 1997)

1.4.4 Induction and regulation of tannase

Tannase was reported to be an induced enzyme. Tannins or phenolic compounds such as gallic acid, pyrogallol, methyl gallate were documented to induce the enzyme production in microorganisms (Bajpai and Patil, 1997; Costa *et al*., 2008). However constitutive levels of enzyme in the absence of tannic acid and in the presence of other substrates such as monosaccharides (glucose), disaccharides (sucrose), polysaccharides (starch) and substrates such as peptone and casein were reported (Aguilar *et al*., 2001b; Bradoo *et al*., 1997). Because of its large size and reactivity, uptake of tannic acid by cell membrane and subsequent tannase induction was questioned by Lekha and Lonsane (1997). Authors further suggested that constitutive levels of tannase hydrolyzed the tannins into glucose and gallic acid which later induced the enzyme. Bajpai and Patil (1997) reported the induction of tannase by gallic acid and pyrogallol. However, gallic acid was shown to inhibit tannase synthesis by end product repression (Aguilar *et al.*, 1999; Bradoo *et al*., 1997). Aguilar *et al*. (2001b) demonstrated catabolite repression by glucose in submerged fermentation while the phenomenon was not observed under solid state fermentation. This indicated that the regulatory mechanisms for tannase may vary for organisms and respective fermentation conditions (Van De Lagemaat and Pyle, 2005b).

1.5 Production of tannase by fermentation

Tannase production by submerged fermentation (SmF) was explored at shake flask level and at fermenter level up to 20 L while solid state fermentation (SSF) studies were carried out using petri dishes, Erlenmeyer flasks, column reactors, continuous solid

state fermenter and modified SSF (Kar *et al*., 1999; Van De Lagemaat and Pyle, 2004; 2005b). The low biomass and consequently low efficiency of enzyme production were documented in liquid surface (LSF) studies of tannase production suggesting it as an unsuitable mode of production. Furthermore, high handling cost, risk of contamination and difficulties in process control were also of a major concern (Lekha and Lonsane, 1997; Rana and Bhat, 2005; Van De Lagemaat and Pyle, 2005b). Various plants containing gallotannins and/or ellagitannins were documented as source of tannins for the production of microbial tannases. The tannin sources, type of tannin and their tannin content in the plant on dry basis used for production of tannase are summarized in Table 1.3.

Table 1.3 Plant sources used for production of tannase and gallic acid

Plant source		**Plant part**	**Type of tannin**	**Tannin %**
Botanical name	Common name			
Anacardium occidentale	Cashew	Testa of seeds	ET	10-40
Caesalpinia cacalaco	Cascolate	Leaves	ET	25
Caesalpinia coriaria	Divi divi	Pods	ET	43
Caesalipinia dignya	Teri pod	Pod cover	GT	45
Caesalpinia spinosa	Tara	Fruit pods	GT	40
Flurensia crenua	Tar bush	Leaves	ET	40
Larrea tridentate	Creosote bush	Leaves	ET	16
Rhus coriaria	Sumac	Leaves	GT	10
Rhus semialata	Chinese galls	Galls	GT	89
Terminalia chebula	Myrobalan	Fruit	ET	40
Quercus infectoria	Turkish galls	Nuts	GT	81

ET: ellagitannin. GT: gallotannin; (Compiled from: Bajpai and Patil, 2008; Banerjee *et al*., 2005; Deschamps and Lebeault, 1984; Kar *et al*., 1999, 2000 and 2002; Lokeswari, 2010; Lokeswari and Jayaraju, 2007b; Paaver *et al*., 2010; Pourrat *et al.*, 1987; Ruiz-Aguilar *et al*., 2004; Ventura *et al*., 2008)

The powdered plant tannin substrates (leaves, fruits, pods, bark) were usually extracted by either with water or with organic solvent like acetone (Barthomeuf *et al*., 1994b; Huang *et al*., 2005; Pourrat *et al*., 1985 and 1987; Sariozlu and Kivanc, 2009; Ventura *et al*., 2008). The total tannin content in the plant material range from as low as 0.5 % to as high as 89 %. The tannin concentration used for production of microbial tannase are in the range from 0.1 % - 15 % (w/v) (Mahapatra and Banerjee, 2009). Though *Aspergillus* sp. was reported to grow at tannin concentration as high as 20 %, there are no reports till date using such as a high concentration of tannin for production of tannase, *per se* (Van Diepeningen *et al*., 2004).

Tannins were documented to be serving as dual purpose as a solid support in SSF and also as a carbon source such as *Larrea tridentate*, *Syzygium cumini, Ziziphus mauritiana* (Kumar *et al*., 2007; Manjit *et al*., 2008; Trevino-Cueto *et al.*, 2007). The use of natural substrates such as wheat bran, palm kernel cake, tamarind seed powder, coffee husk, paddy husk, rice bran, millet husk and ground nut shell as a support in SSF was reported (Battestin and Macedo, 2007a, b; Chattrejee, 1996; Paranthaman *et al*., 2009b; Sabu *et al*., 2006). Some workers used inert carrier such as polyurethane foam impregnated with inorganic salts as a support (Cruz-Hernandez *et al*., 2006; Van De Lagemaat and Pyle, 2001).

There are few reports on the optimization of production of tannase by using statistical tools. Response surface methodology (RSM) was majorly used to elucidate the optimum conditions for tannase production. The general experimental design was based on the variations of the most influencing factors on tannase production. Lekha *et al*. (1994) used RSM for tannase production by SSF. The variables optimized in the study were initial pH, temperature, inoculum ratio, fermentation time and moisture content. Other variables optimized by SSF through RSM were substrate concentration and % residue (Battestin and Macedo, 2007a). Along with pH and temperature, the parameters such as substrate concentration, agitation rate and incubation period were optimized in SmF through RSM for tannase production from *Aspergillus awamori* and *A. niger* (Beniwal and Chhokar, 2010; Seth and Chand, 2000; Sharma *et al*., 2007). Taguchi DOE orthogonal array method was used to optimize tannase production by SmF using *Bacillus licheniformis* to optimize concentrations of tannic acid, KH_2PO_4, NH_4Cl and $MgSO_4$ along with pH and temperature (Das Mohapatra *et al*., 2009).

Though SSF has drawbacks of scale up and large scale operations, SmF is not necessarily advantageous over SSF as per tannase productivity is concerned. A continuous solid state fermenter (CSSF) developed by Van De Lagemmat and Pyle (2004) suggested the possibility of SSF application at large scale. The CSSF design had baffles, cylinders for continuous feeding and sampling devices and facility for gas exchange. It was designed to operate without continuous addition of inoculated feed. Another SSF bioreactor study was carried out using modified SSF (MSSF) in bioreactor which consisted of perforated float for carrying solid substrate and induced *Rhizopus oryzae* inoculum (Kar *et al*., 1999). As shown in the Table 1.4, the yield of tannase was observed to vary greatly by fermentation conditions, type and concentration of tannic acid substrate and microorganism.

Table 1.4 Production of tannase by fungi

Organism	**Tannin g/L**	**Type of fermentation**	**Time (h)**	**Activity U/ml**	**Productivity U/ml/h**
A. awamori	45	SmF	60	5.49	0.09
Aspergillus japonicus	20	SmF/ SSF	24	33.06	1.38
Aspergillus niger	50	SSF	20	2.29	0.11
A. niger	50	SSF	30	0.39	0.01
Aspergillus tamari	10	SmF	144	0.8	0.005
A. tamari	10	SmF	48	12.4	0.26
Aureobasidium pullulans	10	SmF	30	0.8	0.03
Hyalopus sp.	10	SmF	120	1.1	0.009
Paecilomyces variotii	120	SSF	120	1.3	0.01

SmF: submerged fermentation; SSF: solid state fermentation (Compiled from: Banerjee and Pati, 2007; Battestin and Macedo, 2007a; Bradoo *et al*., 1997; Costa *et al*., 2008; Cruz–Hernandez *et al*., 2006; Enemuor and Odibo, 2009; Mata-Gomez *et al*., 2009; Mahapatra and Banerjee, 2009; Seth and Chand, 2000)

Though there was not much variation in the substrate concentration under same fermentation conditions tannase activity was observed to vary depending on the organism. The yield of enzyme by SSF and SmF were not necessary to be the same (Lekha and Lonsane, 1997). The fermentation time for optimum tannase production was found to be 20–144 h by various researchers. Tannase was reported to be growth associated enzyme having its peak activity at the onset of growth (Aissam *et al*., 2005; Raaman *et al*., 2010). On the other hand, Mahapatra and Banerjee (2009) reported maximum extracellular tannase of endophytic fungus *Hyalopus* at the end of exponential phase. Tannase production was faster under SmF due to rapid utilization of growth followed by decrease in activity due to unavailability of substrate while in SSF; it did not decrease indicating higher stability (Raaman *et al*., 2010).

Genetic manipulations of fungal species for improved tannase production were first reported by Hatamoto *et al.* (1996), wherein the researchers cloned and sequenced the tannase encoding gene and found that the *A. oryzae* gene codes for 588 amino acid sequences. The expression of *Aspergillus* tannase in *S. cerevisiae* gave very low yield (Albertase, 2002) while heterologus expression of *A. oryzae* tannase in *Pichia pastoris* yielded high activity. The tannase encoding gene, *tanLp1* from *L. plantarum* was cloned into pURI3 vector and expressed in *E. coli*. Further, the hyper production of recombinant tannase was obtained by one step purification procedure (Curiel *et al*., 2009). Boer *et al*. (2009) isolated tannase encoding *ATAN1* gene from yeast *Arxula adeninivorans* and cloned it in its auxotrophic mutant. The recombinant strain gave four times higher yield of tannase activity.

1.6 Gallic acid production by fermentation

The production of gallic acid is interlinked and dependant on tannase production because tannase is responsible to cleave the acyl bond in hydrolysable tannins to produce gallic acid. Tannase production and gallic acid yield were observed to be directly proportional (Kar and Banerjee, 2000). Production of gallic acid with special emphasis on the optimization of tannase by different combinations of tannin substrates and microorganisms were documented by various researchers (Banerjee, 2004). There are very few reports on the fermenter scale studies of bioconversion of tannins using tannase for gallic acid production because of number of obstacles for the large scale studies of this bioconversion. Limit to the use of high concentrations, because of the sensitivity of

microorganisms to tannic acid, oxidation of tannic acid, lack of sufficient knowledge about tannase properties and optimal production were some of the reasons (Bajpai and Patil, 2008; Van De Lagemaat and Pyle, 2005b).

It can be seen from Table 1.5, that fermenter scale studies were carried out majorly by submerged fermentation using 3 – 20 L of fermenters for tannase and gallic acid production by fungi. Natural gallotannins were the major commercial source for production of gallic acid.

Table 1.5 Production of tannase and gallic acid by fungi

Organism	**Type of fermentation**	**Time (h)**	**Activity U/ml**	**Gallic acid yield[a], %**	**Reference**
Aspergillus awamori	SmF	30	2.97	88	Seth and Chand, 2000
Aspergillus aculeatus	SmF	36	4.12	56.6	Banerjee *et al*., 2007
R.oryzae	SmF	48	23.86	27.5	Kar and Banerjee, 2000
	SSF	72	18.87	30.5	
	MSSF	72	32.76	90.9	
R.oryzae	SSF	48	41.3	94.8	Banerjee *et al*., 2005
Aspergillus niger	SmF	72	28.72	52[b]	Srivastava and Kar, 2009
Penicillium spinulosum	SmF	48	--	93.2	Sariozlu and Kivanc, 2009

SmF: submerged fermentation; SSF: solid state fermentation; MSSF: modified solid state fermentation; a: based on available tannin in the source; b: ug/ml

Ikeda *et al* (1972) showed existence of correlation of different types of tannin and microorganisms for gallic acid production. For instance *A. niger* produced 90 % gallic acid from *R. semialata* gallotannin while *P. chrysogenum* showed most effective gallic acid production (99 %) from *C. spinosa* tannin. On the other hand, low yields of gallic acid by *A. niger* were reported such as 30 % from *C. spinosa* tannin (Pourrat *et al*., 1985)

and 9.75 % from *R. coriaria* (Pourrat *et al*., 1987). Kar *et al.* (1999) reported 90 % yield of gallic acid after 72 h by using 200 g of *C. dignya* tannin for modified SSF by *R. oryzae*. For conversion of high tannic acid concentration, Bajpai and Patil (2008) carried out two stage SmF by *Aspergillus fischeri* using 0.5 % tannic acid as sole source of carbon for gallic acid production wherein growth phase was allowed till 32 h. 2.5 % tannic acid was added at 32 h and later at 50 h a dose of 1.5 % tannic acid was given. The maximum conversion (71.4 %) was achieved at 70 h.

1.6.1 Recovery of gallic acid

The property of less solubility of gallic acid in water (1.14 g /100 ml) and decreased solubility at low temperature was used in its recovery from fermented broth. Gallic acid tends to deposit on fungal mycelia after accumulation in the fermented broth. The recovery of gallic acid from mycelia involved washing of mycelial pellet with hot water. The pH of washings and broth was then adjusted to 2 before concentrating to four fold and the concentrate was kept at 0^0C for crystallization (Bajpai and Patil, 2008; Pourrat *et al*., 1987). The extraction of gallic acid using diethyl ether in 1:1 ratio was reported by Misro *et al*. (1997). Gallic acid was further recovered by the evaporation of diethyl ether.

1.7 Purification of tannase

Cell free broth was directly used as a source of extracellular tannase. While intracellular enzyme was prepared by lysis of cells by grinding in sand or crushing of frozen biomass in mortar or homogenizer or congelation – decongelation and extraction of lysate in appropriate buffer (Aguilar *et al*., 2001b; Barthomeuf *et al*., 1994b; Bhardwaj *et al*., 2003; Sharma *et al*., 2000). Barthomeuf *et al*. (1994a) reported the use of chitinase for the hydrolysis of mycelia to obtain mycelia bound enzyme.

Hamdy (2008) purified tannase from *F. subglutinans* by 65 % ammonium sulphate precipitation followed by ion exchange chromatography on DEAE cellulose and further fractionation on Sephadex G 150. With this method 27 fold purification of the enzyme was achieved. Similarly 19 fold purification was achieved when *Verticillium* tannase was purified by 80 % ammonium sulphate saturation and then by DEAE cellulose column chromatography (Kasieczka-Burnecka *et al*., 2007). Partial purification of tannase from *A. foetidus* was carried out by aqueous two phase extraction using various molecular weights of PEG (polyethylene glycol) (4000-9000) and its concentrations (15–30 %). 25

% PEG 6000 was found to show yield of 82 % with 2.7 fold purification of tannase (Naidu *et al*., 2008). The color impurities contributed by polyphenolic compounds present in *Paecilomyces variotii* tannase preparation was reported to be removed by active charcoal treatment at 4^0C without loss in activity before initial purification step. The broth was then purified 30 fold by 70 % ammonium sulphate precipitation, DEAE celluloase and Sephadex G 200 chromatography (Mahendran *et al.,* 2006). Acetone was used to precipitate and concentrate the *A. awamori* tannase which was then purified using GF 250 column (Mahapatra *et al*., 2005).

A. oryzae tannase was cloned and expressed in *Pichia pastoris*. The recombinant tannase was concentrated by ultrafiltration using 30 kDa cut-off membrane and purified on DEAE sepharose fast flow column with 51 % yield (Zhong *et al*., 2004). Ramirez-Coronel *et al*. (2003) reported a slightly different protocol for purification of A. *niger* tannase where they used preparative isoelectofocusing as initial purification step for 50 kDa ultrafiltered sample, followed by ion exchange chromatography using Mono Q column and Sephadex G-100 chromatography. The SDS PAGE of purified tannase showed two polypeptides with molecular weights 90 and 180 kDa. Two step pH differential protein precipitation using 0.1 % tannic acid and 0.1 % PEG 6000 was successfully reported for purification of *Aspergillus japonicus* tannase. The first step was at pH 3.0 where undesirable proteins precipitated and in the second step at pH 4.5 tannase precipitated with 7.82 fold purification and 50 % enzyme yield (Gupta *et al.*, 1997). Barthomeuf *et al.* (1994b) reported the application high pressure size exclusion chromatography followed by ultrafiltration to purify *A. niger* tannase. Application of affinity chromatography on ConA Ultrogel combined with elution with methyl D glucose was reported for separation of esterase and depsidase activities of tannase by Van De Lagemaat and Pyle (2005). Whereas one step purification of recombinant *Lactobacillus plantarum* overexpressed in *E. coli* was documented by using metal chelate affinity chromatography on poorly activated Ni-IDA 6 % agarose gels by Curiel *et al.* (2010). The purification was 15 fold with 95 % yield.

1.8 Characterization of tannase

Molecular weight

Tannase is a high molecular weight enzyme. All tannases purified so far are multimeric with the molecular weight ranging from 59 - 320 kDa (Boer *et al*., 2009; Hatamoto *et al*.,

1996; Skene and Brooker, 1995; Van De Lagemaat and Pyle, 2005b). Bhardwaj *et al* (2003) reported heterodimeric protein composed of two polypeptide chains of molecular weight 102 and 83 kDa and total molecular weight about 185 kDa. Whereas *A. niger* was found to have smaller active tannase with a molecular mass of 90 kDa, accompanying with less active form of 180 kDa. (Ramirez-Coronel *et al*, 2003). Kasiezcka – Burnecka *et al*., (2007) reported that *Verticillium* tannases were oligomeric enzymes consisting of two kinds of subunits with molecular masses of 39.9 and 45.6 kDa. Table 1.6 lists molecular weight of some of the tannases.

Isoelectric pH of tannase

The pI of tannase was documented in the range of 3.5 to 8.0. The pI of tannase from *Chryphonectria parasitica* was reported as 4.6-5.1 (Aoki *et al*., 1976a). For *A. oryzae* tannase, pI was shown to have a value of near to pH 4.0. (Iibuchi *et al*., 1968) while that of *A. niger* was 3.7-3.9. (Ramirez-Coronel *et al*, 2003). The isoelectric pH was close to 5.8 for tannase I and 6.2 for tannase II from *Verticillium* sp. (Kasiezcka-Burnecka *et al*., 2007).

Glycoprotein nature

All the fungal tannases reported so far are glycoproteins (Lekha and Lonsane, 1997). They contain relatively high carbohydrate content around 25 – 66 % w/w. The high carbohydrate content may help in explaining the high solubility of tannase in water. It also suggests the protection of polypeptide backbone of tannase and probable resistance of tannase to tannin effect (Binding of tannin to proteins and formation of insoluble complex with them) (Van De Lagemaat and Pyle, 2005b).

pH optimum and stability

The optimum pH for tannase was reported to be 5.0-6.0. In case of *A. niger*, two pH optima were recorded at 6.0 and 4.5 (Sharma *et al*., 1999). Tannase was observed to be stable over a pH range of 4.5 – 8.0. Tannases (Tannase I and II) from filamentous fungus, *Verticillium* sp. showed optimum activity at pH 5.5. Whereas Tannase I was observed to be stable over a pH range of 4.5 to 6.0 and tannase II showed stability in the pH range 5.0 to 7.5. Combined preparation of both the enzymes was stable in a broader pH range of 3.5 to 8.0. (Kasieczka–Burnecka *et al*., 2007). The optimum pH and stability values for tannase are summarized in Table 1.6.

Optimum temperature and stability

The optimum temperature for fungal tannase normally ranges between 30-50^{0}C, with a temperature stability ranging from as low as 0^{0}C in case of *F. subglutinans* (Hamdy, 2008) to as high as 80^{0}C (Sharma *et al*., 2008) in the case of *A. oryzae*. Plant tannase from leaves of Penduculate oak had optimum temperature of 35-40^{0}C and stability up to 50^{0}C (Niehaus and Gross, 1997). The lower temperature optimum was reported for tannase I and II of *Vertcillium* sp. P9 isolated from Antarctica. The temperature optima were 25^{0}C and 20^{0}C respectively and stability was at 20-30^{0}C for combined preparation (Kasieczka–Burnecka *et al.*, 2007). The optimum temperature and stability values for tannase from various fungi are listed in Table 1.6.

Kinetic constants (K_m and V_{max})

The Michaelis constants (K_m) of tannase were mostly reported for the substrates, tannic acid and methyl gallate (Table 1.6). Km values for other substrates such as propyl gallate, glucose-1-gallate were also reported by few researchers. Bhardwaj *et al*. (2003) reported lowest affinity of propyl gallate with K_m value of 2.05 mM for *A. niger* tannase whereas tannic acid was found to be the best substrate with K_m value of 0.28 mM and V_{max}/K_m value of 2.53. Hamamelitannin was shown to have K_m of 5.07 mM and V_{max} of 12.41 U/ml for *C. parasitica* tannase (Farias *et al*., 1994).

Activators and inhibitors

Tannase activation and inhibition was studied in presence of metal ions, chelators, detergents and denaturing agents. The inhibition of *A. flavus* and *A. niger* tannase by phenyl methyl sulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DEP) showed that serine involvement at tannase active site (Barthomeuf *et al*., 1994b; Yamada *et al*., 1968). Metal ions such as Ba^{2+}, Zn^{+}, Hg^{2+}, Ag^{+}, Co^{2+}, Cu^{2+}, Fe^{2+} were found to inhibit the enzyme (Chhokar *et al*., 2010; Hamdy, 2008; Mata-Gomez *et al*., 2009; Naidu *et al*., 2008; Rajakumar and Nandy, 1983; Sabu *et al*., 2005). Whereas Ca^{2+}, Mn^{2+} were found to be activators of *F. subglutinans*, *A. foetidus* and *A. awamori* tannase (Chhokar *et al*., 2010; Hamdy, 2008, Naidu *et al*., 2008). In case of *Verticillium* tannase, only Mg^{2+} was observed as enzyme activator (Kasieczka-Burnecka *et al*., 2007).

Table 1.6 Biochemical properties of purified tannases

Source	pH		Temperature (^{0}C)		Km (mM)		Molecular weight (kDa)	Reference
	Optimum	Stability	Optimum	Stability	Tannic acid	Methyl gallate		
Aspergillus flavus	5.0-5.5	5.0-5.5	50-60	Up to 60	0.05	0.86	--	Yamada *et al*., 1968
Aspergillus niger	6.0	5.5-6.0	60	--	0.023	0.041	225	Mata-Gomez *et al*., 2009
Aspergillus niger	6.0 and 4.5	--	60	--	--	0.20	--	Sharma *et al*., 1999
Aspergillus niger	6.0	4.0-8.0	30-40	Up to 60	--	1.03	168	Sabu *et al*., 2005
Aspergillus tamarii	5.0	4.0-8.0	30	20-40	--	--	--	Costa *et al*., 2008
Candida sp.	6.0	3.5-7.5	50	Up to 40	---	--	250	Aoki *et al*., 1976a, b
Chryphonectria parasitica	5.5	4.0-7.5	30	20-45	0.94	7.492	240	Farias *et al*., 1994
Fusarium subglutinans	6.0	6.0-8.0	40	(-)15-70	3.57	--	150	Hamdy, 2008
Lactobacillus plantarm	5.0	--	30	up to 50	--	6.25	--	Rodriguez *et al*., 2008 b
Paecilomyces Variotii	6.0	4.0-8.0	40	30-50	--	--	149.8	Mahendran *et al*., 2006
Penicillium chrysogenum	5.0-6.0	4.0-6.5	30-40	30	0.48	--	--	Rajakumar and Nandy, 1983
Penicillium variable	5.0	3.0-8.0	50	25-80	32	14	310	Sharma *et al*., 2008
Selenomonas ruminantium	7.0	--	30-40	--		1.6	59	Skene and Brooker , 1995

Metal chelating agent such as EDTA was found to inhibit most of the tannases (Battestin and Macedo, 2007b; Kar *et al*., 2003; Kasieczka-Burnecka *et al*., 2007; Mahapatra *et al*., 2005) The surfactants such as sodium dodecyl sulphate (SDS), Tween 80, Tween 60 and Triron X-100 were found to inhibit tannase activity (Battestin and Macedo., 2007b; Kar *et al*., 2003; Mahapatra *et al*., 2005). Denaturing agents like idoacetate, arsenite and arsenate inhibited tannase activity (Hamdy, 2008). Kar *et al.* (2003) found that tannase was inhibited by B-mercaptoethanol and Dimethyl sulfoxide.

The compatibility study of tannase with organic solvents showed complete inhibition of the enzyme by acetic acid, isoamyl alcohol, chloroform, isopropyl alcohol and ethanol while butanol and benzene increased the enzyme activity (Chhokar *et al*., 2010). Mata-Gomez *et al*. (2009) reported enhancement in activity by petroleum ether and complete inhibition by ethanol and acetone at 60 % concentration.

1.9 Immobilization of tannase

In view of the high commercial potential of tannase, attempts were made to obtain a highly active and stable immobilized preparation suitable for commercial applications.

Weetal and Detar (1974) immobilized tannase to glutaraldehyde activated alkylamine glass and used the bound enzyme for the hydrolysis of gallic acid esters of tannic acid. Immobilization brought about a slight shift in the pH for optimum activity viz. pH 6-7 for free enzyme to 7.5. While, tannase immobilized in chitosan showed a decrease in its optimum pH (4.5) than of the free enzyme (pH 5.5) (Abdel-Naby *et al*., 1999). Tannase immobilized in chitosan exhibited good stability to repeated use and retained 85% of its initial activity after 17 cycles. *A. niger* tannase when adsorbed onto Con A-Sepharose showed a decrease in the efficiency with increasing enzyme load. Immobilization brought an increase in the apparent K_m and V_{max} and these were correlated to the formation of a more efficient catalyst. The bound enzyme showed high stability to repeated use and retained 81% of its initial activity after 6 cycles (Sharma *et al*., 2002). *P. variable* tannase when adsorbed onto Amberlite IR-1204 and XAD-7, DEAE-cellulose, celite and silica gel, maximum immobilisation efficiency (69%) was obtained with Amberlite IR-1204. The bound enzyme showed good stability to repeated use and retained 85% of its initial activity after 9 cycles (Sharma *et al*., 2008).

Guo and Yang (2000) entrapped tannase in calcium alginate gels and used the immobilized preparation for the hydrolysis of *R. coriaria* gallotannin to gallic acid. Under optimized conditions, the immobilized enzyme yielded 61% gallic acid. Boadi and Neufeld (2001) entrapped tannase in alginate, chitosan, carrageenan and pectin. Cross-linking of the matrix with glutaraldehyde improved the retention of activity. The immobilized enzyme exhibited good stability to storage at 4^{o}C.

A. niger tannase microencapsulated in a liquid core alginate gel showed an efficiency of 36.8%. Encapsulation brought about an increase in the optimum pH and temperature to 6.0 and 40^{o}C, from that of the soluble enzyme (pH 5.0 and 30^{o}C respectively). Evaluation of the kinetic parameters showed an increase in the apparent K_m and V_{max}. The increase in the apparent K_m was correlated to weak binding of the substrate. Stirred tank reactor studies showed that the bound enzyme could be used for 15 h without any significant loss in its initial activity (Yu *et al*., 2004).

Yu and Li (2005) microencapsulated mycelium bound *A. niger* tannase in calcium alginate gels to study the estrification of propylgallate from gallic acid and propanol in organic solvents. Influence of different organic solvents on the enzyme reaction revealed that maximum conversion (26.8 %) is obtained in presence of benzene. Mahendran *et al*. (2006) entrapped *P. variotii* tannase in calcium alginate gels and used the immobilized system to study the hydrolysis of tannic acid. Time course of tannic acid hydrolysis showed an increase in the rate of hydrolysis with increase in the incubation time. Optimum hydrolysis (98 %) was observed after 6 h of incubation. The bound enzyme was stable and retained approximately 85 % of its initial activity after 8 cycles. Das Mohapatra *et al*. (2007) entrapped *B. licheniformis* cells in calcium alginate gels and used the immobilized system for the production of tannase. Influence of alginate concentration on enzyme production revealed that maximum yield (0.56±0.03 U/ml) was obtained when the cells were entrapped in 3% alginate.

1.10 Overview and future prospects

China is the major manufacturer for gallic acid. The worldwide annual demand of gallic acid is around 8000 tons (Banerjee *et al*., 2007). Gallic acid finds various applications in different sectors of industries from healthcare and food industry to agriculture and photography. Recent findings on therapeutic applications of gallic acid as an antioxidant, anti-inflammatory, anticancer and antiviral molecule make it a promising molecule and

this demands the studies for more efficient, economical and productive process for gallic acid production.

The conventional chemical route of gallic acid production is disadvantageous because of environmental hazards and uneconomical due to poor yield. The bioconversion by enzyme as well as whole cell biocatalyst has immense significance in industry because of high yields, low impurity profiles, environmental safety and process reproducibility. The biocatalyst tannase used for conversion of tannins to gallic acid is itself an industrially important product. Commercial tannase is currently available from very few manufacturers such as Kikkoman (Japan), Biocon (India) and Julich (Germany) to name a few (Van De Lagemaat and Pyle, 2005b). In spite of the commercial importance of both tannase and gallic acid, little work has been done on the large scale production process at fermenter level.

It will be definitely worth searching for different or improved microbial sources which produces high titer of tannase and withstand high concentration of tannic acid. This will make the submerged fermentation an economical option for the production of gallic acid. It will be useful to find out cost effective, productive and efficient bioconversion process for gallic acid production using fungal tannase for industrial exploitation.

Present Investigations: Present investigations were carried out with following objectives:

1. Isolation and characterization of various fungal cultures producing tannase for the conversion of tannic acid to gallic acid
2. Optimization of tannic acid extraction from various plant sources at laboratory level
3. Optimization and strain improvement studies of the strain which can tolerate high concentration of tannin
    a. At shake flask fermentation level
    b. At scale up fermenter level (5 lit, 35 lit and 100 lit)
    c. Effect of morphology of fungal culture on the production of tannase enzyme complex with respect to gallic acid production
    d. Purification and biochemical characterization of tannase enzyme
4. Whole cell and enzyme immobilization for the production of gallic acid

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The materials and chemicals used in the present study were purchased from suppliers listed in the Table 2.1.

Table 2.1 Sources of chemicals and materials

Chemicals / Materials	Suppliers
Gallic acid, bovine serum albumin, N-Methyl N-nitro N-nitroso guanidine (MNNG), β-mercaptoethanol, chitosan, rhodanine, methyl gallate	Sigma Aldrich, India
Acrylamide, N, N- Methylene bis acryamide, tannic acid, tri sodium citrate, citric acid, sucrose, quinine hydrochloride, Folin Ciaoculteau reagent	Merck Specialties Pvt. Ltd, India
Precoated aluminium plates of silica gel GF 254	Merck Specialties Pvt. Ltd, Germany
Ellagic acid, chloramphenicol, peptone, yeast extract, rose bengal agar, Tween 80, Tween 20, Tween 60	Hi Media Laboratories, India
Quinic acid, ammonium persulphate, sodium dodecyl sulphate, Commassie brilliant blue G 250, imidazole, Triton X-100	Sisco Research Laboratories Pvt. Ltd., India
Coomassie brilliant blue R-250	Ubichem Ltd.
Dithiothreitol (DTT)	Genei, Banglore
Molecular weight marker kit	BioRad Laboratories Inc., USA
Bradford reagent	Thermo India Fischer Scientific, USA
DEAE sepharose	Amersham Pharmacia

	Biotech AB, Sweden
Ttriethanolamine	S. D. Fine Chemicals, India
Membrane filters (0.22μ)	Pall Life Sciences

All other chemicals, media, buffers etc. used were of analytical grade, procured from S.D. Fine, Hi Media, SRL, Loba Chemie India.

Sources of tannin used in the present investigations are listed in Table 2.2.

Table 2.2 Sources of plant tannins

Common name	**Botanical name**	**Plant part**	**Source**
Tara	*Caesalpinia spinosa*	Pod	Lima, Peru
Teri	*Caesalpinia dignya*	Pod	Assam, India
Myrobalan	*Terminalia chebula*	Fruit	UP, Maharashtra, India
Beheda	*Terminalia bellerica*	Fruit	Maharashtra, India
Arjuna	*Terminalia arjuna*	Bark	Maharashtra, India
Turkish galls	*Quercus infectoria*	Gall	Himachal Pradesh, India

2.2 Organism

Isolates *Aspergillus allahabadi* and *Aspergillus fumigatus* were used in the present study. Other Aspergilli used in the study were *A. niger* NCIM 620 and *A. oryzae* NCIM 643 procured from NCIM (National Collection of Industrial Microorganisms, NCL), Pune, India.

2.3 Composition of media

Composition of various media used in the present study is given in Table 2.3.

Table 2.3 Media composition

Name of medium	Abbreviation	Purpose	Constituents (g/L)
Potato dextrose broth / agar (2 %)	PDB	Maintenance of culture	Potato infusion 200; dextrose 20; pH 5.5-6.0
Czapek Dox medium with glucose		Production of tannase	$NaNO_3$ 2.5; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; KCl 0.5; pH 5.5
Tannic acid medium / agar (3 %)	TAB	Screening of tannase production	$NaNO_3$ 6.0; KCL 5.0; KH_2PO_4 0.5; K_2HPO_4 0.5; $MgSO_4$ 0.5; tannic acid 10; pH 5.0
Tannic acid-glucose medium / agar (3 %)	TGB	Preparation of inoculum, isolation of tannase producers	$NaNO_3$ 6.0; KCL 5.0; KH_2PO_4 0.5; K_2HPO_4 0.5; $MgSO_4$ 0.5; tannic acid 10; glucose 5; pH 5.0

2.4 Maintenance of organism (s)

All isolates were maintained on PDA slants containing 1 % tannic acid, whereas *A. niger* NCIM 620 and *A. oryzae* NCIM 643 were maintained on PDA slants. Long term preservation was achieved by preparing glycerol stocks preserved at -80^{0}C. All cultures were routinely subcultured every 30 d and incubated at 28^{0}C.

2.5 Isolation and screening of tannase producers

2.5.1 Isolation of tannase producers

For the isolation of tannase producers, animal droppings, bark shavings, leaves, pods, seed cover and fruits of tannin rich plants and soil were used. The soil and animal droppings were serially diluted and plated on TGB medium with agar while leaves, fruits and bark shavings of tannin rich plants were washed gently to remove dirt and soil particles, chopped to small pieces and incubated in TGB medium for 10 d. Medium showing growth was plated on Rose Bengal agar and then isolated colonies were spot inoculated on TGB medium with agar. Pods, galls, seeds and seed cover from different plants were ground to coarse powder, moistened with water to have approximately 50 %

moisture and incubated for 10 d. The samples were then serially diluted and plated on TGB medium with agar. The isolates obtained were numbered as SM 1 through SM 106.

2.5.2 Primary screening for tannase production

All isolates thus obtained were further spot inoculated on TAB medium with agar and incubated for 120 h at 28^0C. Production of tannase was confirmed qualitatively by measuring colony diameter including zone of hydrolysis (clearance) below and around colony every 24 h. The tannase positive isolates were screened for production of tannase under submerged conditions in TAB medium at 28^0C for 72 h. The reference strains *A. niger* NCIM 620 and *A. oryzae* NCIM 643 were also spot inoculated and checked for tannase production.

2.5.3 Secondary and tertiary screening for tannin tolerance

The high tannase producing isolates and reference strains were checked for utilization of tannins as sole carbon source using TAB medium. For this study 2 % (v/v) spore suspension of each isolate (1 x 10^7conidia/ml) was inoculated in TAB (pH 5.0) medium and incubated at 28^0C at 150 rpm for 72 h. The growth of fungal isolate was monitored by microscopy and dry weight after every 8 h. The screening was carried out in stepwise manner. All the isolates were first screened for the growth in tannin at the concentration of 20 g/L, 50 g/L and 100 g/L (w/v). The positive isolates were further screened for growth in 150 g/L, 200 g/L, 250 g/L and 300 g/L tannin as a second step. The third screening was carried out at the concentrations of 350 g/L, 400 g/L and 450 g/L for the positive isolates obtained after second step. Tertiary screening of two strains (SM 70 and SM 88) was carried out using TAB medium with varying concentrations of tannic acid (20 g/L – 200 g/L) as sole source of carbon at shake flask. The cultures were inoculated in TAB (pH 5.0) and incubated at 28^0C at 150 rpm for 72 h. Periodic samples were drawn and analyzed for dry cell weight, tannase activity and residual tannic acid.

2.6 Identification of organism

The best tannase producer (s) was identified by partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene sequencing. In brief, the organism was grown in PDB containing tannic acid (1 %) by using 1x10^7spores/ml. The mycelial growth was harvested and sent for identification at Genei, Banglore.

2.7 Time profile of tannase

Induced and constitutive nature of *A. allahabadi* tannase enzyme production was studied using TAB and Czapek Dox medium with glucose respectively under shaking conditions at 28^0C for 48 h. Production profile of tannase was checked in TAB medium for 60 h. The 100 ml TAB medium was inoculated with suspension of 2 x 10^7 spores or mycelial inoculum (10 % v/v). Samples were drawn periodically to analyze dry cell weight and tannase activity. Similarly growth profile of *A. allahabadi* was studied in PDB and Saboraud's dextrose broth (SDB). Samples were drawn periodically and checked for dry cell weight.

2.8 Development of inoculum

The spores from slant of PDA containing tannic acid were scrapped and suspended in 0.1 % Tween 80 in distilled water. The spore count was carried out using Neubauer hemocytometer. The spore suspension thus obtained was used as a spore inoculum for fermentation. Mycelial inoculum was developed by inoculating 2 ml (1 x 10^7) spore suspension in 100 ml of TGB and incubating it on shaker at 28^0C for 24 h.

2.9 Improvement for tannase production and subsequent gallic acid production

2.9.1 Improvement by coculture

Conidia from 7 d old slants of *A. allahabadi* and *A. parvulus* were harvested in sterile Tween 80 (0.1 % in water). The count was adjusted to 1x10^7 conidia/ml with haemocytometer. One ml of conidial suspension of each strain was inoculated in the 100 ml optimized medium containing 250 g/l tannic acid to prepare cocoulture set. Similarly 1 ml of respective individual strain was inoculated in the optimized medium to prepare monoculture set. The flasks were incubated at 28^0C on a shaker at 150 rpm for 60 h. The samples were periodically drawn to analyze dry weight, tannase activity, residual tannic acid and gallic acid content.

2.9.2 Strain improvement by UV irradiation

Conidial suspension was prepared in sterile Tween 80 (0.1 % in water) by harvesting 10^7 conidia/ml from 7 d old *A. allahabadi* parent strain slant. Five ml suspension containing 0.2 x 10^6 conidia/ml of *A. allahabadi* was taken into petri plate and exposed to UV lamp (15 W) kept at a distance of 15 – 20 cm and was irradiated for different time intervals

between 1 min to 20 min. Untreated and treated conidia were serially diluted in sterile saline and 0.1 ml suspension was spread on TAA medium. Plates were incubated at 28^0C to obtain discrete colonies. Survival curve was plotted with time of exposure (Lotfy *et al.*, 2007; Karnam and Medicherla, 2008).

2.9.3 Strain improvement by MNNG treatment

N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used for mutation studies. 10^6 conidia/ml from 7 d old *A. allahabadi* parent strain slant were harvested in sterile Tween 80 (0.1 %) water. Conidia were centrifuged and resuspended in 5.0 ml sterile citrate buffer (0.5 M, pH 5.5) containing 0.5 mg/ml, 1 mg/ml, 2 mg/ml and 3 mg/ml MNNG respectively for 5 - 20 min under shaking conditions at 28^0C. The mutagen treated conidia were centrifuged, washed 2-3 times with sterile saline and plated on TAA medium. Plates were incubated at 28^0C to obtain colonies (Deshpande, 1981). Survival curve was plotted with time of exposure and constant concentration of MNNG.

2.10 Optimization of extraction of tannin from plant (s)

Tannin rich plant material (bark shavings, pods, seeds and leaves) was cleaned to remove dust and soil particles and dried. Dried material was pulverized to coarse powder (mesh size 20 mm) and fine powder (80 – 100 mm) in a grinder and stored in aluminium bags, properly sealed to avoid absorbance of moisture till further use. The extraction was carried out using different conditions viz. **a**. powdered plant material (coarse as well as fine mesh) was mixed with tap water (pH 7.0) in ratio of 1:5 at static condition at 40^0C for 12 – 16 h and **b**. the fine mesh plant material was mixed with tap water in ratio of 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8 and extracted at 40^0C for 30 min, 60 min, 90 min and 120 min. The mixture was extracted with stirring at 90 rpm in round bottom flask assembly with agitation rod having teflon blades. The effect of temperature on extraction of tannins was checked at 50^0C, 60^0C and 70^0C for 60 min. After extraction, suspended solids were removed by centrifugation at 3000 g for 30 min. or filtration by muslin cloth. The supernatant and residue were used for physicochemical characterization.

2.11 Preparation of plant extract for fermentation

The fine powdered plant material of *C. spinosa* was suspended in tap water at 1:6 proportion and kept at 70^0C for 60 min under stirring conditions. The extract was

allowed to cool and centrifuged or filtered by using muslin cloth. The supernatant was directly used as source of tannins.

2.12 Effect of plant tannin substrate

Effect of plant tannin substrate was studied using optimized extracts of *C. spinosa*, *Q. infectoria*, *C. dignya*, *T. chebula*, *T. bellerica* and *T. arjuna* at equal tannin concentration of 20 g/L. Experiment was set up in TAB medium where tannic acid was replaced by respective plant tannin. Fermentation was carried at 28^0C and 150 rpm for 60 h. Commercial tannic acid at 20 g/L concentration was used as control. Samples were drawn periodically and analyzed for dry weight, tannase activity, gallic acid production by HPLC and residual tannin.

2.13 Gravimetric analysis

2.13.1 Physicochemical characterization

Physicochemical characterization viz. loss on drying, total solids, total ash, acid insoluble ash, total carbohydrates and total nitrogen by Kjeldahl was carried out according to procedures described in AOAC, 1998. Reducing sugars were analyzed as described by Dubois *et al.* (1956). Metal analysis (Na, K, Ca, Mg, Fe, Zn) was carried out by Atomic Absorption Spectrometry.

2.13.2 Biomass estimation

Biomass produced in the fermentation process was filtered, washed twice with sterile distilled water and dried at 60^0C till the constant weight was achieved. The dry weight of biomass was expressed as mg/ml.

2.14 Enzyme assays

2.14.1 Tannase assay

Cell free supernatant/ filtrate were used as a source of extracellular tannase. Intracellular enzyme was prepared by crushing centrifuged cells in liquid nitrogen. The crushed cells were resuspended in 50 mM citrate buffer, pH 5.0 and centrifuged. The supernatant was assayed for intracellular tannase activity. Tannase activity was assayed using methyl gallate substrate as described by Sharma *et al.* (2000). Suitably diluted enzyme (0.25 ml)

was added to substrate (0.25 ml, 0.01 M methyl gallate in 0.05 M citrate buffer, pH 5.0) and incubated at 30^0C for 15 min. The liberated gallic acid was reacted with methanolic rhodanine (0.667 %, 0.3 ml) and further incubation at 30^0C for 5 min, followed by the addition of potassium hydroxide solution (0.5 M, 0.2 ml) and subsequently distilled water (4.0 ml). Absorbance was recorded at 520 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated one micromole of gallic acid per ml per min under standard assay conditions.

2.14.2 Polyphenol oxidase assay

Polyphenol oxidase assay was carried out using pyrocatechol as substrate as described by Shi *et al.* (2005). Test reaction was started by addition of buffer (3.4 ml), substrate (1 ml, 0.1 M in 0.05 M citrate buffer, pH 5.0) and enzyme (1 ml) and the reaction mixture was incubated at 30^0C for 10 min. Reaction was stopped by keeping in boiling water Bath for 2 min. Blank reaction was started with only enzyme (1 ml) kept in boiling water bath for 2 min. followed by addition of substrate and buffer. Absorbance was read at 420 nm. One unit enzyme activity was defined as increase in absorbance by 0.001 per min and calculated by formula U/min = $A_{test} - A_{blank}/0.01$.

2.15 Analytical procedures

2.15.1 Estimation of total tannins

Tannins were estimated by protein tannin precipitation method as described by Haggerman and Butler (1978). The aqueous tannin solution (1 ml of 1 mg/ml or 1 ml crude extract) was added to 2 ml bovine serum albumin (1mg/ml of 0.20 M acetate buffer, pH 5.0 containing 0.17 M sodium chloride). The reaction mixture was mixed properly, allowed to stand at room temperature for 15 min followed by centrifugation (5000 g/15 min). The supernatant was discarded and pellet was washed with buffer. The precipitate was dissolved in 4 ml of SDS-triethanolamine (1 % SDS and 5 % (v/v) triethanolamine). 1 ml of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01N HCl) was added and mixed immediately. The absorbance was read at 510 nm after 30 min.

2.15.2 Thin layer chromatography (TLC)

TLC was performed on precoated plates of silica gel GF_{254} (10x10 mm). Reference standards (tannic acid, ellagic acid, quinic acid and gallic acid) and samples were applied

mechanically using Linomat V applicator (Camag, Switzerland). The TLC was developed with saturated mobile phase chloroform: ethyl acetate: formic acid in twin trough chamber. Substances were identified using UV detection at 254 nm. For visual observation, plates were sprayed with $FeCl_3$ solution (Malbasa *et al.*, 2004).

2.15.3 High Pressure Liquid Chromatography (HPLC) for quantitative determination of gallic acid and quinic acid

Quantitative estimation of gallic acid and quinic acid was carried out by HPLC (Waters 510, UV detector 440) isocratic system using Nucleosil, 250 x 4.6, 5µ, C_{18} reverse phase column. For gallic acid, mobile phase was 5 % acetic acid. Flow rate was maintained at 1.8 ml/min and detection wavelength was 280 nm (Seth and Chand, 2000). Quinic acid was eluted with phosphate buffer (100 mM, pH 2.4) at flow rate 0.8 ml/min. The detection wavelength was 214 nm (AOAC, 1998).

2.15.4 Spectrophotometric estimation of gallic acid

Spectrophotometric estimation of gallic acid was carried out by the method described by Sharma *et al.* (2000). In a standard (gallic acid) or suitably diluted sample, 300 µl of methanolic rhodanine (0.667 % in methanol) was added followed by addition of 200 µl of 0.5 M potassium hydroxide. After incubation at 30^0C for 5 min 4 ml distilled water was added. The absorbance was read at 520 nm after 5-10 min. Standard gallic acid stock (1mg/ml) was prepared in citrate buffer (50 mM, pH 5.0) and calibration curve (5µg-50µg) was plotted.

2.16 Recovery of gallic acid

The fermented broth was filtered to separate the mycelia and mother liquor. Mycelia were washed with hot water to remove the deposited gallic acid. The washings and mother liquor were mixed and adjusted to pH 2.0 before concentrating to 2 fold. The concentrate was then kept at 0^0C for precipitation of gallic acid. The precipitated gallic acid was harvested by filtration and dried under vacuum. The identification and quantification of recovered gallic acid was carried out by TLC, Fourier Transform Infra Red (FTIR) and HPLC.

2.17 Purification of tannase

2.17.1 Precipitation by ammonium sulphate and dialysis

Cell free supernatant was processed for precipitation by ammonium sulphate at 60 % saturation. The solution was centrifuged (10000 g, 20 min, 4^0C) and pellet was discarded. Ammonium sulphate was added slowly in supernatant with constant stirring to 80 % saturation. The mixture was kept for 1 h at 4^0C. The precipitate was obtained by centrifugation and then dissolved in minimum amount citrate buffer (50 mM, pH 5.0). The concentrated enzyme was dialyzed using dialysis membrane (Sigma, 12000 cut off) against same buffer for 8 h with three changes of the buffer. The dialysate was kept at 4^0C till further use.

2.17.2 Precipitation by acetone

Extracellular enzyme was precipitated by 1:3 volumes of chilled acetone (-20^0C). The precipitate was kept at 4^0C for 3 h for complete precipitation and recovered by centrifugation at 10000 g for 20 min at 4^0C. The precipitate was then dried in vacuum desiccator to remove traces of acetone and resuspended in citrate buffer (50 mM, pH 5.0).

2.17.3 Tangential flow filtration

Extracellular enzyme was concentrated using tangential flow filtration assembly (Pall Life Sciences) with 30 kDa cassette (molecular weight cut off 30 kDa) at 4^0C. The concentrated enzyme was lyophilized in freeze dryer (Labconco, USA).

2.17.4 Ion exchange column chromatography

The acetone precipitated protein was applied to DEAE sepharose column (1.5 x 27 cm) previously equilibrated with 50 mM citrate buffer, pH 5.0. The elution was carried out with a linear gradient of 0 – 0.5 M sodium chloride at flow rate 8 ml/h. Fractions (2 ml) were analyzed for protein and tannase activity.

2.18 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was carried out in 8 % (w/v) polyacrylamide slab gel at pH 8.8. Protein bands were detected using commassie blue or silver staining method (Laemmli *et al.*, 1970; Sambrook and Russell, 2001).

2.19 Estimation of protein

The protein was estimated by the method of Lowry *et al.* (1951) and also by the method of Bradford (1976) using Bradford reagent. Bovine serum albumin was used as a standard protein.

2.20 Staining of proteins

2.20.1 Staining by Coomassie blue

The electrophoresed gel was immersed in the staining solution of coomassie brilliant blue R-250 (0.25 % in methanol:acetic acid) and placed on a slowly rocking platform for 3-4 hrs at room temperature. The gel is then removed and destained by soaking the gel into methanol:acetic acid solution on a rocking platform for 3-4 h, changing the destaining solution 3-4 times. After destaining, the gel was stored in deionized water (Sambrook and Russell, 2001).

2.20.2 Staining by silver staining

The electrophoresed gel was incubated in fixing solution (ethanol: acetic acid:water, 30:10:60) overnight at room temperature with gentle shaking to fix the proteins. The fixing solution was discarded and gel was incubated with 30 % ethanol for 30 min at room temperature with gentle shaking. The gel was then removed and incubated deionized water for 10 min with gentle shaking. After the two washes of water, the gel was immersed in freshly prepared silver nitrate solution and incubated for 30 min at room temperature with gentle shaking. The gel was washed with deionozed water and incubated in fresh developing solution (aqueous solution of 2.5 % sodium carbonate and 0.02 % formaldehyde) at room temperature with gentle shaking till the stained bands of protein appeared (Sambrook and Russell, 2001).

2.20.3 Localization of tannase activity in gel

The electrophoresis was carried out at 50 V for 4 h at 4^0C, in Mini Protean II electrophoresis cell (BioRad). The localization of tannase activity in the gel was carried out according to Aoki *et al.* (1979). The gel was washed for 1 h in 100 ml of 2.5 % (v/v) Triton X-100. The gel was then washed with two 45 min washes of 100 ml, 10 mM acetate buffer pH 5.5 with constant shaking. The gel was then incubated in a solution containing 0.5 % (w/v) tannic acid in 0.1 M acetate buffer, pH 5.5 at 30^0C with constant

shaking. The tannic acid solution was then discarded and replaced with 100 ml of 0.5 % (v/v) quinine hydrochloride in 0.05 M acetate buffer, pH 5.5 at room temperature.

2.21 Characterization of tannase

Optimum pH of partially purified tannase was tested at 35^0C in the pH range of 2.0 – 8.0 using 50 mM buffers of range, glycine-HCl (2.0); citrate (3.0, 4.0, 5.0, 6.0); phosphate (7.0, 8.0). Stability at pH was checked by incubating enzyme at 30^0C for 30 min in 50 mM buffers of different pH values. Residual activity of enzyme was calculated considering initial enzyme activity at time zero as 100 %. The temperature stability of enzyme was determined by incubating enzyme at standard assay conditions at different temperatures ranging from 5 – 90^0C for 30 min. The Michaelis constants (Km) and maximum reaction velocity (Vmax) were determined for two substrates i.e. tannic acid and methyl gallate using the range 0.01 mM – 0.1 mM and 0.1mM – 1.0 mM respectively. The total sugar analysis of tannase was carried out by phenol sulphuric acid method.

2.22 Immobilization of tannase

Immobilization was carried out using extracellular enzyme and whole cells. The entrapment method involved mixing of one volume purified enzyme (100 U) in one volume various concentrations (3 – 6 %) of sodium alginate (Kierstan and Coughlan, 1985). The mixture was extruded dropwise to calcium chloride solution (0.2 M) under gentle shaking conditions. The beads thus formed were left in the calcium chloride solution for 2 h to harden. The beads were then washed and kept in 50 mM citrate buffer, pH 5.0 at 4^0C until further use. Similarly warm κ-carragenan (4 %) solution was mixed with enzyme in 9:1 proportion. The mixture was added dropwise in KCl (2 %) solution and beads were left in the solution for 1 h to harden. Immobilization of tannase by covalent binding was carried out using chitosan carrier (Abdel-Naby *et al.*, 1999). Briefly, chitosan (2 g) was dissolved in HCl (0.1 M) containing glutaraldehyde (2.5 %) for 2 h at 30^0C and then solubilized chitosan was precipitated by NaOH (1 M). The precipitate was washed with distilled water. The wet chitosan was mixed with purified enzyme and stirred for 1 h at 30^0C. The immobilized and unbound enzyme was checked for activity and protein. For immobilization of whole mycelia, the biomass obtained after fermentation was washed with sterile distilled water and frozen in liquid nitrogen

followed by lyophilization (Labconco, USA). The lyophilized cells (50 mg) were entrapped in calcium alginate beads as described above.

2.23 Bioconversion of tannins using immobilized tannase

The immobilized enzyme was incubated in the solution (pH 5.0) containing various concentrations of tannic acid (50 g /L, 100 g/L and 200g /L) at 28^0C on shaker. The samples were drawn periodically for gallic acid estimation and residual tannic acid. The beads were washed with citrate buffer (0.05 M, pH 5.0) and estimated for tannase activity and protein content. The beads were then again inoculated in tannic acid solution. The procedure was repeated and reusability of the immobilized enzyme was determined.

CHAPTER 3

ISOLATION AND SCREENING OF FUNGAL STRAINS FOR TANNASE PRODUCTION

Introduction

Tannins generally retard the rate of decomposition of soil organic matter by inhibiting the biodegradative enzymes of microorganisms and inhibit growth of a number of microorganisms (Scalbert, 1991). Isolation of tannin degrading microorganisms capable of producing high tannase was reported by many researchers. The reported work was mainly focused on the screening of high tannase producers which utilized tannic acid as a sole source of carbon. There are no reports on the tolerance and utilization of high tannin concentrations by microorganisms and their application for gallic acid production at fermenter scale. Tannase production was mainly reported by using pure and commercial tannic acid (pentagalloyl glucose). Very few researchers explored the natural plant tannins for the production of tannase and gallic acid.

The focus of the present investigation is the production and application of tannase for conversion of tannins to gallic acid. Therefore there is a need to explore various natural tannin sources, to optimize their extraction and to screen the suitability of fungal strain (s) with tannin source. This chapter describes the work on isolation of fungal strains from tannin rich sources, screening of isolates for production of tannase and for tolerance and utilization of high concentrations of tannin.

Results and Discussion

3.1 Isolation and preliminary screening of tannase producers

To isolate the potential tannase producing microorganisms natural habitat of tannin or polyphenol environment such as forest and garden soil, tannin containing plant parts and fruits of rich polyphenol content were selected. The isolation was carried out on tannic acid –glucose agar as described in section 2.5.1. Table 3.1 shows the number of fungal isolates obtained from collected samples. Total 106 isolates thus obtained were further

Table 3.1 Preliminary screening of tannase producing fungi

Source	Isolates	C.D. in mm	Z.D.in mm	Tannase activity, U/ml
Soil	SM 2	34	-	1.24
	SM 3	23	2	1.29
	SM 4	9	4	0.80
	SM 9	11	7	0.60
	SM 10	18	6	1.56
	SM 11	26	4	1.28
	SM 12	12	2	1.10
	SM 14	16	5	1.14
	SM 15	35	-	1.26
	SM 16	24	3	1.06
	SM 17	16	2	1.00
	SM 18	21	3	0.95
	SM 19	22	3	1.31
	SM 20	14	2	0.97
	SM 21	13	1	1.05
	SM 22	15	3	1.00
	SM 23	18	6	1.23
	SM 24	18	1	1.25
	SM 25	40	-	1.45
	SM 26	19	2	1.20
	SM 28	13	3	0.88
	SM 31	19	1	1.43
	SM 35	21	7	0.09
	SM 36	12	3	0.94
	SM 37	65	-	3.21
	SM 38	75	-	4.18
	SM 39	6	2	1.06
	SM 42	18	4	1.14
	SM 43	9	3	0.65
	SM 44	9	5	0.72

Table 3.1 continued

	SM 46	10	3	0.81
	SM 47	7	4	0.79
	SM 48	24	1	1.76
	SM 49	13	2	1.26
	SM 50	10	5	0.92
	SM 59	12	6	0.93
	SM 60	7	5	0.75
	SM 61	13	1	1.23
	SM 63	19	6	0.97
	SM 64	18	5	0.93
	SM 65	10	1	1.10
	SM 66	9	9	0.65
	SM 67	11	7	0.85
	SM 68	71	-	4.00
	SM 74	50	-	2.56
	SM 75	9	5	0.59
	SM 80	50	-	2.68
	SM 88	78	-	4.70
Plant parts, Fruits	SM 71	3	No zone*	0.09
	SM 72	1.5	No zone	0.05
	SM 73	2.5	No zone	0.21
	SM 81	4	No zone	0.37
	SM 82	3.5	No zone	0.34
	SM 83	3	No zone	0.10
	SM 84	2	No zone	0.05
Bark	SM 29	19	4	1.15
	SM 30	22	3	1.37
	SM 32	6	7	0.77
	SM 34	7	2	0.98

Table 3.1 continued

	SM 41	12	5	0.76
	SM 62	12	6	1.14
	SM 69	70	-	3.98
	SM 76	5	3	0.68
	SM 78	21	4	1.12
	SM 79	13	4	0.36
Leaves	SM 27	16	4	0.89
	SM 33	8	3	0.58
	SM 40	11	6	0.82
	SM 54	13	3	0.61
Pods/seeds	SM 55	12	3	0.75
	SM 56	20	8	0.82
	SM 57	73	-	3.42
	SM 58	18	3	1.17
	SM 70	81	-	5.20
	SM 85	65	-	2.43
	SM 86	17	4	0.90
	SM 87	67	-	3.68
Ref cultures	*A niger* NCIM 620	60	-	2.50
	A oryzae NCIM 643	33	-	1.06

C.D.: colony diameter; Z D.: zone diameter; Diameter of colony and zone of hydrolysis was observed on 2 % TAA medium at 120 h and tannase activity was estimated in 2 % TAB medium after 48 h under shaking conditions.

screened on TAA for their ability to produce tannase by utilizing tannic acid as sole source of carbon by using plate assay method as described in section 2.5.2 (Bradoo *et al.*, 1996). Some potential tannase producers from the culture collection viz. *A. niger* NCIM 620 and *A. oryzae* NCIM 643 were also used for the preliminary screening.

Out of 106 isolates tested, 78 isolates utilized 2 % tannic acid as a sole source of carbon. Among all sources, soil showed 48 fungal tannase producers (48) while comparatively less number was recorded in samples of tannin rich plant parts viz. fruits (7), leaves (5), bark (10) and pods/seeds (8).

The preliminary screening of tannase production was carried out by a simple and rapid plate assay method using tannic acid as sole carbon source described by Bradoo *et al.* (1996). The growth of fungal isolate was determined by measuring colony diameter and zone of hydrolysis (zone of clearance) around the colony after 120 h of incubation (Fig. 3.1 and Table 3.1). It was observed that in case of strains which formed compact colonies, the zone of hydrolysis around the colony was easily measurable. In case of spreaded colonies the reverse side of the plate showed zone of hydrolysis below the colony growth. However in case of few strains such as SM 71, SM 72, SM 81 zone of hydrolysis neither around nor below the colony was observed. Pinto *et al.* (2001) reported correlation between colony diameter and tannase production in the study using *A. niger* strains. Similarly in the present study colony diameter alone was considered as an indicator of higher tannase producer. Though tannase activity did not observe to be directly proportional to the colony diameter in all strains, there was a high correlation with r = 0.93 between colony diameter and tannase activity. These results are in accordance with that reported by Bradoo *et al.* (1996) and Murugan *et al.* (2007), where the correlation was established between tannase production with diameter of colony and zone of hydrolysis.

Tannase producing fungi were reported to be mainly isolated from soil such as tannery soil (Chowdhury *et al.*, 2004), forest and coastal soil (Kawakubo *et al.*, 1991; Lekha *et al.*, 1993; Mondal *et al.*, 2001b). Surfaces of plant parts such as leaves, seeds provide good source of epibiotic fungi (Hunter-Cevera and Belt, 2004). Fungal tannase producers were reported from plants such as white and black gall nuts, *Valonia*, sumac, *Acacia* leaves (Sariozlu and Kivanc, 2009), tissues of *Quercus* sp., *Larrea tridentata*, *Pinus semibroides* (Cruz-Hernandez *et al.*, 2005) and decaying bark or stem of banana, cashew and silk cotton trees (Lekha *et al.*, 1993). In the present study moderate (0.8 – 2.0 U/ml) to high (3.0-5.0 U/ml) tannase producers were isolated from soil samples collected from deep forest and soil around the tannin rich plants. Among the fungal strains isolated from tannin rich plant parts we found the isolates from pods/seeds showed high tannase (3.0 – 5.0 U/ml) production.

These results indicated that soil and pods (of tannin rich plant) were the potential sources of high tannase producers among other sources. Soil microorganisms play an important role in decomposition of organic matter in soil. The presence of tannase in soil fungi isolated from tannin rich soil suggests their active role in decaying of tannin plant

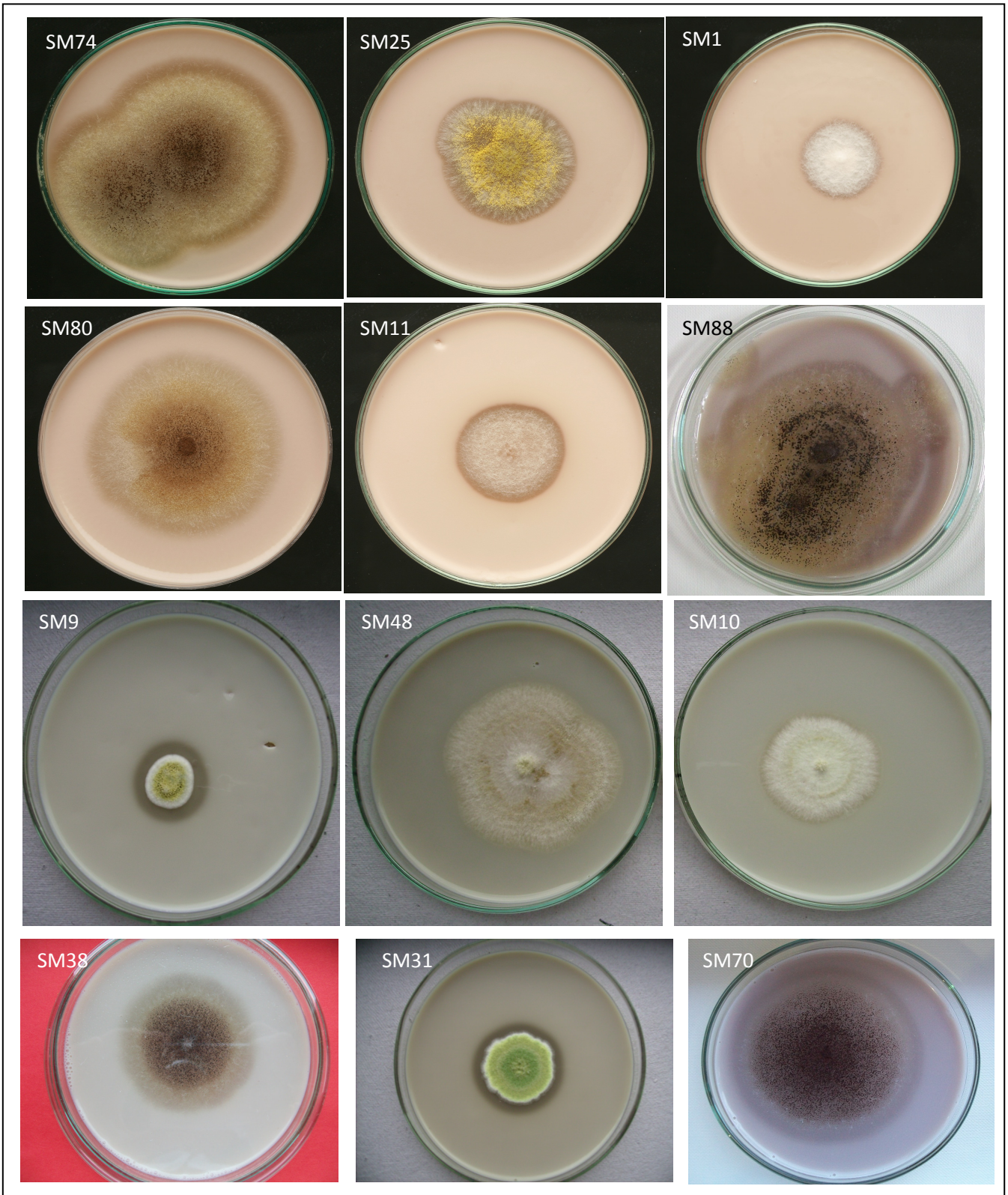


Fig. 3.1 Tannase producing fungal isolates

material. Mondal *et al*. (2001b) reported high number of tannin degrading fungi in soil from Sal (*Shorea robusta*) forest and further attributed it to the high tannin content (12 %) in Sal seed.

Amongst the screened isolates, SM 70 from *C. spinosa* pods produced high tannase (5.2 U/ml) followed by soil isolates SM 88 (4.70 U/ml), SM 68 (4.18 U/ml) and SM 38 (4.00 U/ml) within 50 h. Isolates from bark SM 69 (3.98 U/ml), from pods SM 87 (3.68 U/ml) and SM 57 (3.42 U/ml) and from soil SM 37 (3.21 U/ml) also showed good tannase activity. Thirty three isolates showed moderate tannase activity (1 – 3 U/ml) and twenty nine isolates produced tannase less than 1 U/ml while ten isolates were observed to be poor tannase producers (< 0.5 U/ml) (Table 3.1).

Five moderate tannase producers (SM 2, 15, 25, 74 and 80), nine high tannase producers (SM 37, 38, 57, 68, 69, 70, 85, 87, 88) and reference strains (*A. niger* and *A. oryzae*) were then screened for their ability to tolerate and utilize high concentration of tannins under submerged conditions. The study was carried out as described in section 2.5.3. All the nine high tannase producing strains were able to degrade and utilize tannin up to 200 g/L. It was observed that SM 70 and SM 88 tolerated and utilized tannin concentration up to 400 g/L under submerged conditions. There was no growth observed when the strains screened for concentration more than 400 g/L. SM 85, SM 38 and SM 69 were able to tolerate tannin concentration up to 300 g/L and 250 g/L respectively but the growth was 20 – 40 % less than that of SM 70 and SM 88. Reference strain *A. niger* NCIM 620 was the low tannin tolerant (70 g/L) among this group (Table 3.2).

Cruz-Hernandez *et al*. (2005) reported similar study on degradation of high tannin levels (10 g/L – 100 g/L) by *Penicillium* and *Aspergillus* sp., wherein all the strains could utilize tannic acid up to 50 g/L (dry weight range 1.5- 3.5 g/L in 50 h), while only two *Aspergilli* strains utilized tannic acid up to 100 g/L.

In the present study, the isolates which utilized such high levels of tannic acid were all black (70 g/L – 400 g/L) in color while other strains which utilized low concentrations of tannic acid (20 – 50 g/L) were non black *Aspergilli* and species of *Penicillium* and *Fusarium*. These results are in accordance with the effective degradation of tannic acid (up to 200 g/L) by black *Aspergilli* (Van Dipeningen *et al.*, 2004). The authors have demonstrated that only black colored *Aspergillus* strains among all *Aspergilli* and *Penicilli* were able to grow on liquid cultures containing 200 g/L tannic acid. There are no reports available on the tolerance of fungal isolates to 400 g/L tannic acid which was recorded in the present study.

Table 3.2 Secondary screening of tannase producing fungi for growth in tannic acid*

Isolate / Strain	Colony color	Dry weight mg/ml	Growth in Tannic acid (g/L)	Tannase activity (U/ml)
SM 2	Violet pink	1.35	50	2.53
SM 15	Violet pink	0.90	50	1.98
SM 25	Greenish yellow	0.72	50	3.26
SM 74	Green	3.30	20	5.03
SM 80	Green	1.46	50	5.38
SM 37	Black	1.47	200	2.64
SM 68	Black	4.7	200	1.84
SM 38	Black	4.34	250	2.36
SM 88	Black	3.54	400	2.30
SM 69	Black	4.18	250	3.92
SM 85	Black	4.30	300	2.71
SM 87	Black	2.88	200	5.34
SM 57	Black	3.71	200	6.98
SM 70	Black	5.30	400	3.12
***A oryzae* NCIM 643**	Green	3.31	70	4.64
***A niger* NCIM 620**	Black	1.53	70	3.8

*Fungal strains were checked for their growth in various concentrations of tannic acid as described in section 2.5.3.

3.2 Selection and Identification of culture

Based on faster growth, high tannase activity and utilization of high tannic acid concentration, isolates SM 70 and SM 88 were further studied for the morphological characters and identified based on partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene (section 2.6) sequencing. Both the strains showed white aerial mycelia and black colored conidia on PDA and TAA (Fig. 3.2). There was no pigmentation on reverse side of the plate. The zone of hydrolysis around the colony was difficult to observe because of spreaded growth but it was clear from reverse side of the plate. Under the microscope, globose vesicle with radiate, biseriate conidiophores and septate hyphal morphology was observed. Conidia were light grey, rough and of < 6 µm size (Fig. 3.3).

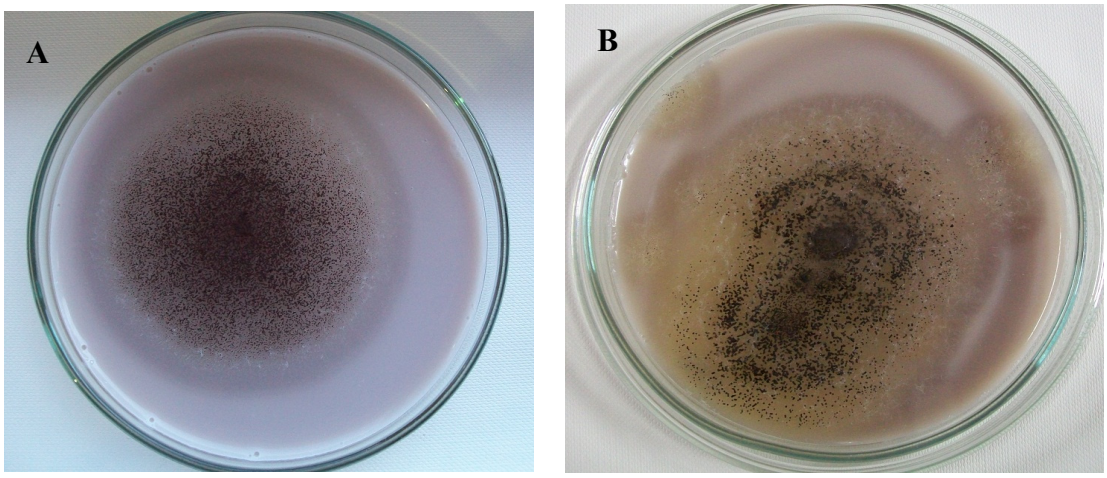


Fig. 3.2 Growth of **A**. *A. allahabadi* **B**. *A. parvulus* on TAA

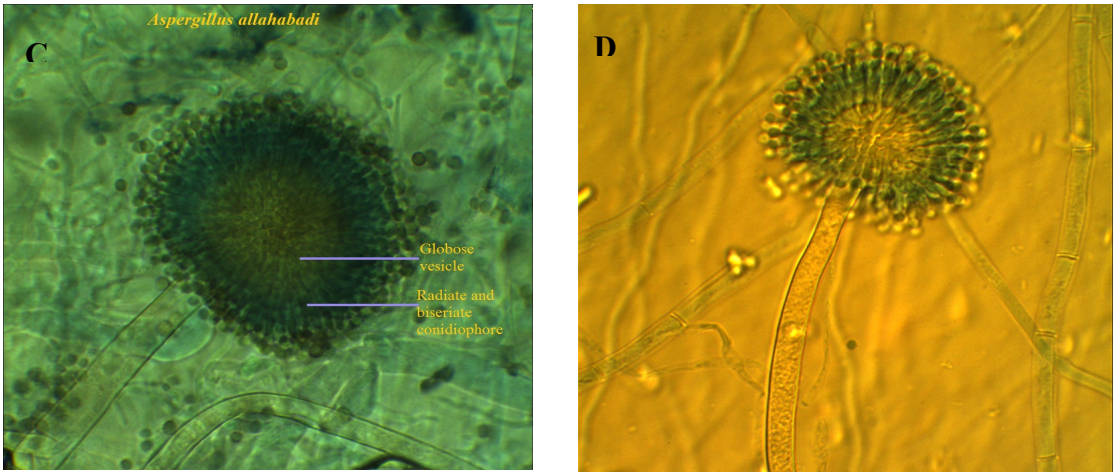


Fig. 3.3 Light microscopy of ***C***. *A. allahabadi* and **D**. *A. parvulus* (400 X)

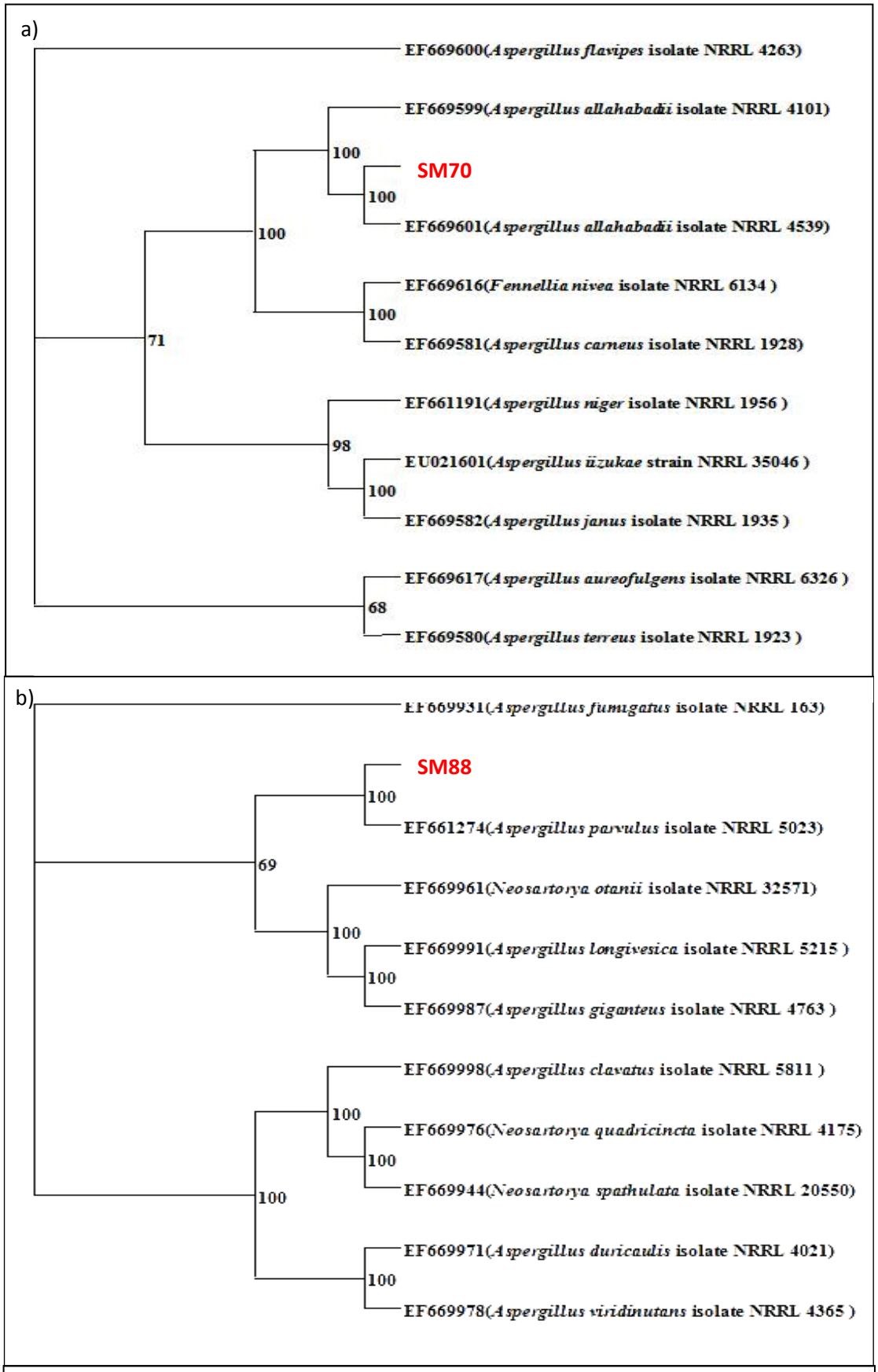


Fig. 3.4 Phylogenetic relatedness of a) SM 70 with *A. allahabadi* NRRL 4539 and b) SM 88 with *A. parvulus* NRRL 5023

Based on the nucleotide homology and phylogenetic analysis, the strain SM 70 was identified as *A. allahabadi* and strain SM 88 was identified as *A. parvulus*. Fig 3.4 a and b show 100 % homology of SM 70 with *A. allahabadi* NRRL 4539 and of strain SM 88 with *A. parvulus* NRRL 5023. These species have not been reported previously for tannase production and high tannic acid utilization.

3.3 Selection of strain by tertiary screening

The effect of tannic acid concentration on tannase production by *A. niger* was reported by Aguilar *et al.* (2001a) under SSF and SmF conditions. In case of SSF, the authors reported increase in tannase activity with increase in tannic acid concentration from 20 g/L to 160 g/L. Further, in case of SmF, the tannase production increased with tannic acid concentration up to 50 g/L and decreased at higher concentration of tannic acid up to 100 g/L.

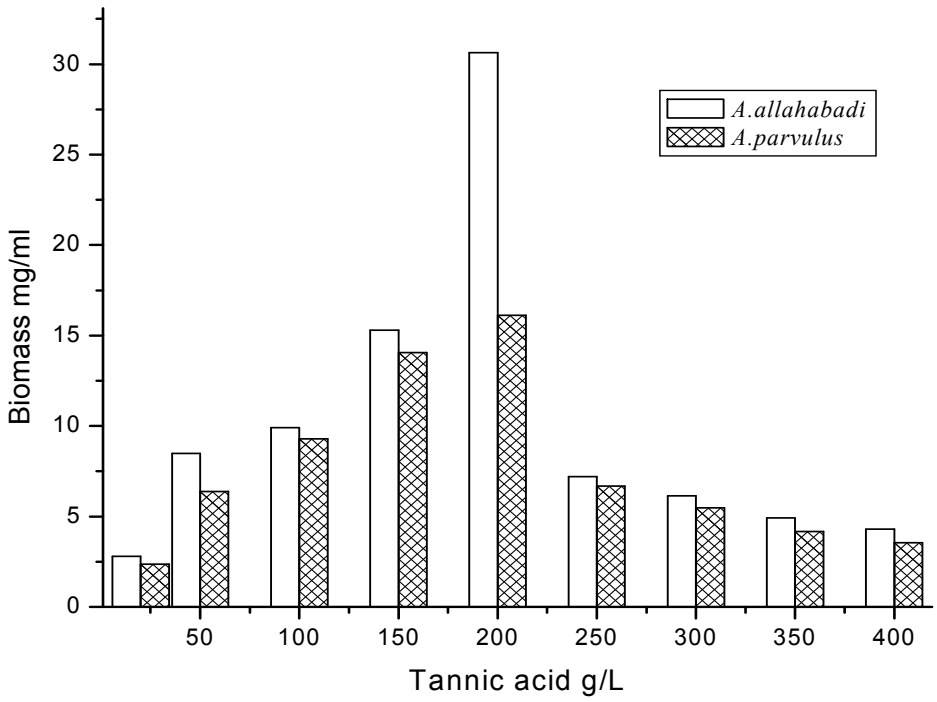


Fig. 3.5 a Effect of tannic acid concentration on the growth of *A. allahabadi* and *A. paruvulus*

The strains were grown in TAB medium with varying concentrations of tannic acid as indicated under SmF at 28^0C for 60 h.

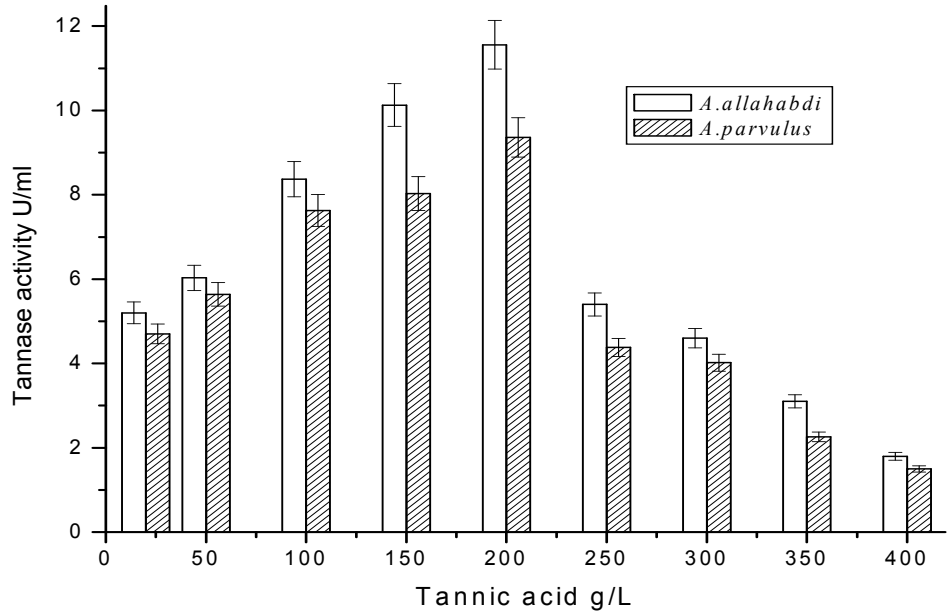


Fig. 3.5b Effect of tannic acid concentration on tannase activity of *A. allahabadi* and *A. parvulus*

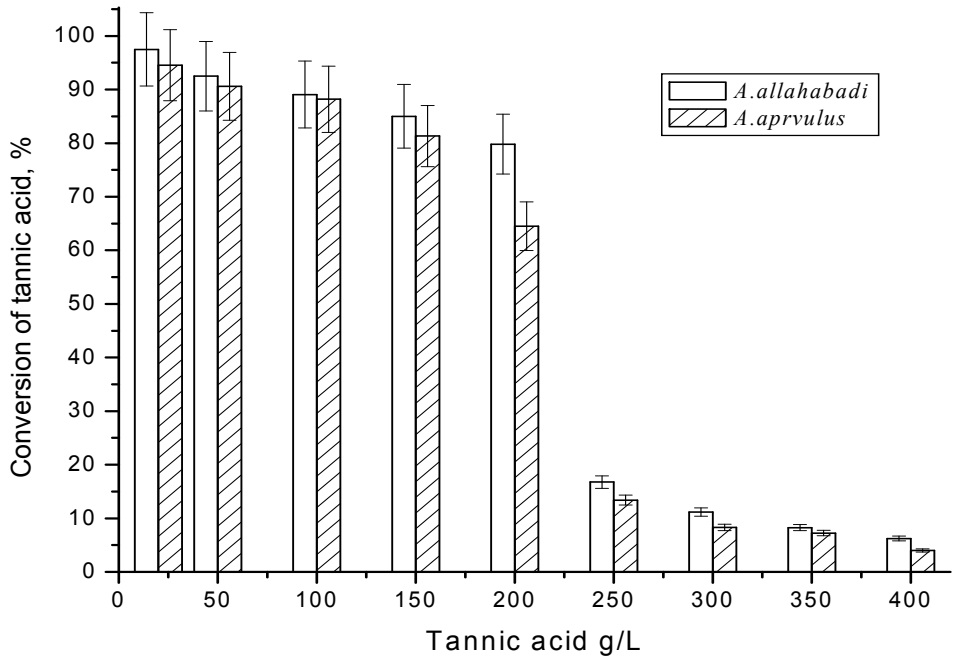


Fig. 3.5c Effect of tannic acid concentration on utilization of tannic acid by *A. allahabadi* and *A. parvulus*

The strains were grown in TAB medium with varying concentrations of tannic acid as indicated under SmF conditions at 28^0C for 60 h. Conversion efficiency was calculated by measuring residual tannic acid in the medium.

In the present investigation two isolates viz. *A. allahabadi* and *A. parvulus* withstood the high tannic acid concentration (400 g/L). These two strains were further studied for the tannase production and for their efficiency of conversion of tannic acid using concentrations in the range of 20 g/L to 400 g/L under SmF conditions as described in section 2.5.3. The conversion efficiency of tannic acid by both the strains was determined by measuring mycelial biomass (dry cell weight), tannase activity and residual tannic acid in the medium. It was observed that in both the cultures mycelial biomass and tannase activity gradually increased when tannic acid concentration increased from 20 g/L to 200 g/L. Tannic acid conversion efficiency was observed to be highest (94 - 97 %) when tannic acid was 20 g/L in the medium in case of both the strains whereas at 200 g/L, *A. allahabadi* utilized more tannic acid (80 %) as compared with *A. parvulus* (64 %). The profile of tannic acid utilization was different when concentration of tannic acid was in the range of 250 g/L to 400 g/L than that at 20 to 200 g/L (Figure 3.5 a, b and c).

The mycelial biomass, tannase activity and tannic acid conversion efficiency decreased with increase in tannic acid concentration from 250 to 400 g/L in case of both the strains. However both of them utilized tannic acid concentration up to 400 g/L with efficiency of conversion of 6.23 % in case of *A. allahabadi* and 4 % in case of *A. parvulus*. At 400 g/L, the utilization by both the strains was drastically reduced by 90 % than that at 20 g/L tannic acid concentration. The decrease in biomass, tannase production and utilization can be explained on the basis of reduced hydrolysis of tannic acid and hence less availability of glucose/quinic acid as carbon source for both the fungi. Based on the tannase production (11.56 U/ml) and conversion efficiency (79.8 %) of high tannic acid (200 g/L) concentration, *A. allahabadi* was found to be a better strain than *A. parvulus* and therefore selected for further studies of tannase production by submerged fermentation.

CHAPTER 4

STUDIES AND OPTIMIZATION OF PRODUCTION OF TANNASE AND GALLIC ACID BY FERMENTATION

Introduction

Tannase (E.C. 3.1.1.20) hydrolyses polyphenolic structure of hydrolysable tannins to produce simple phenolic acid i.e. gallic acid and / or ellagic acid. It acts on galloyl residues of galloyl esters as well as hexahydroxydiphenoyl residues of ellagitannins. However, galloyl residues were reported to be more easily hydrolyzable than other groups (Scalbert, 1991).

Hydrolysable tannin degradation was best understood in fungal systems (Bhat *et al*., 1998). Commercially *Aspergillus* sp. was employed for tannase production whereas SmF was most commonly used fermentation for laboratory level studies of tannase production and also exploited at commercial scale production (Van De Lagemaat and Pyle, 2005). Various studies were reported for the production of tannase and the optimization of nutritional parameters (carbon, nitrogen), physical (pH, temperature) and operational (agitation and aeration) parameters (Das Mohapatra *et al*., 2006; Natarajan and Rajendran, 2009). However very few studies reported effect of different process and operational parameters on tannase production at fermenter level (Pourrat *et al.*, 1982; Seth and Chand, 2000). Tannic acid concentration was also observed to be significantly affecting the tannase production profile. Higher tannic acid concentrations decreased tannase activity in *Aspergillus japonicus* and *Rhizopus oryzae* (Bradoo *et al*., 1997; Hadi *et al*., 1994). The highest reported concentration of tannic acid used for fermentation of tannase was 160 g/L in solid state fermentation (SSF) of *Paecilomyces* sp. while 100 g/L in submerged fermentation (SmF) of *A. niger* (Aguilar *et al*., 2001; Battestin and Macedo, 2007).

Present investigation was aimed at studying the effect of various nutritional (C, N, Mg, Ca, Zn, Fe, Mn, Co) and process parameters (agitation, aeration) on tannase production by *A. allahabadi* at shake flask and fermenter level (35 L) and taken at 100 L scale. The

tannase production using coculture of *A. allahabadi* with *A. parvulus* was also attempted.

4.1 Time course of extracellular tannase production by *A. allahabadi* under submerged conditions

Time course of tannase production in relation with growth of *A. allahabadi* was studied using TAB medium as described in section 2.7. Tannase was observed to be growth associated enzyme and the maximum activity was detected at late exponential phase of growth after 48 h. Similar results were reported for *Aspergillus tamarii* (Costa *et al.*, 2008). On the other hand, Enemour and Odibo (2009) reported production of highest extracellular tannase by *A. tamarii* during stationary phase of growth at 144 h .under submerged conditions.

A. allahabadi had its early exponential phase up to 18 h and the growth was slowly and steadily increased after 18 h till 54 h and remained constant thereafter. The specific growth rate (μ) was 0.025 h^{-1} . Tannic acid was utilized up to 97 % over 48 h. The pH

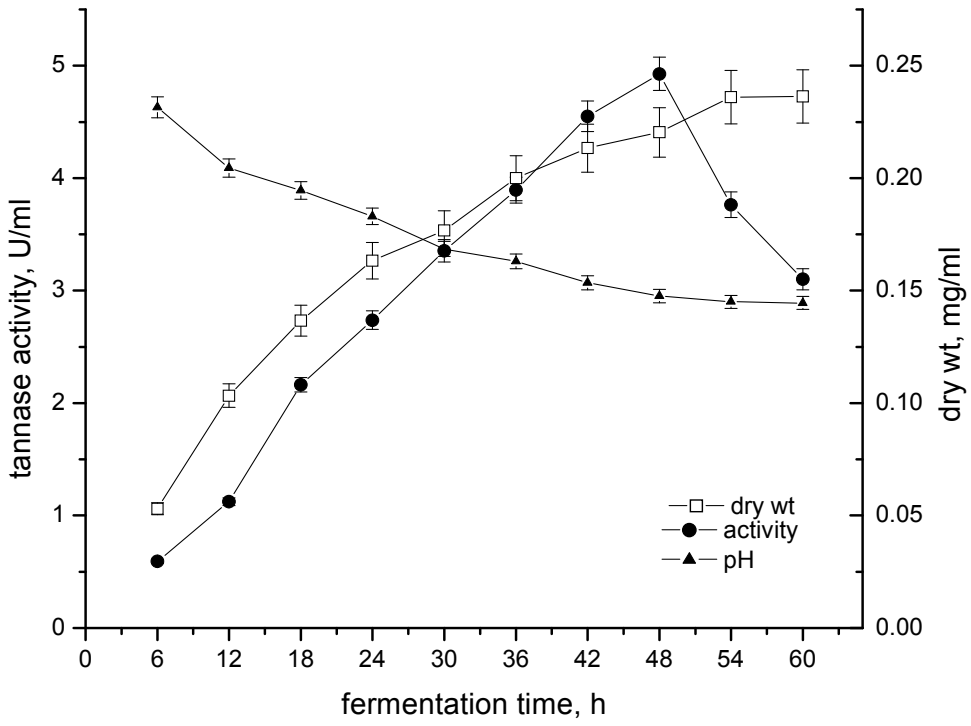


Fig. 4.1 Time course of tannase production by *A. allahabadi* in TAB medium (20 g/L tannic acid) under shaking conditions up to 60 h

decreased steadily from 5.0 to 2.6 over 60 h (Figure 4.1). Tannase activity was first detected at 6 h, reached its peak at 48 hrs and started decreasing thereafter. The decrease in the tannase activity was suggestive of possible inhibition of the enzyme by end product and/ or no substrate availability. The increase in biomass suggested that the organism was able to utilize the liberated glucose and gallic acid after tannic acid degradation for cell growth. However in an experiment conducted separately during this study, the strain was observed not to utilize gallic acid as sole source of carbon. Therefore it can be concluded that only glucose was utilized by *A. allahabadi* and hence decrease in tannase activity after 48 h could be due to substrate unavailability.

Basal levels of tannase (0.12 U/ml) were estimated when *A. allahabadi* was grown in glucose. Tannase activity was increased to 0.39 U/ml when grown in tannic acid. The constitutive production of tannase by *A. allahabadi* might be due to its natural habitat in tannin rich pods of *Caesalpinia spinosa*. The findings corroborate with Bradoo *et al*. (1997).

4.2 Effect of plant tannin substrate on tannase production by *A. allahabadi*

Natural plant (hydrolysable) tannins, abundantly available in the nature in wild as well as cultivated plants such as *Caesalpinia spinosa*, *Terminalia chebula* or tannin rich agro forest residues such as pods of *Quercus infectoria*, *Caesalpinia dignya*, leaves of *Syzygium cumini*, *Phyllanthus emblica* and *Acacia nilotica* provide a good economical option for production of tannase and gallic acid. There are very few reports on the use of plant tannins such as *C. spinosa*, *C. dignya* for tannase production (Kar *et al.*, 1999; Pourrat *et al.*, 1985). However, tannic acid present in different sources are structurally different and tannase exhibit different affinity towards these tannin substrates. It hydrolyses only those substrates that contain at least two phenolic OH groups in the acid component (Curiel *et al.*, 2009). Manjit *et al.* (2008) have reported that tannin rich leaves of *S. cumini* was found to be the best substrate for tannase production (141.34 U/g) by *A. fumigatus* as compared to other tannin containing plant substrates viz. *P. emblica*, *Zyzyphus mauritiana*, *Syzygium* sp. and *A. nilotica* under SSF.

In the present investigation, the objective was to screen the natural tannin substrate for production of high tannase and gallic acid by *A. allahabadi*. Therefore the effect of various plant tannin substrates was studied by growing *A. allahabadi* in the media containing sources of abundantly and easily available gallotannin, ellagitannin and

commercial tannic acid as control. All plant sources were first optimized for extraction of highest tannin content in water. The extracts were further characterized quantitatively for presence of organic nitrogen, reducing sugars and mineral content (data shown in Annexure I**).** The tannin content of water extract and type of tannin of each source is summarized in Table 4.1. Schematic representation shows the structural differences of plant tannin sources (Fig. 4.2). Among the different plant substrates, *C. spinosa* was found to be the best plant tannin source based on highest total tannin content (47.76 % w/w) in crude plant material and its water extract (9 %, w/v).

Table 4.1 Chemical nature and tannin content of plant tannin sources

Plant source#	Chemical nature of tannin	Tannin content in crude plant material (w/w), %*	Tannin content in water extract (w/v), %*	Products of hydrolysis
C. spinosa (Tara)	Pentagalloyl quinic acid	47.76	9	Gallic acid, quinic acid
C. dignya (Teri)	Mono or di galloyl glucose	33.93	4.81	Gallic acid glucose
Q. infectoria (Oak galls)	Hepta galloyl glucose	27.59	4.47	Gallic acid, glucose
T. chebula (Chebulic myrobalan)	1,3,6 tri-O-galloyl-2,4-chebuloil–B-D-glucopyranoside (chebulinic acid)	16.57	3.13	Gallic acid, ellagic acid, chebulic acid
T. bellerica (Beleric myrobalan)		6.03	0.98	Gallic acid, ellagic acid
Tannic acid	Pentagalloyl glucose	--	--	Gallic acid, glucose

Compiled from: Howes-Kew (1952); Paaver *et al* .(2010); http://www.worldagroforestry.org/sea/Products/AFDbases/af/index.asp.

#: Common name of plants are given in bracket.*: Results obtained in the present study (Annexure I). Tannin content was determined by method of Haggerman (1978) as described under materials and methods.

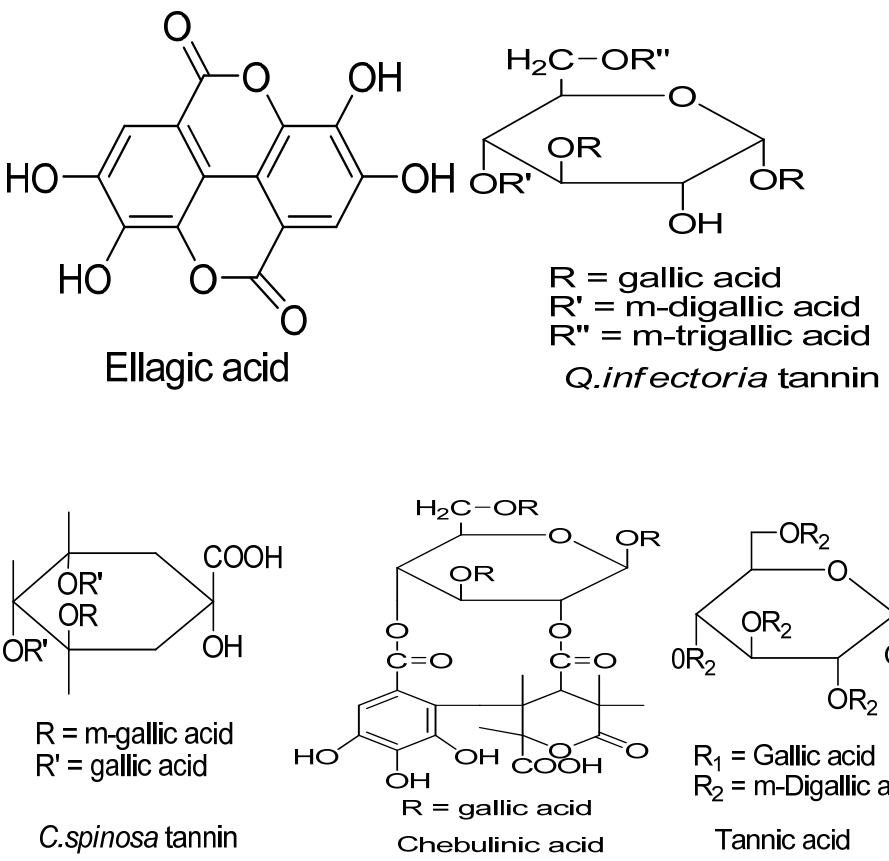


Fig. 4.2 Schematic representation of different plant tannin

To study the effect of plant tannin substrate, *A. allahabadi* was grown in the TAB medium under submerged conditions where tannic acid (20 g/L) in the medium was replaced with plant tannin extract at 20 g/L tannic acid equivalence. The results are given in the Table 4.2 and Figure 4.3 a, b. *A. allahabadi* showed highest tannase activity (8.10 U/ml) in 48 h in the medium containing *C. spinosa* followed by 4.67 U/ml tannase activity in *Q. infectoria* tannin among all plant tannins. Medium containing *C. dignya* tannin showed highest tannase activity at 24 h followed by decrease in activity. Highest gallic acid was detected at 48 h in all plant tannins. The effect of both ellagitannins (*T. chebula* and *T. bellerica*) was observed to be different from that of gallotannins. They showed highest gallic acid content at 36 h and tannase activity at 48 h. In both ellagitannins, gallic acid and tannase activity were found to be decreased after reaching the peak. In gallotannins and commercial tannic acid, gallic acid remained constant after reaching its maximum. However tannase activity was increased in case of *C. spinosa* and *Q. infectoria* and remained constant in *C. dignya* and commercial tannic acid. Kar *et al.* (1999) reported effect of *C. dignya* tannin (5 – 40 g) concentration on tannase production

Fig. 4.3 a Effect of natural tannin substrate on tannase production by *A. allahabadi*

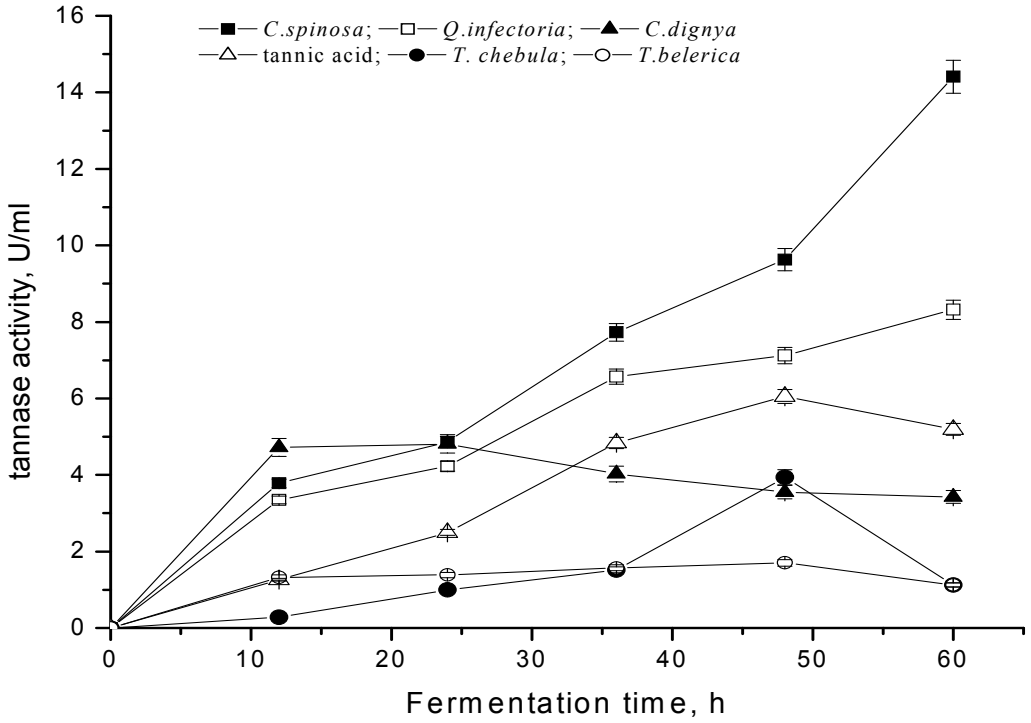
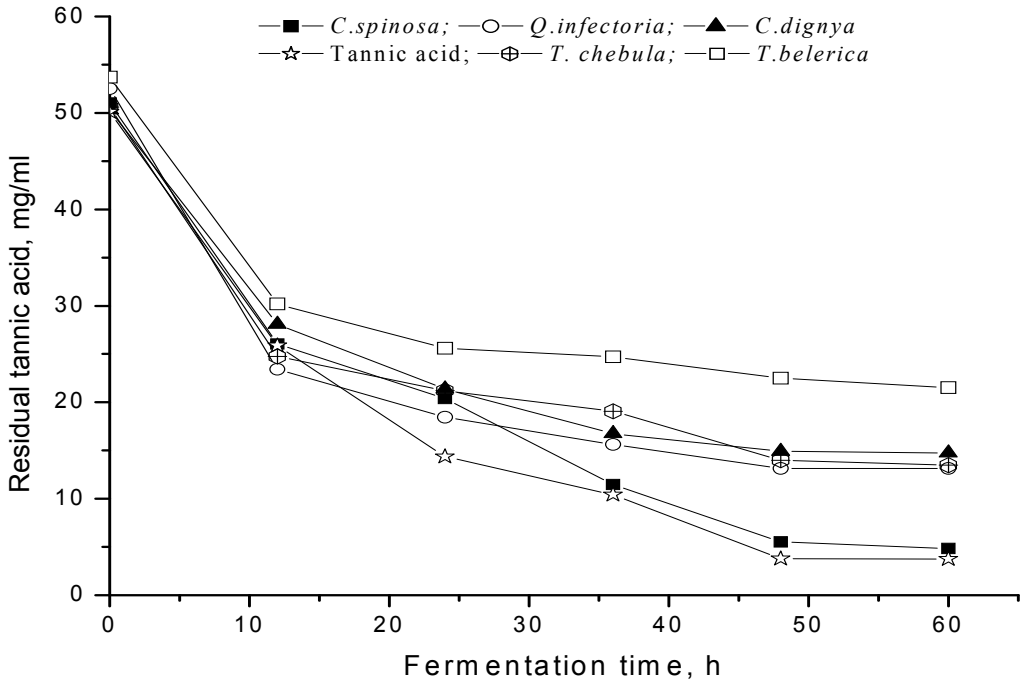


Fig. 4.3 b Conversion of plant tannin by *A. allahabadi* tannase



of *R. oryzae* over 72 h under SSF. The authors found that 20 g substrate gave optimum tannase activity after which it decreased.

In the study of effect of pure tannic acid and plant tannin extract on tannase production by Deschamps and Lebeault (1984), strains of *Klebsiella* and *Corynebacterium* were shown to have different tannase activities and tannin utilization profiles when grown in *C .spinosa* crude extracts as well as purified *C. spinosa* tannin. *Klebsiella* sp.OM 26 was found to be best strain with 93 % tannin degradation in first 6 h in crude extract. However 75 % degradation was observed in first 8 h in purified *C. spinosa* tannin with tannase activity of 0.045 U/ml. Determination of tannase activity was not carried out after 6 – 8 h because of the rapid darkening of the medium. The authors also reported the best tannase activity (0.066 U/ml) obtained with another *Klebsiella* strain TM 21 with tannin degradation (21 % in 8 h in TM 21). Similarly in the present investigation tannase activity obtained with different plant tannins did not show correlation with tannin conversion (Table 4.2). However in case of *C. spinosa* tannin medium, highest tannase activity with high tannin conversion was obtained and hence was selected for further studies.

Table 4.2 Effect of plant tannin substrate on production of tannase and gallic acid

Plant tannin Source	Tannase activity[a], U/ml	Conversion of tannin, %	Gallic acid mg/ml
Tannic acid (Control)	4.89	97.49	13.52
C. spinosa	8.10	90.50	16.56
Q. infectoria	4.67	78.80	16.41
C. dignya	2.73[b]	78.31	1.71
T. chebula	2.25	77.31	5.90
T. bellerica	1.01	90.31	0.86

a: the activity at 48 h; b: activity at 24 h A. allahabadi was grown in TAB medium with 20 g/L plant tannin extract indicated at 28^0C under SmF conditions. Crude plant tannin material was extracted at 70^0C for 1 h using 1:6 (plant tannin: water) ratio. Conversion of tannin was calculated by measuring residual tannin content.

The tannin conversion and tannase production was well studied using commercial tannic acid (Lokeswari and Jayaraju, 2007a). Plant tannin extract itself is a complex medium containing water soluble extractables other than tannins. The interaction of fungal isolate with natural tannin might lead to different behavior as compared with commercial tannic acid in terms of growth profile, tannase production, substrate utilization and gallic acid production. In the present study of effect of plant tannin, specific growth rate (μ) of 0.437 revealed that *A. allahabadi* hydrolyzed *C. spinosa* tannin efficiently and used quinic acid released for growth. There was increase in biomass till 48 h and no decrease in gallic acid content over 60 h. Therefore probably organism could be utilizing quinic acid, the core molecule of *C. spinosa* as the carbon source. The postulate was confirmed by studying *A. allahabadi* for the utilization of quinic acid as sole source of carbon and found to be utilizing quinic acid with biomass 9 mg/ml in 54 h. Highest gallic acid production with substrate conversion efficiency at 90.50 % in case of *C. spinosa* defined it as the best substrate among all plant tannins followed by *Q. infectoria*. Increase in tannase activity in case of *C. spinosa* (8 U/ml) than that of commercial tannic acid (6 U/ml) suggests the possibility of induction of tannase by low molecular weight phenolic acids present in the extract. The organism certainly showed different profiles with gallotannins and ellagitannins. In case of ellagitannin, *T. bellerica* the conversion efficiency was 90.31 % which was comparable to that of *C. spinosa* and commercial tannic acid. Tannase breaks the ester bond between HHDP (hexahydroxy diphenoyl) group and glucose in ellagitannins. Polyphenol oxidase is the enzyme which oxidizes HHDP groups to ellagic acid. Polyphenol oxidase and tannase together degrade the ellagitannins (Shi *et al.*, 2005). In both *Terminalia* species, polyphenol oxidase was detected (12 - 54 U/ml) at 48 h. Gallic acid and tannase activity was observed to decrease after 48 h while biomass was increased gradually up to 60 h. The polyphenol oxidase activity of *A. allahabadi* was not observed when it was grown in *C. spinosa* medium under submerged conditions.

4.3 Optimization of culture conditions for tannase production by *A. allahabadi* at shake flask level

The objective of this investigation was to study and optimize the various culture conditions viz. effect of nutritional parameters, effect of initial pH, effect of agitation and aeration on tannase production by *A. allahabadi* under submerged conditions. *C. spinosa* tannin was found to be best substrate for tannase production by *A. allahabadi*. The effect

of (20 and 200 g/L) *C. spinosa* (pentagalloyl quinic acid) tannin and tannic acid (pentagalloyl glucose) with and without different nutritional additives such as carbon and nitrogen sources and minerals on tannase production was studied (Table 4.3). The control medium was kept containing equivalent concentrations of tannic acid and *C. spinosa* tannin without additives.

Table 4.3 Various media used for optimization of tannase production at shake flask

Media	Composition of medium
TC-1	TAB + *C. spinosa* extract 20 g/L
TC-2	TAB + *C. spinosa* extract 200 g/L
TT-1	TAB + tannic acid 20 g/L
TT- 2	TAB + tannic acid 200 g/L

4.3.1 Effect of additional carbon source on tannase production by *A. allahabadi*

Some researchers have reported degradation of tannin increased with addition of other substances. Bradoo *et al.* (1997) showed enhanced tannase activity by *A. japonicus* in presence glucose as additional carbon source. Similarly *Aspergillus* and *Penicillium* was shown to grow more efficiently in the medium containing glucose and tannic acid (Ganga *et al.*, 1977). Albertase (2002) postulated that additional nitrogen and carbon sources enhanced the tannase production.

In the present investigation, eight different carbon sources were used as additional carbon sources and results obtained are shown in Table 4.4. All carbon sources were added on the basis of carbon equivalent to 1 % glucose except ellagic acid. Due to very low solubility of ellagic acid in water, it was added at concentration of 0.1 %.

In the TT-1 medium (TAB + tannic acid 20 g/L), addition of glucose resulted in increase in mycelial biomass but decrease in tannase activity. Though there was no major change in mycelial biomass, addition of sucrose, starch, glycerol and mannitol decreased the

Table 4.4 Effect of additional carbon source on tannase production under SmF by *A. allahabadi*

Carbon source	**Final pH**	**Dry wt. (mg/ml)**	**Tannase activity (U/ml)**
Control (only with tannic acid)	2.92	2.10 ± 0.09	4.89 ±0.24
Tannic acid + Glucose (1 %)	2.94	2.35 ± 0.08	4.08 ± 0.08
Tannic acid + Sucrose (0.95 %)	3.01	2.14 ± 0.75	2.34 ± 0.05
Tannic acid + Starch (1 %)	3.15	2.16 ± 0.08	4.15 ± 0.07
Tannic acid + Glycerol (1 %)	3.08	1.89 ± 0.06	4.10 ± 0.04
Tannic acid + Mannitol (1 %)	3.06	1.75 ± 0.05	3.40 ± 0.17
Tannic acid + Gallic acid (0.8 %)	2.91	2.04 ± 0.09	5.72 ± 0.35
Tannic acid + Quinic acid (0.93 %)	2.89	2.11 ± 0.1	4.80 ± 0.18
Tannic acid + Ellagic acid*	2.97	2.68 ± 0.06	4.81 ± 0.19
Control (only with *C. spinosa*)	2.90	9.51 ± 0.33	8.12 ± 0.16
C. spinosa + Glucose (1 %)	2.93	9.54 ± 0.25	8.05 ± 0.10
C. spinosa + Sucrose (0.95 %)	3.06	9.46 ± 0.24	7.97 ± 0.22
C. spinosa + Starch (1 %)	3.18	9.42 ± 0.34	7.95 ± 0.10
C. spinosa + Glycerol (1 %)	3.10	9.37 ± 0.38	7.85 ± 0.07
C. spinosa + Mannitol (1 %)	3.07	9.45 ± 0.27	7.90 ± 0.12
C. spinosa + Gallic acid (0.8 %)	2.96	10.0 ± 0.21	9.50 ± 0.15
C. spinosa + Quinic acid (0.93 %)	2.84	11.74 ± 0.38	8.05 ± 0.12
C. spinosa + Ellagic acid*	2.90	9.10 ± 0.27	8.0 ± 0.08

Culture was grown at 28^0C in TAB medium containing $NaNO_3$ (0.6 %) with tannic acid as TT-1 medium and *C. spinosa* extract (20 g/L tannic acid equivalence) as TC- 1 medium for 48 h. Control contained tannic acid or *C. spinosa* as sole carbon source. All additional carbon sources were used at carbon equivalence of 1 % glucose. *Ellagic acid was used at 0.1 % concentration.

tannase activity by 15 – 40 % than that of control. Probably all the four sources could were utilized as preferential carbon sources over tannic acid by the strain and hence the decrease in activity was observed. Effect of sugars as additional carbon source was reported by some researchers. Glucose, fructose, sucrose and arabinose (10 g/L to 30 g/L) were shown to improve the tannase production by *A. niger* (Belmares *et al.*, 2004). Dependence of tannase activity on glucose concentration was documented (Lokeswari and Jayaraju, 2007a). The authors reported that high tannase activity was observed in presence of low glucose (0.5 g/L) and it was suppressed at high concentration of glucose in *A. niger*. Strong catabolite repression with glucose (20 g/L) in SmF of *A. niger* (Aguilar *et al.*, 2001b) and with 10 g/L glucose for *A. tamari* (Costa *et al.*, 2008) have also been documented. Results of the present investigations have shown no appreciable change in tannase activity with use of 10 g/L glucose by *A. allahabadi*.

In the present study, in the TT-1 medium (TAB + tannic acid 20 g/L), there was no appreciable change in tannase activity when quinic acid and ellagic acid were used as additional carbon source. This may be due to ability of the culture to use these sources as carbon source as these are the degradation products of hydrolysable tannins.

However gallic acid addition as a carbon source along with tannic acid was found to induce tannase activity in *A. allahabadi*. This can be a reason for the ability of *A. allahabadi* to hydrolyze high concentration of tannic acid. The findings were supported by reports of high levels of tannase induction by gallic acid in *A. tamarii* (Costa *et al.*, 2008). On the other hand, repression effect of gallic acid on tannase activity was reported in *A. niger* (Aguilar *et al.*, 2001b) and in *A. japonicus* (Bradoo *et al.*, 1997).

In the TC-1 (TAB + *C. spinosa* extract 20 g/L) medium, gallic acid showed increase in tannase activity (9.5 U/ml) as compared with that of the control (8.12 U/ml). This is a typical instance of end product activation of the tannase. The remaining seven carbon sources did not show appreciable change in the tannase activity. *C. spinosa* extract itself serves as complex medium containing reducing sugars, organic nitrogen and minerals along with tannins (Annexure I). Therefore additional carbon sources viz. glucose, sucrose, glycerol, mannitol and starch might not be preferentially utilized by the organism. Battestin and Macedo (2007a) reported that when starch was used along with coffee husk, due to rich nutrient source of coffee husk starch was not utilized by *P. variotii* under SSF conditions for tannase production.

Effect of additional carbon source was also studied in presence of higher concentrations of tannic acid/ C. *spinosa* tannin (200 g/L) respectively in media TT- 2 and TC-2. It was observed that additional carbon source showed no appreciable effect on tannase activity in both the media. The polyphenols (ellagic acid), phenolic acids (gallic acid), aliphatic acid (quinic acid) mono and disaccharides (glucose, sucrose), polysaccharide (starch) and sugar alcohols (glycerol, mannitol) showed the activity comparable to that of control. At high concentration, tannic acid was observed to control the expression of tannase while at low concentration (20 g/L) of tannic acid, only gallic acid as additional carbon source was found to induce the activity. In view of the results obtained, no additional carbon source was used in further studies for optimization of the fermentation medium.

4.3.2 Effect of nitrogen sources on tannase production

The study of effect of different nitrogen sources on tannase production was carried out using organic and inorganic nitrogen sources on equal nitrogen basis.

Tannins have a natural tendency to bind to proteins to form water insoluble complex. All organic nitrogen sources (peptone, yeast extract, corn steep liquor, and casein hydrolysate) showed precipitation and turbidity in all media and were therefore not used further.

Out of inorganic nitrogen sources investigated, ammonium nitrate was observed to be the best source among all followed by urea in both TT-1 (TAB + tannic acid 20 g/L) and TC-1 (TAB + *C. spinosa* extract 20 g/L) media. In case of all other inorganic nitrogen sources, tannase activity was less (Table 4.5).

In the present study, the physicochemical characterization of *C. spinosa* extract showed 0.3 % nitrogen content (Annexure I). The experiment carried out using TC-1 medium without addition of nitrogen source showed less tannase activity (7.02 U/ml) than that of control (8.05 U/ml) containing 0.6 % $NaNO_3$ as nitrogen source. This emphasized that available nitrogen in *C. spinosa* extract was not sufficient or not available for the organism to utilize and there was requirement of appropriate nitrogen source in the TC-1 medium.

When the effect of nitrogen was checked on the basis of equal nitrogen, ammonium nitrate was observed to be better source of nitrogen. The probable reason might be the presence of both reduced as well as oxidized form of nitrogen in ammonium nitrate.

Table 4.5 Effect of inorganic nitrogen source on tannase production of *A. allahabadi*

Nitrogen source	**Final pH**	**Dry wt mg/ml**	**Tannase activity U/ml**
Control (Tannic acid + Sodium nitrate 0.6 %)	2.95	2.10 ± 0.05	5.02 ± 0.23
Tannic acid + Ammonium nitrate (0.3 %)	2.89	2.76 ± 0.06	6.67 ± 0.18
Tannic acid + Ammonium sulphate (0.47 %)	2.98	1.54 ± 0.03	4.45 ± 0.22
Tannic acid + Ammonium di-hydrogen phosphate (0.82 %)	3.06	1.23 ± 0.03	3.70 ± 0.17
Tannic acid + Potassium nitrate (0.72 %)	2.96	1.75 ± 0.04	4.51 ± 0.23
Tannic acid + Urea (0.21 %)	2.94	1.87 ± 0.04	5.57 ± 0.24
Control for *C. spinosa* (*C. spinosa* + Sodium nitrate 0.6 %)	2.93	9.47 ± 0.33	8.05 ± 0.3
C. spinosa + Ammonium nitrate (0.3 %)	2.85	11.82 ± 0.39	11.18 ± 0.36
C. spinosa + Ammonium sulphate (0.47 %)	2.94	7.12 ± 0.29	7.01 ± 0.28
C. spinosa + Ammonium di hydrogen phosphate (0.82 %)	2.95	7.12 ± 0.31	5.95 ± 0.27
C. spinosa + Potassium nitrate (0.72 %)	2.97	6.78 ± 0.27	7.16 ± 0.19
C. spinosa + Urea (0.21 %)	2.95	8.84 ± 0.34	9.13 ± 0.28

Culture was grown at 28^0C using TAB medium with tannic acid as TT-1 medium and *C. spinosa* extract (at 20 g/L tannic acid equivalence) as TC- 1 for 48 h. Control contained $NaNO_3$ (0.6 %) and replaced with nitrogen source indicated in the table with respective concentration on equal nitrogen basis.

Similar findings were documented by Battestin and Macedo (2007b) where highest tannase production was obtained when *P. variotii* was grown in ammonium nitrate as nitrogen source in SSF. The authors have attributed high tannase yields to the

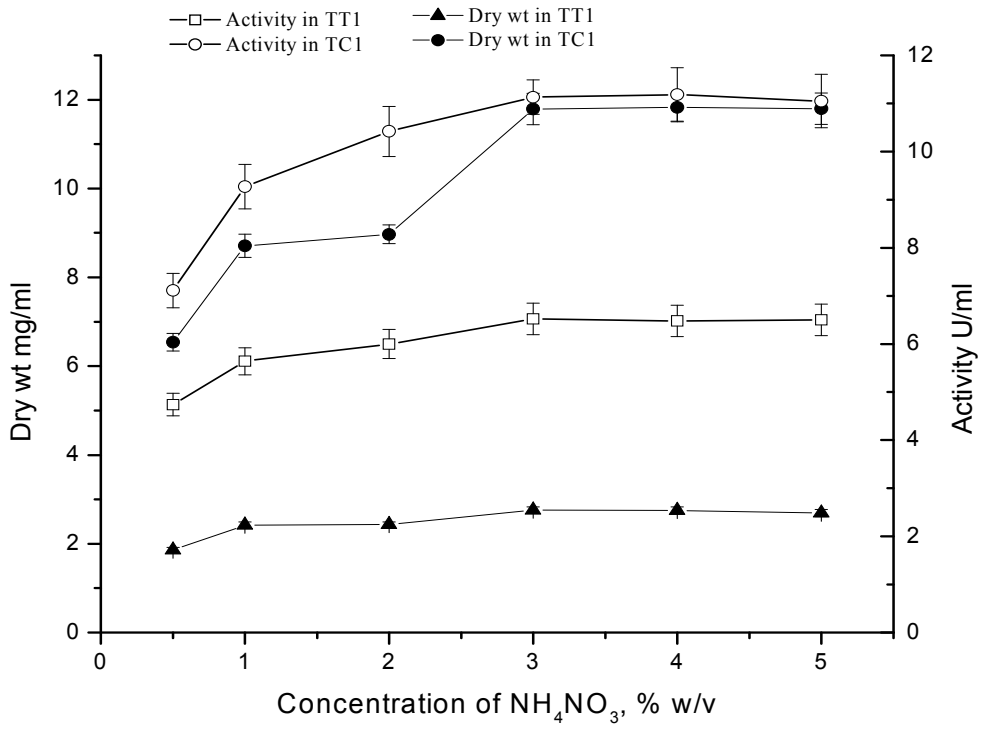


Fig. 4.4 Effect of ammonium nitrate concentration on tannase production

contribution of ammonium ions stimulating synthesis of proteins which in turn were available as ready nitrogen source.

In the next set of experiments, concentration of NH_4NO_3 was optimized in the media, TT-1 and TC-1 (Fig. 4.4). The concentration of NH_4NO_3 was increased from 0.05 % - 0.5 % in both the media. Tannase production increased with the increasing concentration of NH_4NO_3 up to 0.3 % (w/v) and it remained constant thereafter. NH_4NO_3 concentration at 0.3 % (w/v) was found to be optimum to achieve high tannase production in both the media. The C:N ratio was 4:1 in case of media TT-1 and TC-1. The C:N ratio was maintained when higher tannic acid (200 g/L) concentration was used in the medium for further experiments.

4.3.3 Effect of magnesium ions on production of tannase by *A. allahabadi*

Magnesium ions were reported to have pronounced effect of enzyme activities. The tannase production was checked in the media viz. TT-1 (TAB + tannic acid 20 g/L) and TC-1 (TAB + *C. spinosa* extract 20 g/L) without magnesium. Controls were kept with

addition of magnesium ions (at 0.05 g /L) in both the media. Tannase activity was 5.14 U/ml and 9.44 U/ml in TT-1 without magnesium and TC-1 without magnesium respectively. In both the media without magnesium salt tannase production was decreased by 16 – 22 % than that containing magnesium (6.42 U/ml in case of TT-1 and 11.21 U/ml in case of TC- 1). The findings validated the requirement of magnesium ions for optimum tannase activity.

4.3.4 Effect of minerals on tannase production by *A. allahabadi*

The effect of minerals (Zn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}, Mo^{3+}) on tannase production was studied using salts of these minerals at effective concentration of 0.02 g/L in the media viz. TT-1 and TC-1 both individually and in combination (Table 4.6).

Tannase production by *A. allahabadi* was observed to be increased with addition of $ZnSO_4$ and $CaSO_4$. $CaSO_4$ showed only marginal increase in the activity than that of control. There was no appreciable change in activity in both the media when a combination of $ZnSO_4$ and $CaSO_4$ was used as compared with addition of $ZnSO_4$ alone. Zn metal was reported to enhance tannase activity in the experiment conducted using purified tannase (Lokeswari and Jayaraju, 2007a). Increase in mycelial growth of *A. allahabadi* might in turn have showed increase in the activity. $FeSO_4$ did not show any change in activity when the culture was grown in TT-1 with $FeSO_4$. The media viz. TT-2 (TAB + tannic acid 200 g/L) and TC-2 (TAB + *C. spinosa* extract 200 g/L) turned blackish purple causing hindrance in determination of activity . Gallic acid / polyphenols are known to form complex with Fe called as iron gallates. Presence of iron may in turn impart the colour to gallic acid. Therefore $FeSO_4$ was not used for further studies.

C. spinosa extract was nutritionally complex medium (Annexure I). The physicochemical characterization of *C. spinosa* extract carried out in present study showed elemental zinc and calcium at concentration of 0.0012 g/L and 0.003 g/L respectively. However Table 4.6 shows that additional zinc and calcium resulted into comparable mycelial growth and tannase production in TC-1 with that of control. It can be seen that there was no inhibition because of the additional zinc and calcium on the culture growth as well as tannase production in TC-1. TC-1 showed around 70 % more activity (11.25 U/ml) than that in TT-1 (6.49 U/ml). *C. spinosa* medium itself is rich with micronutrients (Annexure I) and the increase in enzyme activity can be attributed to the presence of trace minerals in the *C. spinosa* extract.

Table 4.6 Effect of minerals on tannase production by *A. allahabadi*

Mineral salt	**Final pH**	**Dry wt. (mg/ml)**	**Tannase activity (U/ml)**
Control (only Tannic acid)	2.94	2.68 ± 0.11	6.49 ± 0.23
Tannic acid + $ZnSO_4$	2.91	3.04 ± 0.09	7.64 ± 0.26
Tannic acid + $CaSO_4$	2.92	2.74 ± 0.12	6.99 ± 0.19
Tannic acid + $ZnSO_4$+$FeSO_4$	2.95	2.58 ± 0.07	6.78 ± 0.15
Tannic acid + $ZnSO_4$ + $CaSO_4$	2.92	2.91 ± 0.06	7.68 ± 0.15
Tannic acid + $ZnSO_4$+Na_2MoO_4	2.90	3.0 ± 0.08	7.16 ± 0.12
Tannic acid + $ZnSO_4$ +$CoCl_2$	2.95	2.96 ± 0.07	7.60 ± 0.21
Tannic acid + $ZnSO_4$+ $CaSO_4$ + Na_2MoO_4 +$CoCl_2$	2.93	2.99 ± 0.09	7.58 ± 0.19
Control (only *C. spinosa*)	2.93	11.81 ± 0.43	11.25 ± 0.32
C. spinosa + $ZnSO_4$	2.95	11.70 ± 0.36	11.09 ± 0.16
C. spinosa +$CaSO_4$	2.96	11.58 ± 0.42	11.17 ± 0.14
C. spinosa +$ZnSO_4$+$FeSO_4$	2.93	10.64 ± 0.29	ND
C. spinosa + $ZnSO_4$ + $CaSO_4$	2.90	11.79 ± 0.51	11.13 ± 0.18
C. spinosa +$ZnSO_4$+Na_2MoO_4	2.91	11.65 ± 0.36	11.05 ± 0.12
C. spinosa +$ZnSO_4$ +$CoCl_2$	2.92	11.67 ± 0.43	10.98 ± 0.15
C. spinosa + $ZnSO_4$ + $CaSO_4$ + Na_2MoO_4 +$CoCl_2$	2.91	11.80 ± 0.38	11.21 ± 0.13

ND: not detected. Culture was grown at 28^0C using TAB medium containing tannic acid as TT-1 and *C. spinosa* extract (at 20 g /L tannic acid equivalence) as TC-1. Both the media contained NH_4NO_3 (0.3 %), $MgSO_4$ (0.005 %) and addition of respective salts**.** Control was without mineral salts. All salts were used at 0.02 g/L effective concentration.

Other mineral salts (Na_2MoO_4, $CoCl_2$) individually or in combination with $ZnSO_4$ did not show any appreciable change in the activity or in the mycelial biomass. In view of

the results, addition of only Zn salts and Ca salts was included as parameters for optimization studies.

4.3.5 Optimization of environmental conditions and process parameters for tannase production by *A. allahabadi*

Effect of initial pH on tannase production by *A. allahabadi*

An effect of initial pH over the range of 2.0 – 8.4 on *A. japonicus* tannase under submerged conditions was shown to have pH 6.0-7.0 as optimum for tannase activity (Bradoo *et al.*, 1997). Similarly studies on the effect of initial pH of the growth medium on tannase production by *A. allahabadi* were carried out within a pH range of 3.0 – 7.5 in media viz. TT-1 (TAB + tannic acid 20 g/L) and TC-1 (TAB + *C. spinosa* extract 20 g/L). The initial pH 5.0 (after autoclaving) was found to be most optimal for high tannase production in case of both the media. For both the media tannase activity and mycelial biomass were increased as the pH increased from 3.5 to 5.0 and decreased gradually as the pH increased from 6.5 to 7.5 (Figure 4.5). This may be because either there is

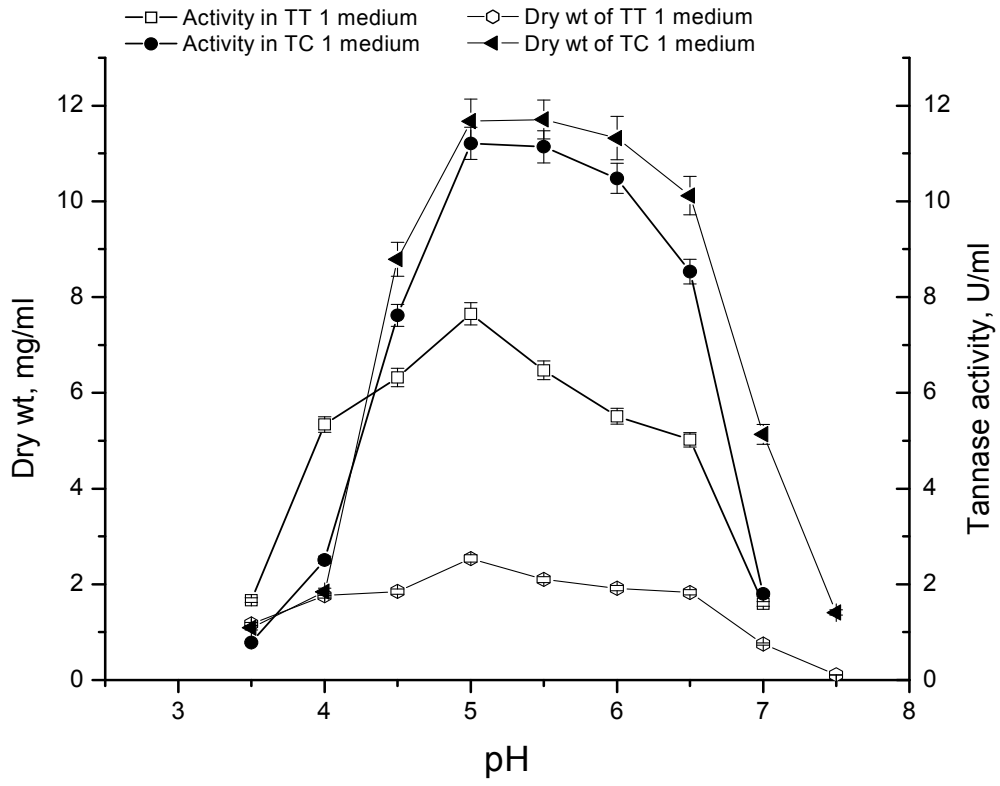


Fig. 4.5 Effect of initial pH on tannase production by *A. allahabadi*.

possibility of salt formation of tannic acid at higher pH, or possibility of low activity of tannase at higher pH which might have led to unavailability of tannic acid for the utilization and hence the tannase activity was observed to be less at pH 7.0 and above than that at pH 5.0. Hadi *et al.* (1994) observed effect of initial pH on *R. oryzae* tannase production in the range of 4.0 – 6.0. Tannase activity (4.5 U/ml) was found to be highest at pH 5.0 in 120 h under submerged conditions. Similar findings were documented for *A. niger* tannase (optimum pH 5.0) under submerged conditions (Lokeswari and Jayaraju, 2007a).

Tannase activity in the TC-1 medium was stable over a pH range 5.0-6.0. The pH did not seem to affect the mycelial growth in this pH range and there were comparable activities in this range of pH. Probably the complex and micronutrient rich TC-1 medium might have supported the growth of the organism providing buffering action to the change in pH.

Effect of media volume /Flask volume ratio on tannase production by *A. allahabadi*

In the present investigation, effect of media volume over range of 1:5 (100 ml/ 500 ml of Erlenmeyer flask) to 1:2 (250 ml/ 500 ml of Erlenmeyer flask) for tannase production by *A. allahabadi* was studied in TT-1 (TAB + tannic acid 20 g/L) and TC-1 (TAB + *C. spinosa* extract 20 g/L).

It can be seen from Fig. 4.6 as the media volume ratio changed from 1:5 to 1:2, the decrease in tannase activity in case of TT-1 was steep while the same for TC-1 was gradual. Media volume at half the volume of the flask resulted in very less growth, in the form of clumps and in turn poor enzyme production. The decrease in dry weight in case of both the media and rate of decrease was steep and nearly same. The tannase activity was highest at media/flask volume ratio 1:5 for both the media, TT-1 (8 U/ml) and TC- 1 (11 U/ml). The decrease in activity and in dry weight with increase in media/flask volume ratio is due to lower oxygen availability. This indication confirms aerobic nature of the organism. Atkinson and Mavituna (1991) reported oxygen transfer rate of 54.6 mM O_2/L/h in shake flask having media to volume ratio of 1:5 and similar trend of results. Banerjee and Pati (2007) have also reported highest tannase activity for media volume ratio of 1:5. In view of the results obtained during the present investigation, effect of aeration was studied for optimizing parameters for larger scale fermenters (35 L and 100 L).

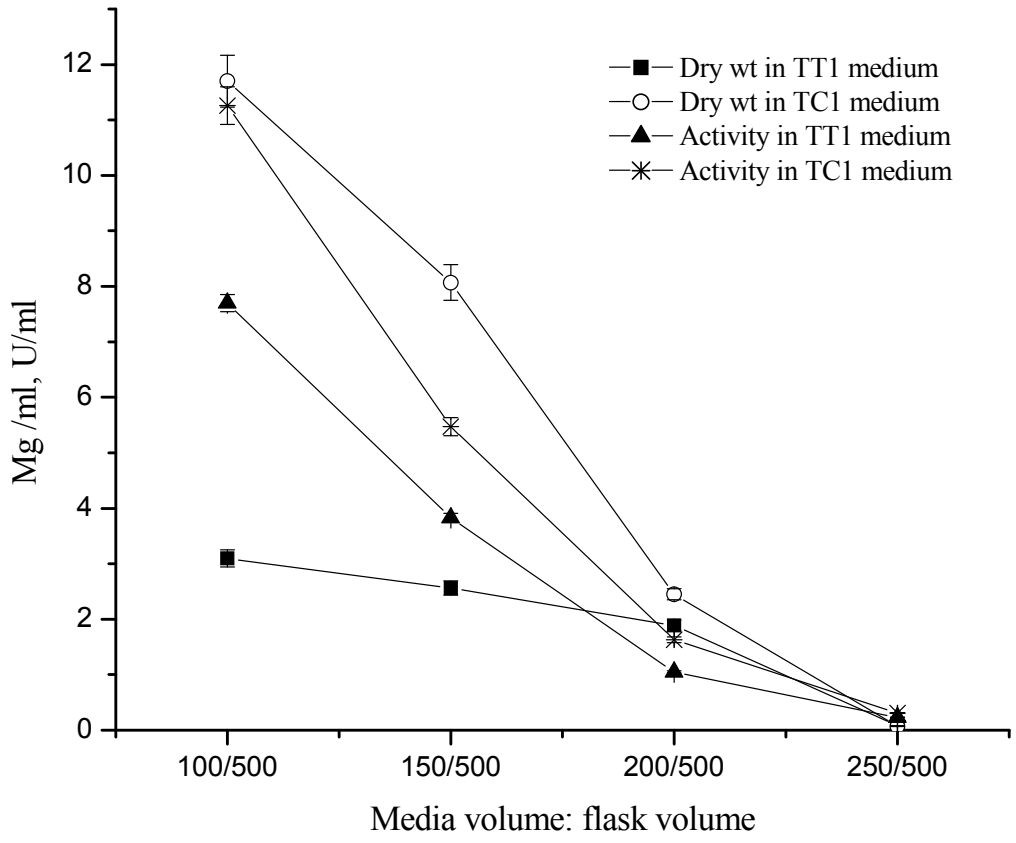


Fig. 4.6 Effect of media volume/flask volume ratio on tannase production of *A. allahabadi*.

Effect of shaking speed (agitation) on tannase production by *A. allahabadi*

Agitation is useful in enhancing the availability of nutrients as well as oxygen to the microorganisms. *A. allahabadi* was grown in 500 ml Erlenmeyer flask containing 100 ml TAB medium under different shaking speed viz. 100, 150 and 200 rpm. With available highest rpm of 200, both mycelial biomass (2.96 mg/ml) and tannase production (7.61 U/ml) of *A. allahabadi* was observed to be highest. While biomass (1.06 mg/ml) and tannase production (4.14 U/ml) was 50 % less at 100 rpm and 10 – 13 % less at 150 rpm than that at 200 rpm. The increase in the tannase activity was a function of higher mycelial biomass as it was a growth associated enzyme. This indicated the requirement of higher oxygen availability and good mixing efficiency to get good activity of tannase. In a similar type of study, Natarajan and Rajendran (2009) showed that at 125 rpm, tannase production was higher with 9.4 U/ml by *L. plantarum* under submerged

conditions. The authors attributed the reason of lesser activity at rpm less than 125 to inadequate mixing.

4.3.6 Optimization of inoculum for tannase production by *A. allahabadi*

Effect of form of inoculum (spore or mycelial inoculum) on tannase production

The effect of spore and mycelial inocula on tannase production was studied in TAB medium for 60 h. The inocula were prepared as described in section 2.8. The enzyme activity (7.23 U/ml) and mycelial biomass (3.0 mg/ml) obtained was comparable in both the forms of inocula (Figure 4.7). In spore inoculum, spores started germinating after 6 – 8 h and hence spore inoculum showed a lag in growth up to 12 h. By 18 h, there was 100 % germination of the spores. The tannase production peaked at 6 h later in spore inoculum than that of mycelial inoculum. Despite the lag due to spore inoculum, the mycelial growth was found to be comparable with that of mycelial inoculum after 36 h.

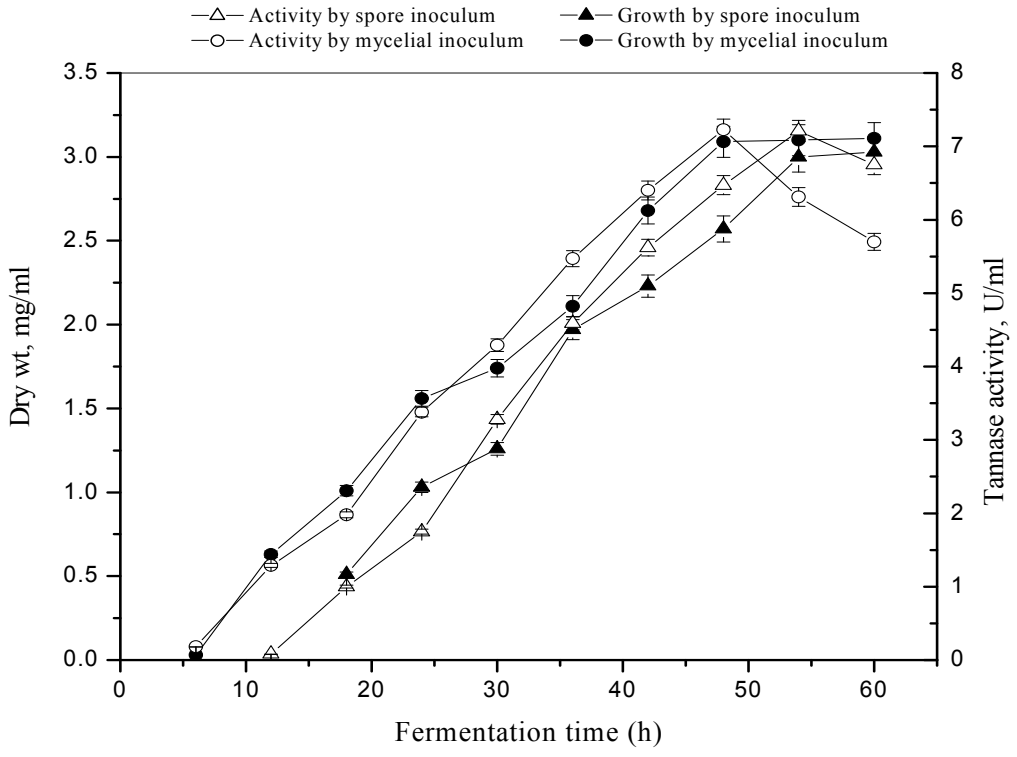


Fig. 4.7 Effect of spore Vs mycelial inoculum on tannase production by *A. allahabadi* Culture was grown in TAB medium under shaking conditions at 28^0C. Inocula were prepared as described in section 2.8.

The specific growth rate [μ] of *A. allahabadi* in spore inoculum was found be higher (0.702 h^{-1}) than that of mycelial inoculum (0.502 h^{-1}). However the initial biomass in case of mycelial inoculum was much higher than that of spore inoculum. This led to higher biomass production and higher tannase production in lesser time by *A. allahabadi*. Mycelial inoculum was therefore used for further experiments to minimize the lag period in growth and tannase enzyme production by *A. allahabadi*.

Effect of inoculum age on tannase production by *A. allahabadi*

Inoculum age governs the extent of growth of culture in the production medium. In case of *A. allahabadi*, 24 h old mycelial inoculum showed high tannase production (7.14 U/ml) at 48 h while lag of 12 h to produce the same level of enzyme activity in TAB medium was seen with 48 h old inoculum (Figure 4.8). This can be attributed to the higher level of mycelial cells in exponential growth phase for 24 h old inoculum. In inoculum of 48 h age, organism entered into late exponential phase and hence there was

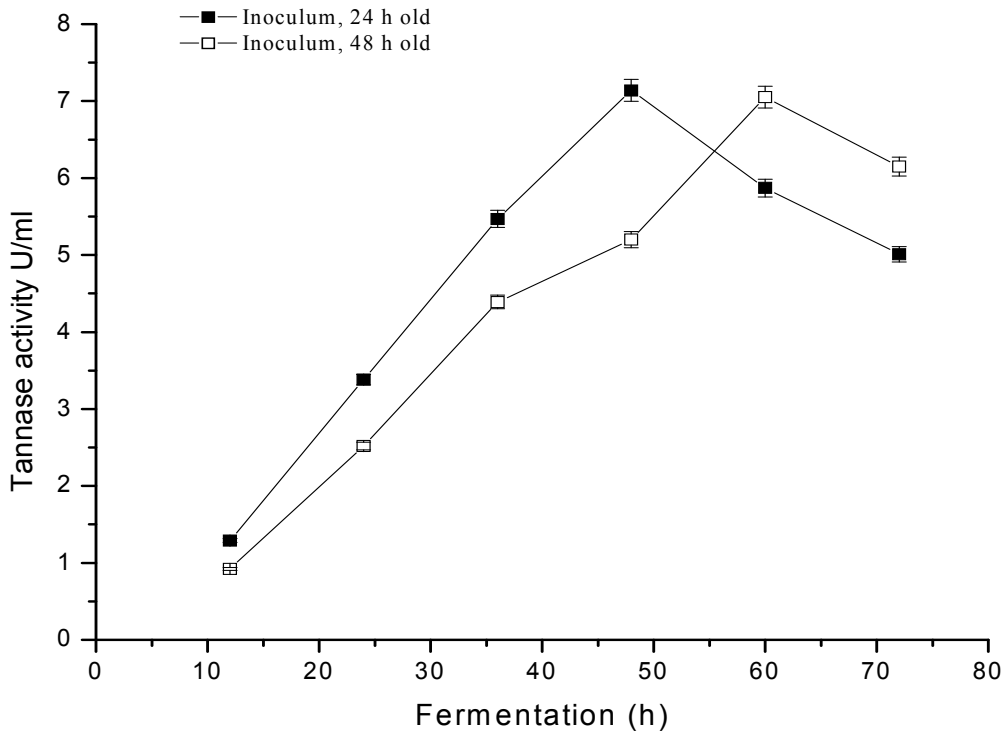


Fig. 4.8 Effect of inoculum age on tannase production by *A. allahabadi* (Culture was grown in TAB medium containing tannic acid under shaking conditions at 28^0C. Mycelial inoculum was prepared as described in Section 2.8).

lag in the initial growth of organism. Therefore 24 h old inoculum was used for further experimentation unless otherwise mentioned.

4.3.7 Profile of tannase production by *A. allahabadi* in optimized medium and at optimized process parameters at shake flask

Production profile of tannase was studied in optimized medium (tannic acid, 20 % or *C. spinosa* extract 20 %; NH_4NO_3, 3 %; $MgSO_4$, 0.05 %; $ZnSO_4$, 0.002 %, $CaSO_4$, 0.002 %, pH 5.0) at 28^0C under shaking conditions at 200 rpm for 60 h using 24 h old mycelial inoculum. In both the media (media with tannic acid as well as *C. spinosa* extract) mycelial growth remained constant after 48 h as can be seen from Figure 4.9. Mycelial biomass and tannase production were high in *C. spinosa* tannin medium than the medium

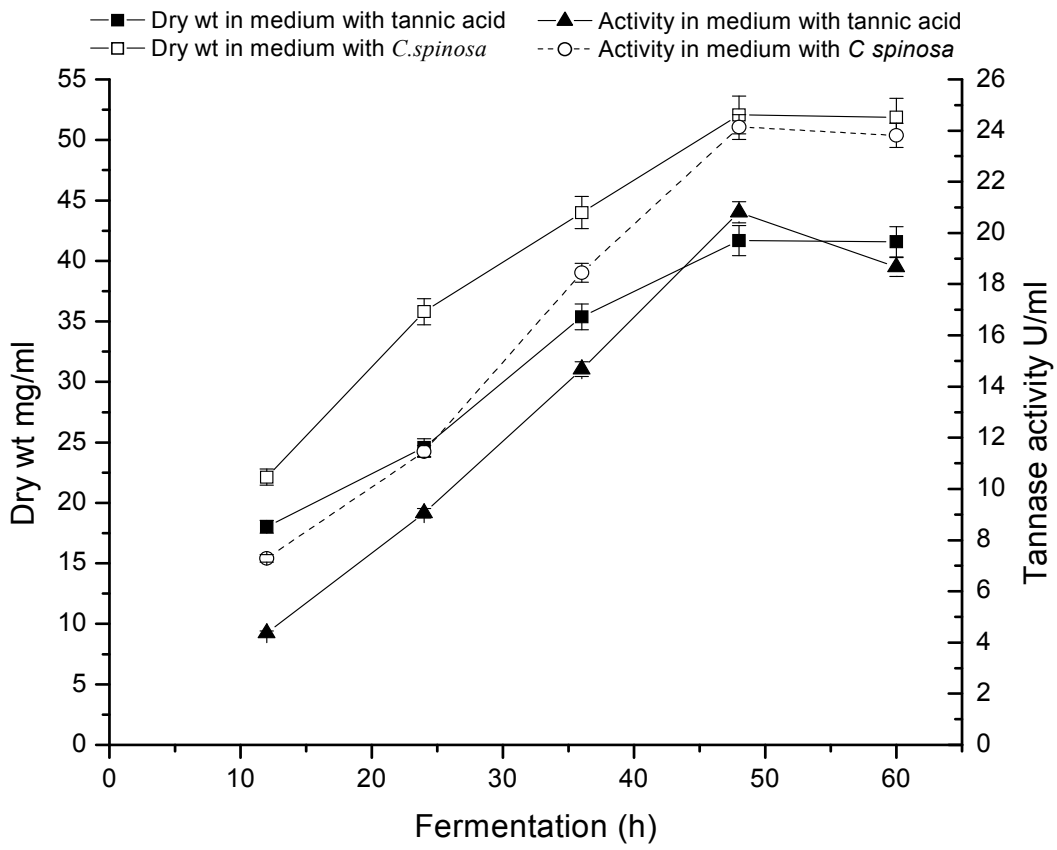


Fig. 4.9 Tannase production profile by *A. allahabadi* in optimized medium using optimized process parameters in shake flask

Culture was grown in mediaTT-2 and TC-2, containing tannic acid and *C. spinosa* tannin at 200 g/L tannic acid equivalence under shaking conditions at 28^0C for 60 h.

with tannic acid. This can be attributed to the residual sugars, trace minerals and organic nitrogen present in *C. spinosa* extract playing supportive role for enhancing the growth and tannase activity. Also the low molecular weight polyphenols in the *C. spinosa* extract might be contributing to the tannase activity induction along with residual tannic acid and accumulated gallic acid thus maintaining the enzyme activity levels constant. The productivity of tannase production in medium with tannic acid was 433 U/L/h and that in medium with *C. spinosa* tannin was 503 U/L/h at 48 h.

4.4 Studies and optimization of tannase production by *A. allahabadi* at fermenter level

In shake flask studies, *A. allahabadi* produced 24.14 U/ml tannase in 48 h under optimized conditions (*C. spinosa* tannin, 200 g/L, ammonium nitrate, 30 g/L, MgSO4, 0.5 g/L, ZnSO4 and CaSO4, 0.02 g/L each, initial pH 5.0) at 28^0C. Using these conditions, further fermentation studies were conducted at 35L stirred tank reactor /fermenter (STR) and was also scaled up to 100L capacity.

In case of fermenter level studies, the parameter of media volume/flask volume ratio does not remain strictly relevant and effect of this ratio gets represented by the aeration rate. The parameter of shaking speed gets represented by impeller tip speed.

In the present investigation, studies were carried out to check the effect of process parameters on fermenter performance. The aforementioned process parameters were agitation and aeration. Fermentation scale up was carried out in 35 L and 100 L fermenters (Bio Chem Engineering Ltd., Pune). The working volume of 35 L fermenter was 30 L whereas for 100 L fermenter it was 80 L. Both the fermenters were insulated with jackets and had automated controls for pH, temperature and dissolved oxygen (Ingold). The sterile air was passed through 0.22μ membrane filters (Pall).

Studies on intensity of agitation in fermentation were reported by Atkinson and Mavituna (1991). The intensity of agitation is generally accepted as being governed by impeller type and impeller tip speed. The impeller tip speed in the present investigation was varied over the range of 0.5 m/s to 3.0 m/s. The type of impeller used in the present investigation was what is commonly referred to as “Rushton turbine.” The Rushton turbine type is preferred type of impeller for aerobic fermentation since it helps in good and uniform distribution of air bubbles in the fermentation medium. Results on use of

Rushton turbine in STR of varying capacities [7 L to 9000 L] were reported by Schell *et al.* (2001). The authors have reported findings regarding oxygen transfer in cellulase production from *Trichoderma reesei.*

In the present investigation, sparger was used for introducing air flow into the fermenter [STR]. The oxygen concentration in the fermentation medium was measured by a measuring system using probes for dissolved oxygen [DO]. In the measurement system used for DO, the probe, transmitter and indicator were integral and inseparable part of the system. The measuring system used indicated oxygen concentration in the fermentation medium in terms of percentage saturation.

Effect of back-pressure was studied at 1 volume/volume/min (vvm) aeration. At this level of aeration DO in the medium was measured, at back-pressure magnitudes of NIL [no back pressure] and 0.5 bar,g keeping all other parameters unchanged. The DO was 48 % and 54 % at NIL and 0.5 bar back pressure, respectively. Increase in back pressure more than 0.5 bar,g did not yield any significant further improvement in DO level in the medium.

4.4.1 Optimization of tannase production by *A. allahabadi* at 35 L STR

Effect of agitation (impeller tip speed) on tannase production at 35 L STR

The effect of tip speed of impeller on tannase production was studied by varying the tip speed over a range of 0.5 m/s to 3.0 m/s. At tip speed of 2 m/s (368 rpm) the optimum tannase activity (25.98 U/ml) and mycelial biomass (52.16 mg/ml) was obtained. It can be seen from Figure 4.10 that at lower tip speed (< 2m/s) the mycelial biomass and tannase production were low. The lower tip speed affected production of biomass and tannase significantly compared to that at 2 m/s. Further increase in the tip speed to 2.5 m/s and 3 m/s reduced biomass as well as enzyme activity. At lower tip speed (<2 m/s) the DO concentration decreased significantly at 16 h of fermentation and did not change thereafter. The DO concentration was increased and was above 45% of air saturation throughout the fermentation when tip speed was at 2 m/s and above. At increased tip speed of 2.5 m/s and above, the oxygen saturation level remained same but the shear rate was higher and fragmented mycelial growth with thinning of mycelia were observed. This led to reduced biomass and in turn reduced tannase activity.

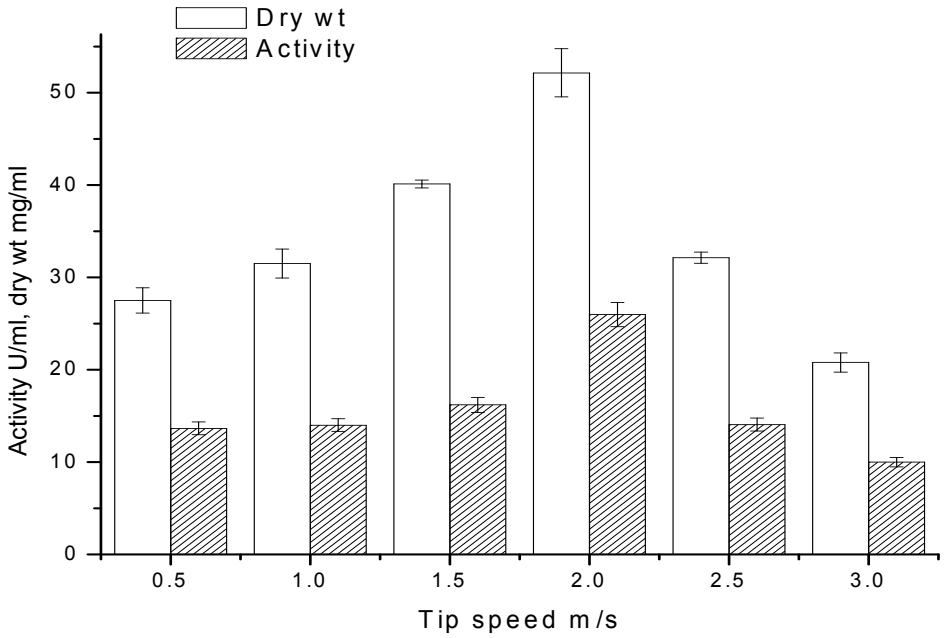


Fig. 4.10 Effect of agitation with respect to impeller tip speed on tannase production by *A. allahabadi* in 35 L STR

(*A. allahabadi* mycelial inoculum was inoculated in 35 L STR and fermentation was carried out at 28^0C for 48 h. The corresponding rpm for tip speed (m/s) were maintained in the bracket: 0.5 m/s (97), 1 m/s (138), 1.5 m/s (207), 2 m/s (276), 2.5 m/s (345), 3 m/s (415))

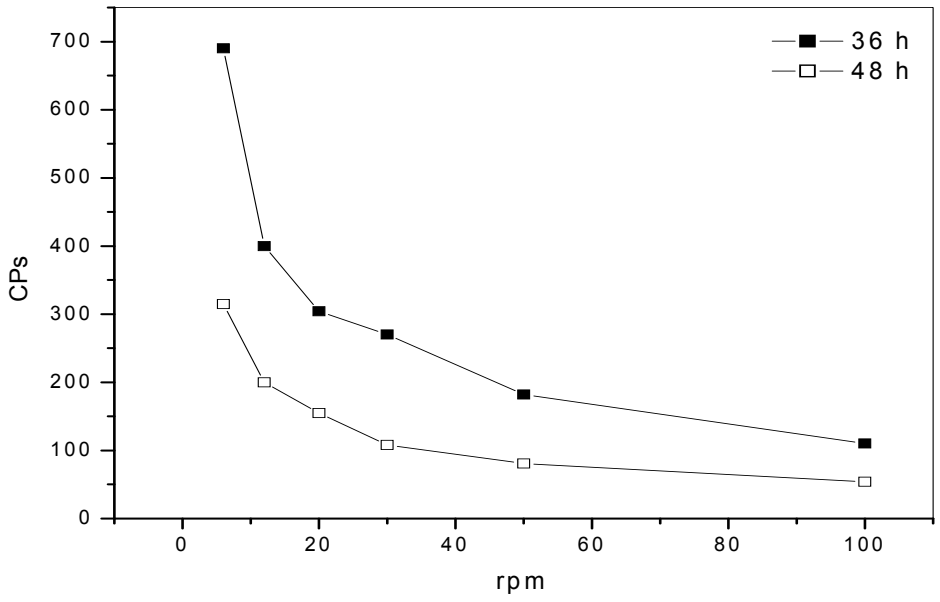


Fig. 4.11 Effect of agitation on viscosity of *C. spinosa* fermentation broth of 36 h and 48 h. Viscosity was checked at various rpm in increasing order.

Samples were drawn at eight h interval to check the rheological properties of the fermented broth. The samples were checked for viscosity using Brookfield Viscometer at varying rpm. The broth showed higher apparent viscosity (315 CP) at lower viscometer (6) rpm and lower apparent viscosity (54 CP) with higher viscometer (100) rpm. This is typically non Newtonian pseudo plastic behavior (Atkinson and Mavituna, 1991) (Fig. 4.11). The authors have reported that at lower rpm, the apparent viscosity was high and therefore overall mass transfer coefficient and oxygen transfer rate would be low. In the present investigation it was observed that mycelial growth and tannase activity bear a relationship with different morphological behavior at different agitator speeds. The effect of agitator speed on morphology of *A. allahabadi* and its effect on tannase production are further discussed in Section 4.5.

Study and optimization of aeration

vvm is a typical parameter employed to quantify extent of aeration. 1 vvm means airflow of 1 vol/min for every 1 vol of fermentation broth. Aeration is most energy consuming process and contributes to major operating cost of submerged fermentation. Reduction in aeration by optimization in turn leads to optimizing operating cost. Other objectives of the present investigation were to determine effect of aeration (vvm) on morphology and

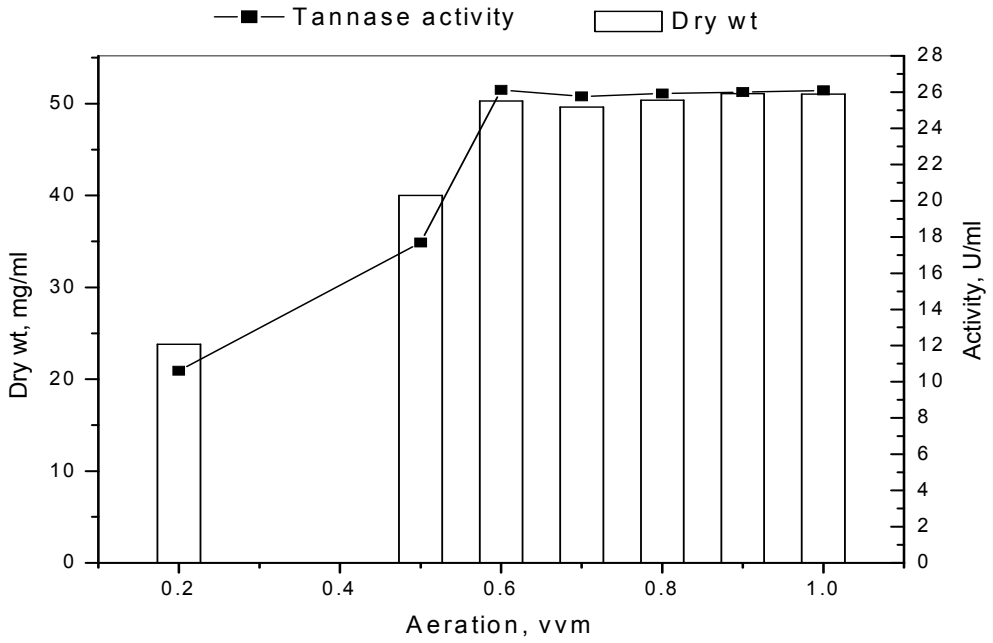


Fig. 4.12 Effect of aeration on tannase production by *A. allahabadi* in 35 L STR

dissolved oxygen concentration (DO levels) in the fermentation medium.

In the present study, initially aeration was kept highest (1 vvm) to check the effect on growth and tannase activity. Though the aeration at 1 vvm and 0.5 kg back pressure showed maximum saturation limits of oxygen, it was necessary to determine minimum required air flow to achieve equal saturation of oxygen from the point of view of optimization. At 0.6 vvm and 0.5 kg back pressure, the oxygen saturation was observed to be equal to that at 1vvm (Fig. 4.12). In the earlier study of agitation (Section 4.2.1), it was shown that the oxygen saturation limit of the medium before inoculation was 54 % of the air saturation limits. Similarly Pourrat *et al.* (1982) demonstrated that constant air flow at 0.4 vvm gave better yield of *A. niger* mycelia and higher tannase activity than constant dissolved oxygen at 30 %. Whereas DO of 50 % showed complete hydrolysis of *Rhus coriaria* tannin by *A. niger* after 40 h in a fermentation carried out in 6 L STR (Pourrat *et al.*, 1987). At 0.2 vvm air flow, the specific growth rate of *A. allahabadi* was reduced and pellet morphology was predominant. The tannase titer was also less by 40 % than that at the optimized conditions. This was due to reduced biomass and pellet form of morphology. At optimum aeration (0.6 vvm, 0.5 kg back pressure) and agitation (2 m/s, 368 rpm) conditions, O_2 concentration dropped to 40 % of the solubility of oxygen in first 20 h and then gradually increased and remained constant at 45% of the air saturation limits till end of fermentation. Tannase activity and growth were observed to be comparable to that at 1vvm air flow. For instance, in a study on the effect of oxygen levels on biomass of S*treptomyces griseaus,* DO concentration was observed to be dropped by 60 % in initial 10 h and then gradually increased to saturation level over a period of 60 h. At the end of 70 h, it reached to the maximum saturation level (Atkinson and Mavituna, 1991). These findings are different than observations during the present investigations. In the present studies, the compact pellet morphology was observed at low aeration rate (<0.6vvm) and mycelia morphology at higher aeration rate. The later was favorable for higher biomass and tannase production. However, few researchers have reported the different behavior such as studies of citric acid fermentation using *A. niger* showed that there was no direct relationship exist between the morphology and DO in the fermented broth, though the pellet morphology had better productivity over mycelial morphology (Papagianni, 2004). Similar results were observed in penicillin fermentation using *P. chrysogenum*. Irrespective of the fact that the oxygen requirement for the productivity of penicillin and biomass was different, there was no direct correlation between dissolved oxygen and morphology of the culture (Vardar and Lilly,

1982). The present investigation indicates 0.6 vvm aeration as optimum. This observation of constant aeration is in accordance with the findings of Seth and Chand (2000) wherein constant aeration at 0.75 vvm was demonstrated as optimum in fermenter studies of tannase production by *A.awamori*. It was postulated that excess oxygen leads to oxidation of tannins which then become unavailable for microbial utilization resulting in reduced tannase production (Barthomeuf *et al.*, 1994a; Seth and Chand, 2000).

4.4.2 Profile of tannase and gallic acid production by *A. allahabadi* at 35 L STR

Profile of tannase activity, gallic acid production and tannic acid conversion efficiency was carried out using optimized medium (*C. spinosa* tannin, 200 g/L) and optimized conditions (0.6 vvm, 0.5 kg back pressure and tip speed 2 m/s). The peak of tannase (26.09 U/ml) and highest gallic acid (86.82 % of available *C. spinosa* tannin) yield were obtained at 40 h. Corresponding tannin conversion efficiency was 95.70 % and gallic acid productivity was 3.5 g/L/h (Fig. 4.13). In 35 L STR, the productivity of tannase

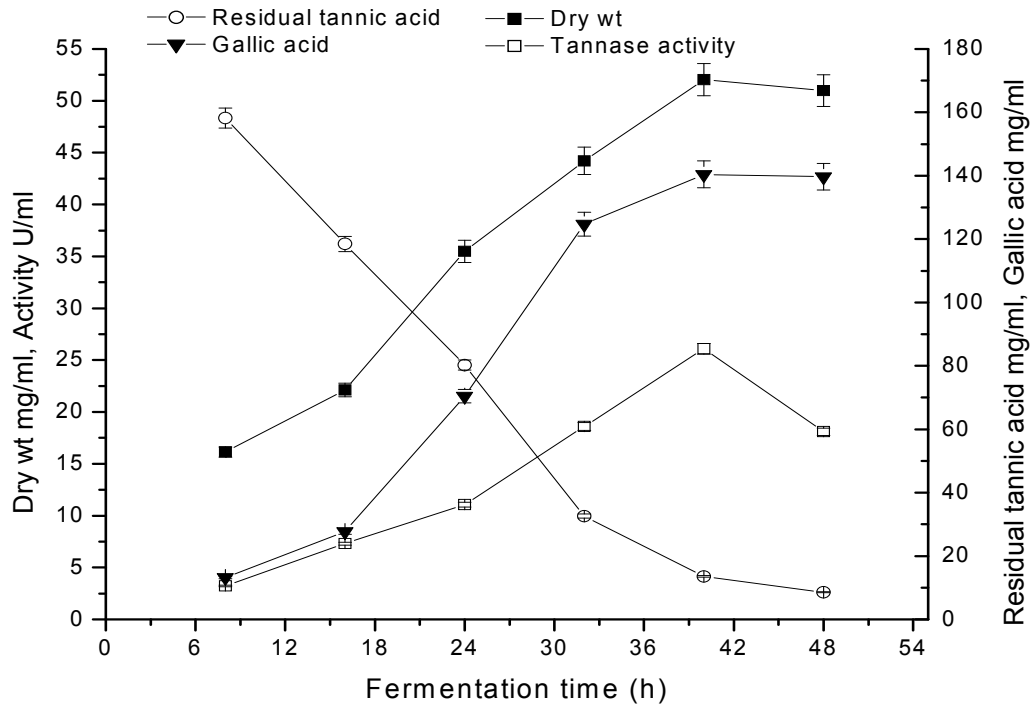


Fig. 4.13 Profile of tannase and gallic acid production by *A. allahabadi* in 35 L STR.

Profile was carried out using *C. spinosa* medium (200 g/L tannic acid) using optimized conditions (0.6 vvm, 2 m/s tip speed i.e. 276 rpm) at 28^0C.

(652 U/L/h) was increased by 1.29 fold as compared with the results of shake flask studies (502 U/L/h). The improved performance in 35 L fermenter as compared with shake flask is attributable to better mixing efficiency by virtue of better aeration and agitation. The TLC and HPLC profile of *C. spinosa* fermentation was carried out as described under section 2.15.2 and 2.15.3 which showed the decreasing concentration of tannin along with disappearance of other polyphenols as the fermentation progressed (Fig. 4.14 a, b and c).

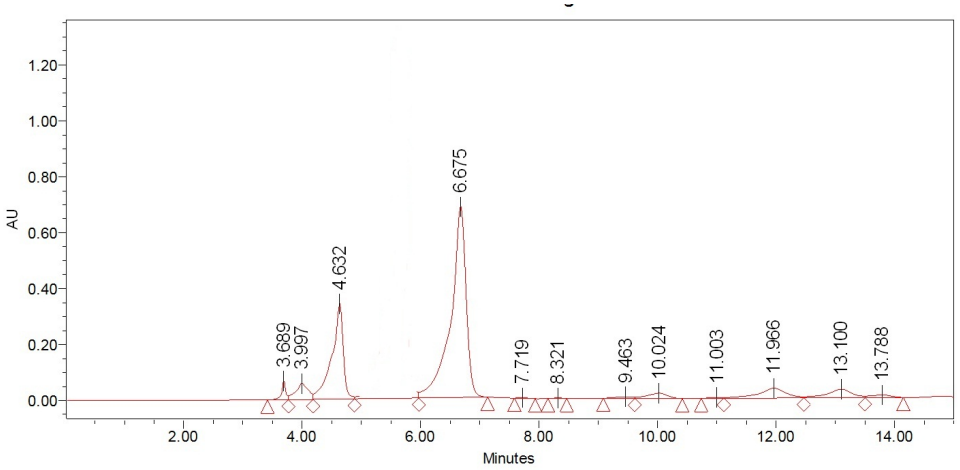


Fig 4.14 a HPLC of *C. spinosa* tannin before conversion by *A. allahabadi* tannase

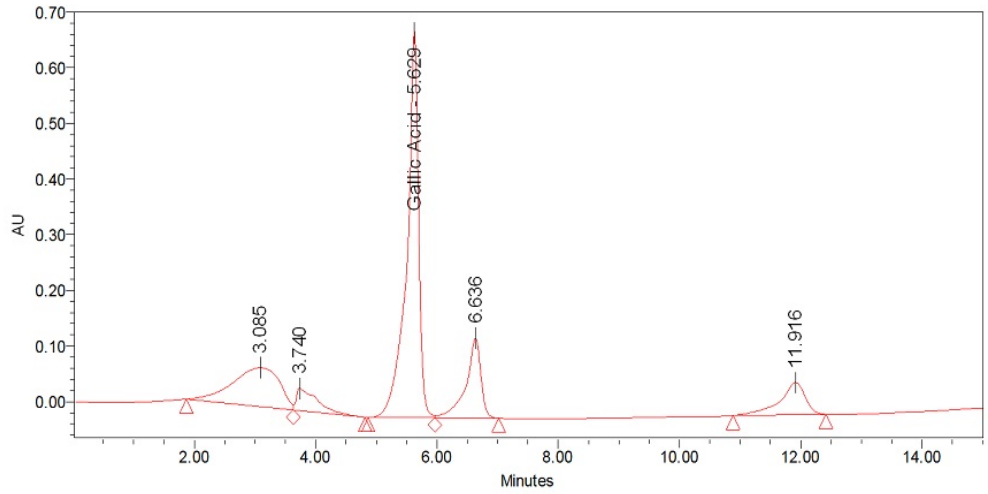
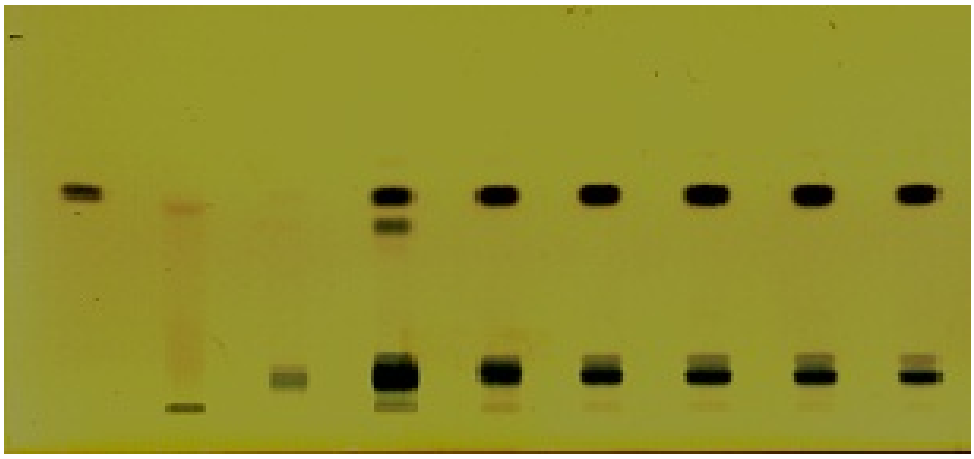


Fig 4.14 b HPLC of *C. spinosa* tannin conversion by *A. allahabadi* tannase at 48 h



1 2 3 4 5 6 7 8 9

Fig. 4.14c TLC profile of *C spinosa* fermentation in 35 L STR (1.Gallic acid ; 2. Ellagic acid; 3. Tannic acid; 4. *C spinosa* extract medium; Fermentation of C. spinosa at 5. 16 h; 6. 24 h 7. 36 h; 8. 42 h; 9. 48 h)

4.4.3 Studies on scale up from 35 L to 100 L STR

The performance of 100 L fermenter was evaluated using the parameters optimized at 35 L fermenter scale (agitator tip speed 2 m/s, aeration 0.6 vvm with 0.5 kg back pressure). The impeller diameter to tank ratio in 35 L STR was 0.38 and the same in 100 L STR was 0.42. Hence the corresponding rpm in 35 L STR was 368 rpm and in 100 L was kept at 206 rpm to maintain the constant tip speed. There are no published reports seen on the scale up of tannase production at larger STR however, Kim *et al.* (2005) reported the scale up of enterokinase fermentation using *Saccharomyces cerevisiae* from 5 L to 300 L maintaining constant impeller tip speed (700 rpm for 5L and 150 rpm for 300 L) and minimum DO level at 60 % or at 2 vvm air flow.

During operation under these conditions the morphology was observed to be same as in case of 35 L fermenter predominantly filamentous growth of *A. allahabadi*. After 40 h, the tannase activity was 26.2 U/ml and gallic acid yield was 87 % (Table 4.7).

On the other hand, a fermentation of *R. coriaria* tannin by *A. niger* in 6 L STR was reported at constant DO of 50 % showed complete hydrolysis of tannin after 40 h. However the total gallic acid yield was 9.75 % of available tannins (Pourrat *et al.*, 1987).

The precipitation and harvesting of gallic acid was carried out as described in section 2.16. The total crude gallic acid yield was 4.20 kg from 35 L STR and 11.2 kg from 100

L STR with 93- 94 % recovery. Further purification was resulted into 98 % pure gallic acid with 6 % loss in the yield. There are no published reports on the scale up of tannase production at large scale fermentation. The SmF studies were carried out using STRs up to 20 L. Tara tannin fermentation (20 g/L) at shake flask by *Klebsiella pneumoniae* showed 93 % utilization of tannins with 55 % yield of gallic acid (Deschamps and Lebeault, 1984).

Table 4.7 Scale up of tannase and gallic acid production by *A. allahabadi* in 100 L STR

H	Dry wt, mg/ml	Tannase, U/ml	Residual tannic acid, mg/ml	Gallic acid, mg/ml
8	16.36	3.25	158.34	13.21
16	22.14	7.28	116.41	28.02
24	35.49	11.1	80.56	70.45
32	44.43	18.72	32.61	124.76
40	52.12	26.2	12.98	140.84
48	50.78	18.32	8.45	139.58

Scale up of tannase and gallic acid production was carried out from 35 L to 100 L STR using optimized conditions (0.2 vvm, 2 m/s tip speed i.e. 206 rpm) at 28^0C for 48 h.

The productivity of tannase production (655 U/L/h) and that of gallic acid production (3.52 g/L/h) was comparable with that in 35 L STR. It can be seen from the results that the scale up of tannase and gallic acid production from 35 L to 100 L was achieved with a reasonable degree of success.

4.5 Effect of various nutritional factors and process parameters on morphology of *A. allahabadi* and tannase production

During fungal fermentation, under submerged conditions, filamentous fungi exhibit broadly two morphological forms viz. densely woven mycelia masses (pellets) and dispersed or free mycelia.

The morphology of fungi is influenced by number of factors such as carbon and nitrogen source, metal ions, pH of the medium, shear rate and inoculum. The morphology of filamentous fungi is an important parameter related to productivity of several industrial processes (Dobson 2008; Vilena and Gutirrez-Correa, 2007). For instance, El-enshasy *et al.* (2006) showed increase in protein production and glucose oxidase activity when the morphology of *A. niger* was changed from pellets to dispersed mycelia.

However there are no reports available on the correlation of fungal strain morphology and its effect on tannase production except that by Purwanto *et al.* (2009) where it was shown that with increased agitation morphology of *A. niger* changed to dispersed mycelia and there was increase in tannase activity .

In the present study the effect of tannic acid concentration together with various nutritional factors and process parameters on the morphology of *A. allahabadi* and its effect on tannase production were studied. Four different types of morphologies viz. (a) free or dispersed mycelia (b) big fluffy pellets (5-6 mm), (c) medium sized compact pellets (2-3 mm) and (d) mycelial clumps were observed (Fig 4.15 a and b).

Effect of carbon and nitrogen on morphology

The morphology of *A. allahabadi* was influenced by concentration of tannic acid. When a culture was grown in potato dextrose broth, loose fluffy pelleted morphology was predominant whereas when grown in TAB medium with 20 g/L tannic acid, compact pellets were observed. With increase in tannic acid concentration (50 g/L), loose fluffy pellets were observed. When the concentration was increased further to 200 g/L, dispersed mycelial growth was predominant. Presence of carbon source in addition to tannic acid did not change the morphology except for the starch in which occasional fluffy pellets along with dispersed mycelia were observed at higher concentration of tannic acid. There was no change in morphology when tannic acid concentration was above 50 g/L in presence of different nitrogen sources and only loose fluffy pellets to dispersed mycelia were predominant. The culture showed compact small pellets at low tannic acid irrespective of the nitrogen source. In case of *R. oryzae*, the study showed that peptone as nitrogen source produced much smaller and heavier pellets than other nitrogen sources such as urea (Liao *et al*., 2007).

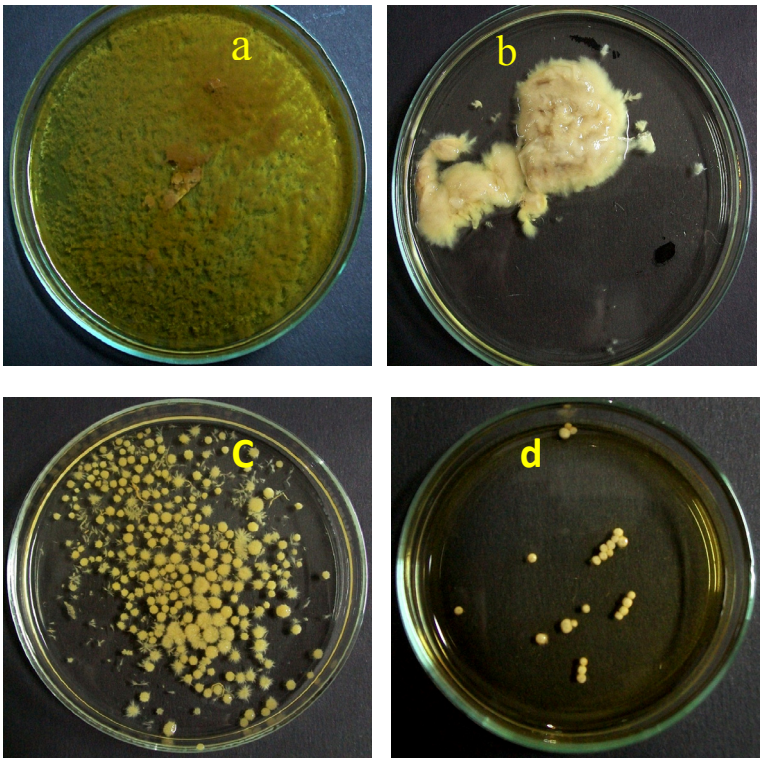


Fig. 4.15 a Morphology of *A. allahabadi* observed in SmF for tannase production: a. dispersed mycelia b. mycelial clump c. loose, fluffy pellets d. mixture of loose and compact pellets.

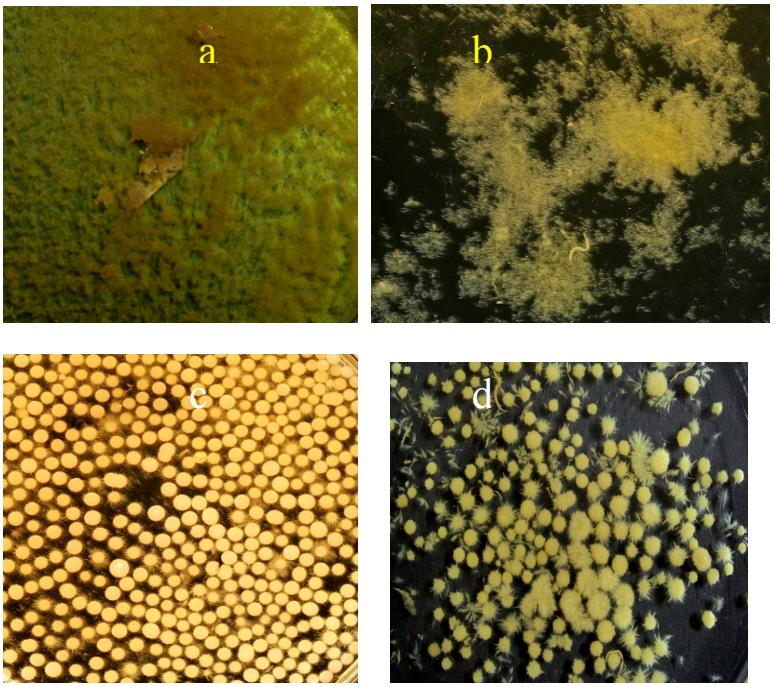


Fig. 4.15 b Close view of a. and b. dispersed mycelia c. compact pellets d. fluffy pellets

Ca^{2+} ion (at 0.25 - 0.5 g/L) was reported to favor pellet formation in case of *A. niger* (Dobson, 2008). However in the present studies, the morphology of the strain was comparable to that of control and did not show any significant change when grown in presence of different concentrations of calcium.

When C. *spinosa* tannin was used in place of tannic acid, the morphological behavior did not change in case of *A. allahabadi*.

Effect of initial pH

Significant effect of initial pH was found on the morphology of *A. allahabadi* under submerged conditions. At pH 3.5, irrespective of concentration of tannic acid from 20 g/L to 200 g/L, compact pellets were formed. From pH 4.0 to pH 6, when tannic acid concentration in the medium was 20 g/L, loose, fluffy pelleted morphology was predominant. However in the same pH range, dispersed mycelia were observed when tannic acid concentration was 50 g/L and above (Table 4.8).

Table 4.8 Effect of initial pH on *A. allahabadi* morphology and tannase production

Initial pH	Morphology	Tannase U/ml
3.5	Compact pellets	1.67
5.0	Big fluffy pellets	7.65
7.0	Clumps	1.59
7.5	Clumps	ND

The culture was inoculated with 1 x 10^7spores/ml grown in TAB medium (with 20 g/L tannic acid) and various pH as indicated at 28^0C for 48 h. At 200 g/L and pH 5.0 morphology was in the form of dispersed mycelia while there was no change in the morphology at pH 3.5, 7.0 and 7.5.

As the pH increased to 6.5 and 7.5, growth was reduced and color of the medium turned to dark brown. The morphology was changed from mixture of big fluffy pellets and dispersed mycelia at pH 6.5 to clumps at pH 7 and pH 7.5 irrespective of the tannic acid concentration. Oncu *et al.* (2006) observed small and compact dense pellets in case of *A.*

sojae under submerged conditions at pH 6 and loose fluffy pellets under uncontrolled pH conditions where exact pH was not mentioned in the report.

Effect of vegetative and spore inoculum

When mycelial inoculum was used to study the effect on morphology, tannic acid concentration in the fermentation medium defined the morphology. At low concentration of tannic acid, small pelleted morphology was predominant and at higher concentrations loose fluffy to dispersed mycelial morphology was observed.

In case of the spore inoculum, when tannic acid concentration of 20 g/L was used in the fermentation medium, remarkable morphological patterns were observed with different concentrations of the spore inoculum. The spore concentration as low as 1 x 10^3 per 100 ml did not show growth in TAB medium even after 60 h of incubation. Spore concentration at 1x10^4 and 1x10^5 per 100 ml showed compact pellets when tannic acid concentration was 50 g/L or less. Further higher spore concentration of 1x10^6 showed loose, fluffy pellets at a concentration of tannic acid less than 50 g/L. At still higher spore concentration 1x10^7, a mixture of dispersed mycelial morphology was observed even at lower concentration (<50g/L) of tannic acid after 60 h.

When the tannic acid concentration in the medium was above 50 g/L, at 1x10^4 and 1x10^5 spores /100 ml spore concentration, loose fluffy pellets were observed at 60 h whereas at 1x 10^6/ml and 1 x 10^7/ml spore inoculum, dispersed mycelia were predominant. The tannase activity was less by 45 % while growth was 36 % less in case of spore concentration 1x 10^6/ml as compared with spore concentration of 1 x 10^7/ml at 60 h.

Effect of spore inoculum on morphology and tannase production was studied as described in section 2.8. Table 4.8 indicates that both the type of inocula supported the growth of organism in tannin containing media. The morphology was changed from loose fluffy pellets to dispersed mycelia when tannin concentration was changed from 50 g/L to 200 g/L.

Similar effect of spore inoculum on glucosamine was reported in case of *A. niger* where pellet morphology was observed when the strain was inoculated with lower spore concentration viz. 10^4 and 10^5/ml however glucosamine formation was high in pellet morphology (Papagianni and Mattey, 2006).

Effect of aeration and agitation

At shake flask level, effect on morphology was checked for media volume to flask volume ratio and for shaking at different rpm.

The present study showed that the media volume to flask volume ratio significantly affected the morphology and growth. In the present study it was observed that 250 ml medium in 500 ml flask resulted in very less growth in the form of clumps where tannase activity was 3.28 U/ml after 48 h. Probably reduced mixing efficiency resulted in the formation of clumps of mycelia. Similarly 200 ml medium in 500 ml flask also showed pellet growth. 100 ml and 150 ml medium in 500 ml flask exhibited fluffy pellets and free mycelial growth which can be attributed to increased mixing efficiency.

Purwanto *et al.* (2009) have reported that at shaking speed of 130 rpm at shake flask level, the morphology of *A. niger* was in the form of loose pellets which gave highest tannase production (at 20 g/L tannic acid). On the other hand, *A. allahabadi* in the present investigation showed free mycelia with highest tannase production at 200 rpm. While tannase activity was found to be reduced at 100 rpm where morphology was in the form of big fluffy pellets. Similarly morphology of *A. niger* was demonstrated to be influenced by shaking speed when grown under submerged conditions at shake flask, for phytase production. At 150 rpm, morphology was in the form of small pellets and entangled mycelia. Total phytase production was higher at 300 rpm where free filamentous form was present (Papagianni *et al.*, 2001).

In the STR, tip speed of impeller was observed to play an important role. In *A. allahabadi* at tip speed of 0.5 m/s (69 rpm) in 35 L STR, fluffy pellets with clumps were observed while at 1 m/s and above, only free mycelial morphology was observed. The predominance of pellet morphology can be attributed to lower tip speed and that of free dispersed mycelia to higher tip speed. Dispersed filamentous broth mostly show non Newtonian behavior and apparent viscosity found to be quite high. They generally exhibit a decrease in viscosity with increasing shear rate (Atkinson and Mavituna, 1991). At lower tip speed, tannase activity was found to be lower than that at higher tip speed. This can be directly correlated with the pellet morphology and reduced biomass at low tip speed which resulted into low tannase activity.

Similar behavior was observed when *A. niger* was grown in 5 L STR for production of glucose oxidase. The study was carried out at various rpm (200, 500 and 800). The culture showed pellet morphology at lower rpm and free filamentous morphology at higher rpm. The biomass was demonstrated to be highest (30 g /L) at 200 rpm while enzyme activity (800 mukat/L) was highest at 500 rpm. At 800 rpm, both the biomass and enzyme activity were observed to be reduced to 15 g/L and 500 mukat/L respectively. The morphology was also changed from pellets to filamentous (El-Enshasy *et al.*, 2006). The change in pellet morphology was reported in case of production of lovastatin using *A. terreus*. With impeller tip speed up to 1.02 m/s, pellets of approx 2.3 mm were predominant while with increased tip speed at 2.03 m/s pellet size was reduced to 0.9 mm. Small and dense pellets were formed under intense agitation when tip speed was increased above 2.03 m/s. There was change in the size of pellets but there was no change in morphology from pellets to free mycelia (Rodriguez Porcel *et al.*, 2005).

In the fermentation where free mycelia form is predominant, cell growth and productivity was higher. However there were other disadvantages such as high viscosity which changed the rheology of the broth causes difficulty in mass transfer of oxygen and nutrients (Oncu *et al.,* 2006). In the present study it was demonstrated that dispersed mycelia showing high tannase activity than other morphology forms. In case of dense, compact pellets as well as in case of clumps biomass and tannase activity both were very low. Though number of factors influences the morphology, tannin concentration and initial pH were the most important factors which controlled the morphology. At very low pH of 3.5, irrespective of the tannic acid concentration, the small pelleted morphology was predominant. For very high pH above 6.5, *A. allahabadi* was in the form of clumps. Both these forms were associated with low tannase activity whereas dispersed mycelia showed highest tannase activity. Activity by loose, fluffy pellets was observed to be comparable with that by dispersed mycelia.

Effect of tannic acid concentration on the hyphal growth

The tannin as a sole carbon source was observed to induce effect on the mycelium morphology. A. *allahabadi* showed change in width of hyphae when grown in different tannic acid concentration. The hypha width of 24 h old hypha in potato dextrose medium measured 2.85 µ while that in TAB (20 g/L) showed 2.02 µ. When tannin concentration was 200 g/L, hypha width of 24 h old hypha was 6.15 µ. In high tannin concentration

medium (100 g/L and above), the mycelial morphology was in the heterogenous forms (Fig 4.16). Growing hyphae were observed with extending hyphal tips. A few hyphae were observed with reduced distance between two septa along with increased hyphal width. Some hyphae showed abnormality in the hypha. White *et al* (2006) has termed this type of morphology as cryptic growth, a type of autolysis especially under submerged conditions. This portion of the hypha can be utilized by the extending hyphal portion for growth thus maintaining the biomass and preventing the reduction in dry cell weight. In the present studies, inspite of heterogenous mycelial morphology and hyphal autolysis, there was no reduction in dry cell weight and tannase production at higher tannin concentration. The high efficiency of tannin utilization despite high tannin concentrations can be attributed to the phenomenon of cryptic growth.

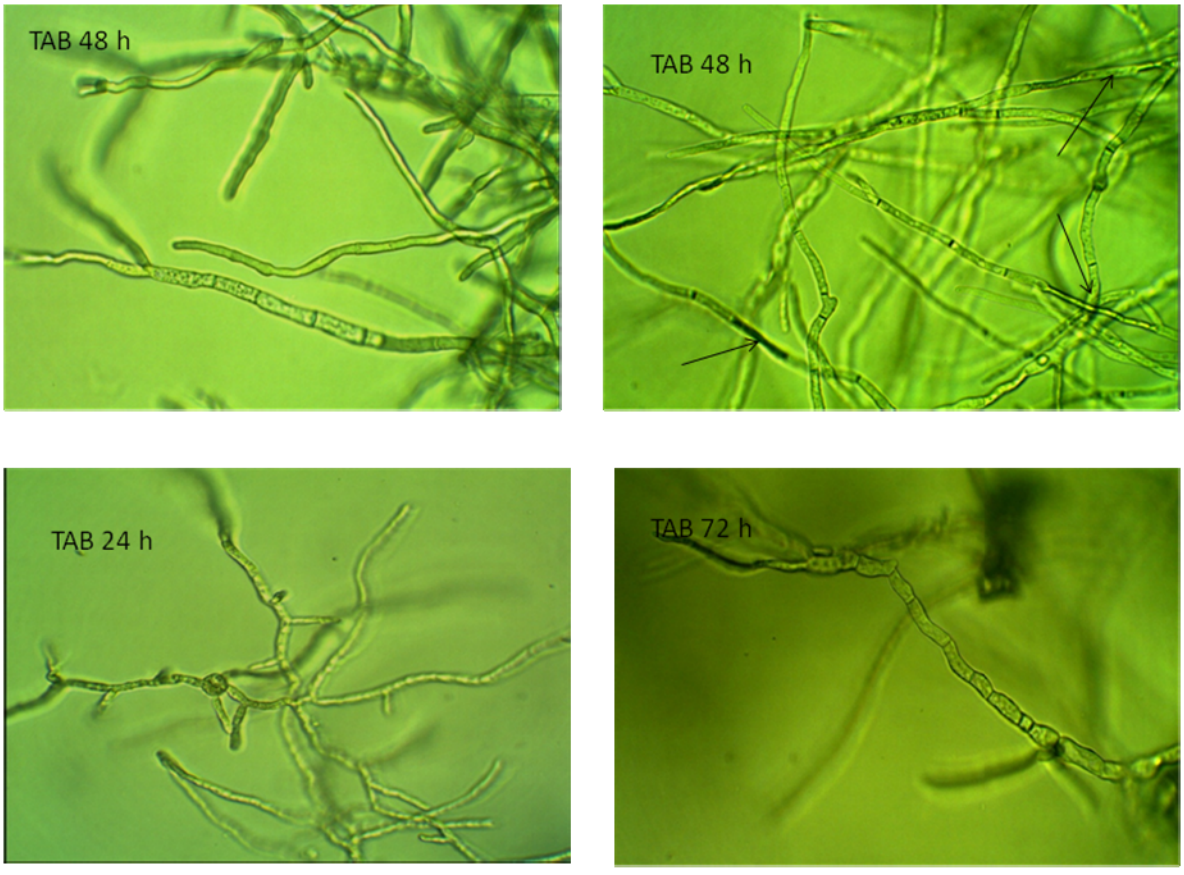


Fig. 4.16 Effect of tannin on morphology of *A. allahabadi* (Black arrow indicates cryptic growth)

4.6 Studies to improve tannase production and tannic acid bioconversion efficiency

Improvement in tannase activity with concomitant increase in tannin bioconversion efficiency was evaluated for synergistic effect of coculture and strain improvement methods.

4.6.1 Improvement in tannase production by coculture

The aim of the study was to carry out bioconversion of tannins by improving conversion efficiency of cultures. The biodegradation of natural products was reported to be effectively enhanced using cocultures (Shi *et al.*, 2005). Both the strains under investigations viz. *A. allahabadi* and *A. parvulus* had tolerance to the tannins at 400 g/L. However they did not utilize tannin more than 20 % when the concentration of tannin was more than 200 g/L. Moreover biomass was less in a medium containing tannin more than 200 g/L. The coculture of both the strains as well as monocultures were carried out as described under materials and methods section 2.9.1 using 300 g/L *C. spinosa* tannin under SmF. A lag of 12 h in the growth was observed in case of coculture as well as monocultures of *A. allahabadi* and *A. parvulus*. However the specific growth rate of coculture was found to be 30 % more than that of *A. allahabadi* and *A. parvulus*. The biomass increased gradually in both monocultures and coculture till 48 h and then remained constant. The tannin utilization efficiency in coculture was found to be higher (46 %) in 48 h whereas it was 10 % in *A. allahabadi* and 8 % in *A. parvulus* monocultures. The enzyme activity in case of coculture was increased (7.5 U/ml) than that of monoculture of *A. allahabadi* (4.59 U/ml) and that of *A. parvulus* (4.02 U/ml) in 48 h. Similarly gallic acid yield by coculture was increased by 32 % of that of *A. allahabadi* and 40 % of that of *A. parvulus* (Fig. 4.17).

The coculture of *A. allahabadi* and *A. parvulus* has demonstrated the enhanced conversion efficiency by synergistic action of both the strains. In a similar work on SmF of valonea tannins, coculture of *A. niger* and *Candida utilis* (both isolated from tannin contaminated soil) was found to degrade 25 g/L tannins in 168 h with 21 % ellagic acid (EA) yield and tannase (T) and polyphenol oxidase (PPO) activity of 66 U/ml and 47 U/ml respectively. The synergistic effect of coculture of both fungi improved overall productivity along with tannase activity and ellagic acid yield than that of monoculture of *A. niger* (EA, 14.3 %, T, 63 U/ml in 216 h) and *C. utilis* (EA, 11.48 %, T, 32 U/ml, PPO,

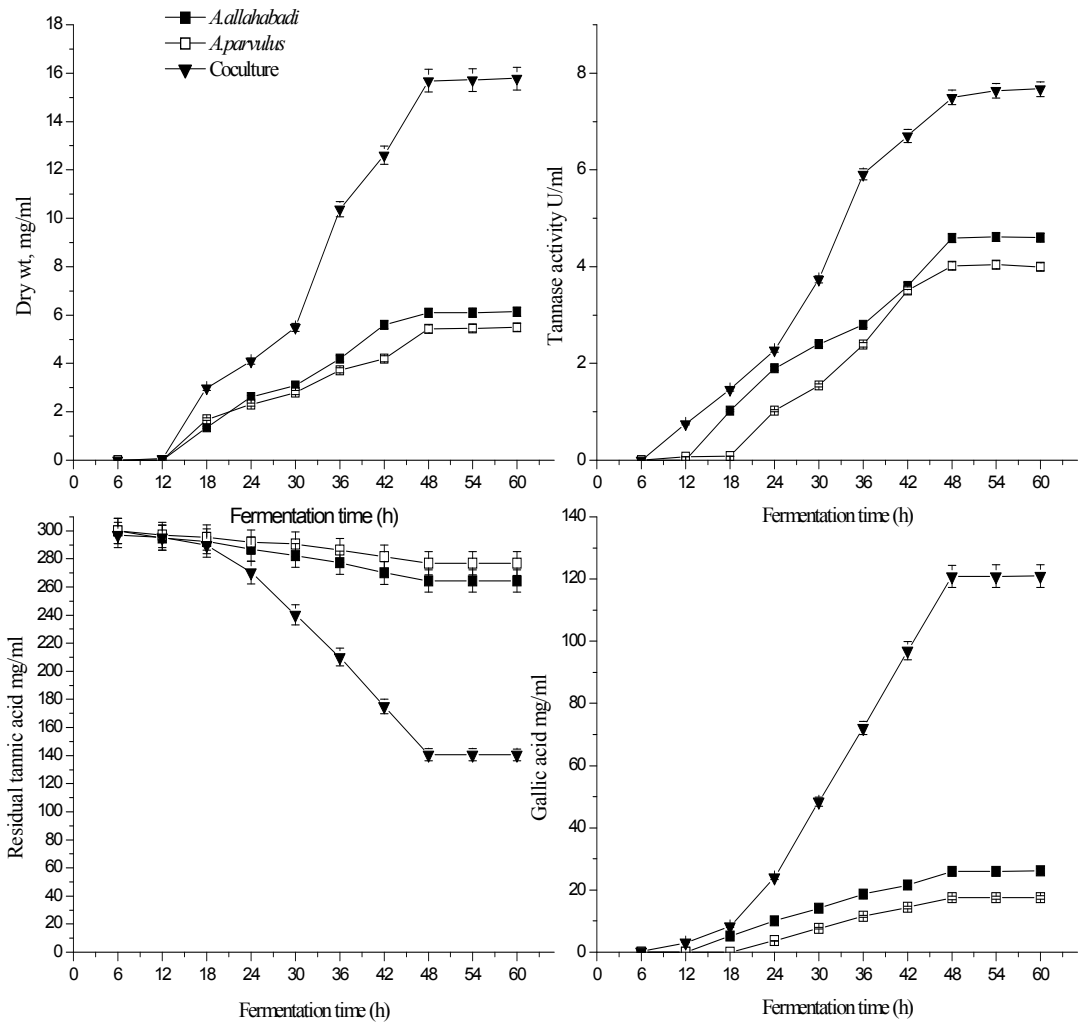


Fig. 4.17 Bioconversion of *C. spinosa* tannin (300 g/L) by coculture of *A. allahabadi* and *A. parvulus*

29 U/ml in 216 h) by 50 – 60 % (Shi *et al.*, 2005). Cocultures of *R. oryzae* and *A. foetidus* showed tannase activity of 41.3 U/ml and 94.8 % gallic acid yield of available tannin by SSF. However the activity and yield of cocultures have not been compared with that of individual cultures in the study (Banerjee *et al.*, 2005). Paranthaman *et al.* (2009a) demonstrated enhanced tannase production by SSF of grape peel using coculture of *P. chrysogenum* and *Trichoderma viridae* (39 U/g) than that of monocultures of *Penicillium* (21 U/g) and *Trichoderma* (24 U/g).

The coculture of *A. allahabadi* and *A. parvulus* showed promising results compared to their respective monocultures. However further investigation at fermeneter scale is

required to study the behavior and interaction of both the cultures in stirred tank reactor (STR).

4.6.2 Strain improvement by UV irradiation and MNNG treatment

Improvement of strains for enhanced tannase and gallic acid production was documented first in 1972 by Ikeda *et al*. The authors used UV irradiation, sodium nitrite and N-methyl-N-nitro-N-nitroguanidine (MNNG) for *Aspergilli* and *Penicilli* mutations. Though the detailed methodology was not discussed, mutated strains accumulated more gallic acid than the parent strains (*Aspergillus* strain V-82 more than 90 % gallic acid from Chinese gallotannin and that of *P. chrysogenum* WH-3, 83 % gallic acid from tara tannin).

Here in the present investigations, the strain improvement of *A. allahabadi* was attempted by using UV and MNNG mutagens (section 2.9.2 and 2.9.3). The survival curves for *A. allahabadi* spores exposed to the UV (15 W, up to 20 min) and MNNG (0.5 – 2 mg/ml) were plotted as shown in Figure 4.18. The surviving isolated colonies from the plates (UV 20 min, MNNG 15 min) were further subcultured and tested for tannase production using optimized conditions. It can be seen from Table 4.9, all UV and MNNG mutants showed activity and growth comparable with that of parent strain. There was no change observed in the tannic acid utilization efficiency of mutant strains.

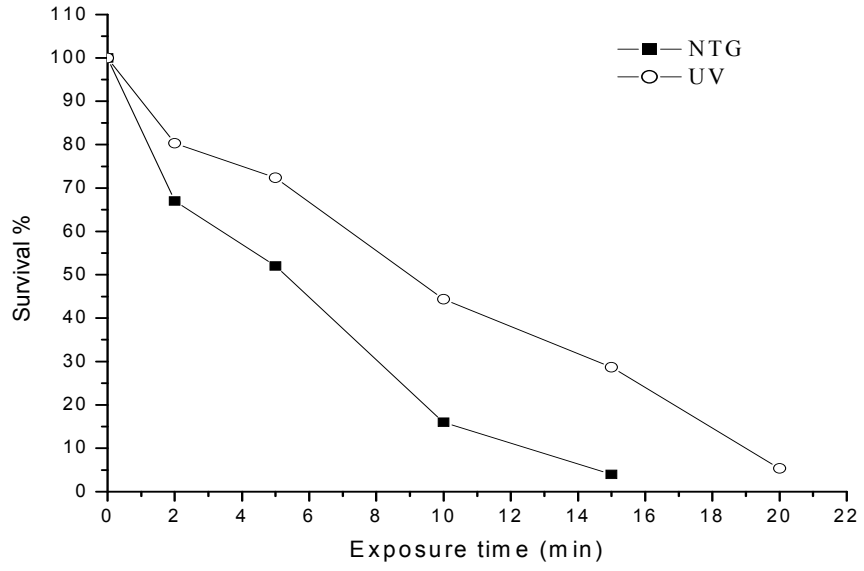


Fig. 4.18 Survival curve of *A. allahabadi* spores exposed to UV and MNNG

Table 4.9 Tannase production by mutants of *A. allahabadi*

Mutant / isolate	Dry cell weight, mg/ml	Tannase activity U/ml
UV 1	2.55	7.30
UV 2	2.48	6.84
UV 3	2.37	6.75
UV 4	2.81	7.59
UV 5	2.63	7.02
MNNG 1	2.79	7.57
MNNG 2	2.50	6.91
MNNG 3	2.77	7.61
MNNG 4	2.61	7.43
Wild	2.93	7.68

All isolates were grown in optimized medium containing tannic acid (20 g/L) at 280C for 48 h under shaking conditions

Similar results were reported by Purohit *et al.* (2006) for enhanced tannase and gallic acid production by mutagenesis in *R. oryzae* and *A. foetidus* cocultures. Mutations were carried out by treatment of heat (60^0C), UV (40 W) and MNNG (10 %) for various time period. The hyper producers based on faster growth and 5 % survival were selected as putative mutants. However similar to the observations in the present study, there were no hyper tannase producer obtained by UV and MNNG mutations. The stable mutant (for 13 generations) was achieved by 60 min heat treatment, showed 21 % enhanced tannase activity and 1.27 % increased gallic acid yield than that of wild cocultures.

In view of the results obtained, different approach to improve the strain for higher tannic acid utilization will be explored in further course of work.

CHAPTER 5

PURIFICATION, CHARACTERIZATION AND IMMOBILIZATION OF TANNASE FROM *A. allahabadi* AND BIOCONVERSION STUDIES USING IMMOBILIZED TANNASE

Introduction

Tannase (Tannin acyl hydrolase E.C.3.1.1.20) acts on acyl bond of hydrolysable tannins releasing glucose/ quinic acid and gallic acid. Number of fungi and bacteria were reported to produce tannase extracellularly. In the present study, *A. allahabadi* was found to produce intracellular tannase (0.14 U/mg) also.

This chapter presents the purification and characterization of extracellular tannase. The immobilization of extracellular tannase was carried out to evaluate its reusability in high tannin concentration. Further, immobilization of whole mycelia was also carried out as it offers possibility of advantage over immobilization of free tannase. Efficacy of both immobilizations was investigated.

Results and Discussion

5.1 Purification of tannase

Purification of tannase from *A. allahabadi* was carried out as described under materials and methods. Initially low titer broth was obtained by using shake flask. The broth was then further concentrated by using three different methods viz. precipitation by ammonium sulphate and acetone and cross flow filtration as described in Section 2.17. The specific activity and fold purification was 15.72 U/mg and 2.11 respectively when tannase was concentrated by fractional ammonium sulphate precipitation and subsequent dialysis.

Low molecular weight impurities were removed when crude enzyme was passed through 30 kDa membrane in tangential flow filtration system but the colour of enzyme concentrate became dark brown which interfered in the estimation of enzyme activity by spectrophotometer at 520 nm. Therefore, alternatively, precipitation with chilled acetone

(-20^{0}C) was carried out. Protein precipitation by chilled acetone retained 37 % of the original tannase activity with specific activity of 46.19 U/mg and 6.2 fold purification. The acetone precipitate was dissolved in citrate buffer (50 mM, pH 5.0) and subjected to ion exchange column chromatography on DEAE sepharose. The elution profile is given in Fig. 5.1. Total 45 fractions were collected. The active fractions (no. 26-29) having significant tannase activity were pooled and lyophilized. This step showed 20 fold

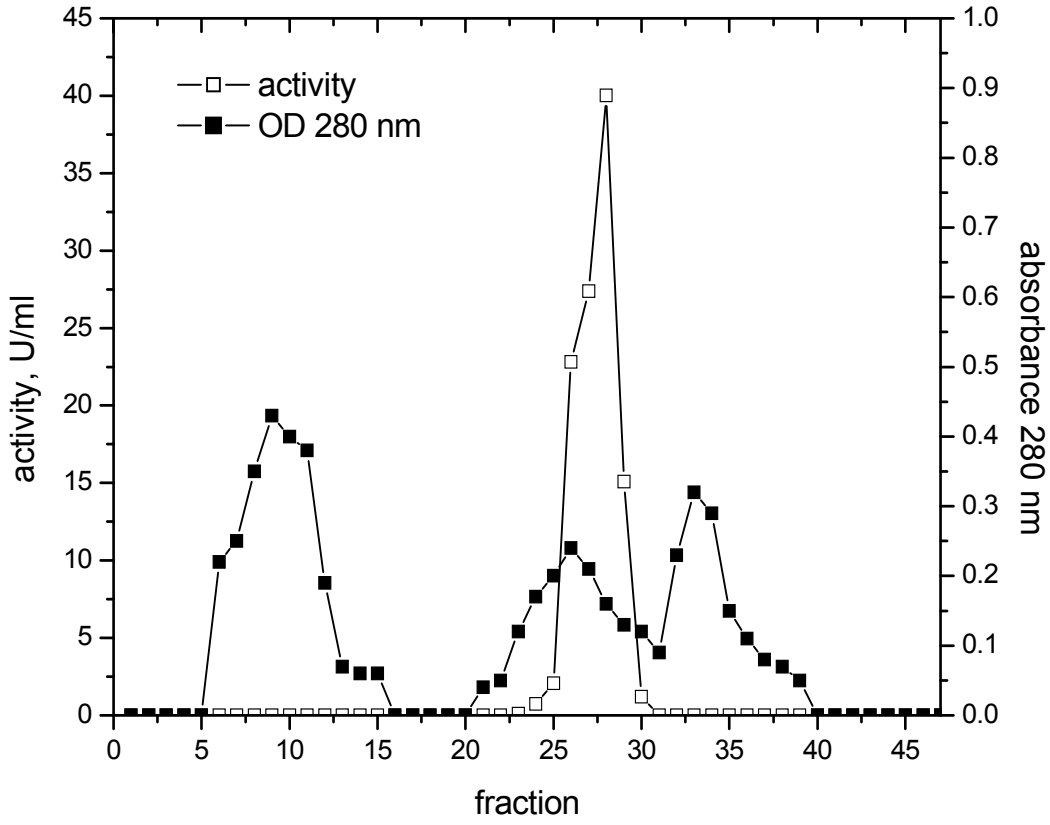


Fig. 5.1 Elution profile of tannase on DEAE sepharose column using 0-0.5 M NaCl

purification with specific activity 147.88 U/mg and 24.64 % yield (Table 5.1). Similarly the purification of *P. variotii* tannase, by ammonium sulphate precipitation followed by DEAE cellulose column chromatography showed fold purification of 19.4 but with higher yield of 56 % (Mahendran et al., 2006). Zhong *et al.* (2004) reported use of ultrafiltration followed by DEAE sepharose for purification of *A. oryzae* tannase. The purified tannase showed specific activity of 50 IU/mg of the protein. The enzyme was further purified by Sephadex G-200 filtration with 30.5 fold purification and 17% yield.

The purified fraction of specific activity of 147.88 U/mg was further subjected to native PAGE and found single band. Therefore this fraction was used for further studies.

Table 5.1 Summary of *A. allahabadi* tannase purification

Step	Total volume, ml	Total protein, mg	Total activity, U	Specific activity, u/mg	Fold purification	Yield, %
Cell free supernatant	172	189.2	1410.4	7.45	1	100
Acetone ppt	12	11.2	521.13	46.19	6.2	36.95
DEAE sepharose column	8	2.16	319.42	147.88	19.85	22.64

5.2 Characteristics of *A. allahabadi* tannase

5.2.1 Molecular weight

The PAGE of purified tannase was carried out as described under materials and methods section 2.18. Both native PAGE and SDS PAGE showed single band of purified tannase from *A. allahabadi*. The tannase was also analyzed for activity staining within the gel. Transparent band of tannase against opaque background of tannic acid- quinine hydrochloride complex confirmed the presence of tannase. The SDS PAGE revealed a single band with minimum molecular weight of 100 kDa (Fig. 5.2). Similarly *A. awamori* tannase was reported to be of 101 kDa molecular weight with single band on SDS PAGE (Chhokar et al., 2010) and *A. niger* tannase as monomeric protein of 168 kDa molecular weight (Sabu et al., 2005). Smallest active tannase of 90 kDa was reported along with its less active form of 180 kDa weight from *A. niger* (Ramirez-Coronel et al., 2003). However, *Verticellium sp.* was found to produce two types of tannases TAH I and TAH II, both of which were found to be oligomeric proteins consisting of two kinds of sub units with molecular weights of 39.9 and 45.6 kDa (Kasiesczka-Burnecka et al, 2007).

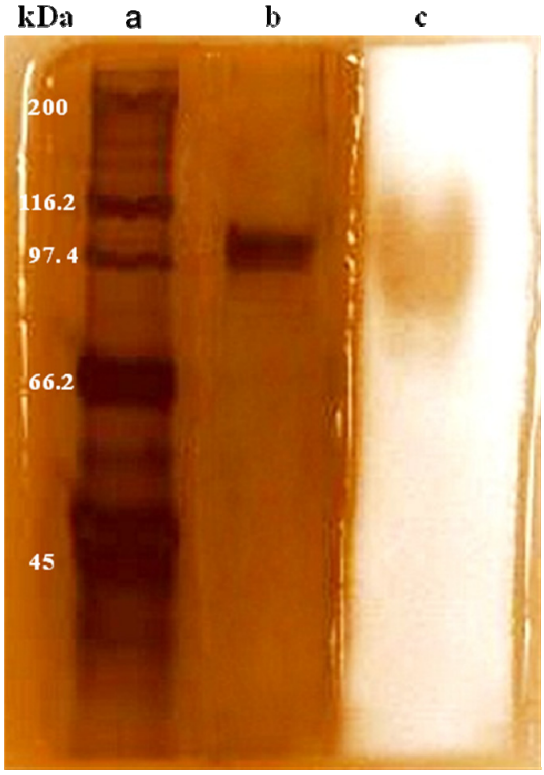


Fig. 5.2 SDS PAGE of a) molecular weight marker b) DEAE purified tannase of *A. allahabadi* and c) activity staining of tannase

5.2.2 Effect of pH on tannase activity and stability

The *A. allahabadi* tannase was found to have high activity at pH 5.0 and the activity substantially decreased as the pH increased in alkaline range (Fig. 5.3). This change could be due to enzyme inactivation at high pH. The stability of enzyme at different pH was carried out by incubating enzyme at different pH for 30 min. The residual tannase activity indicated the stability of enzyme over a pH range of 4.5 – 6.0. Similarly *A. awamori* tannase showed optimum activity at pH 5.0 (Mahapatra *et al*., 2005) and that of *A. niger* at pH 6.0 with stability over wide range of pH 4.0 – 8.0 (Sabu *et al*., 2005). Yamada *et al.* (1968) showed optimum pH of 5.0-5.5 and narrow range of pH stability (5.0-5.5) for *A. flavus*.

5.2.3 Effect of temperature on tannase activity and stability

The optimum temperature for *A. allahabadi* tannase was observed to be 35^0C while the thermo stability was from 25 – 45^0C (Fig. 5.4). The optimum temperature for tannase typically ranges between 30-50^0C (Mahapatra *et al*., 2005; Mahendran *et al*, 2006; Kasieczka-Burnecka *et al*, 2007; Sabu *et al*., 2005) however the tannase from

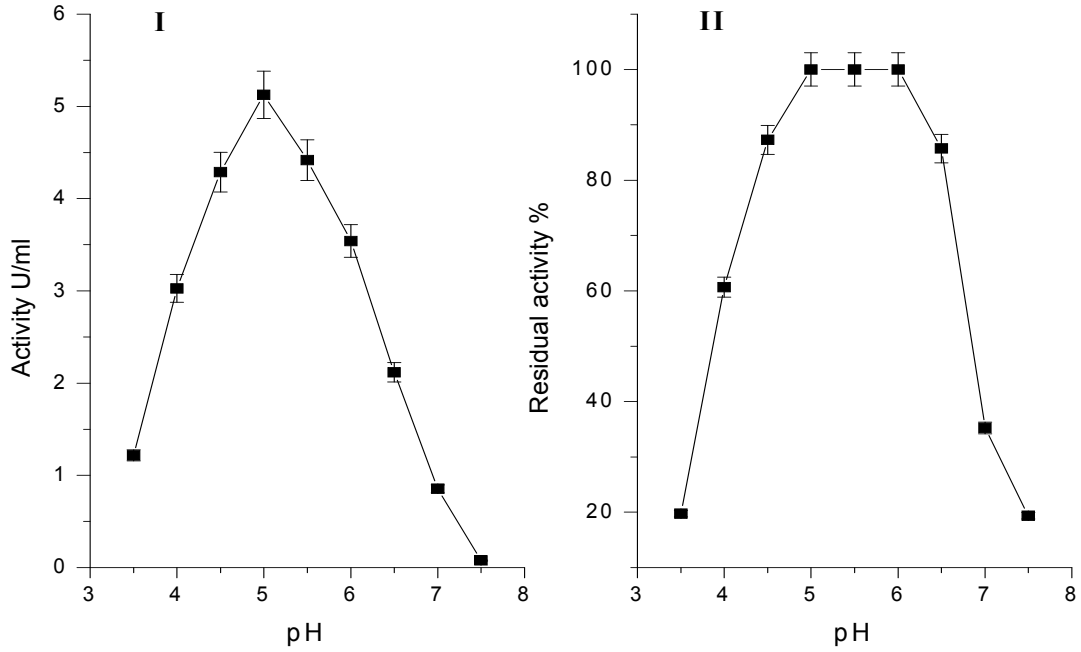


Fig. 5.3 The pH optima (I) and pH stability (II) of *A. allahabadi* tannase

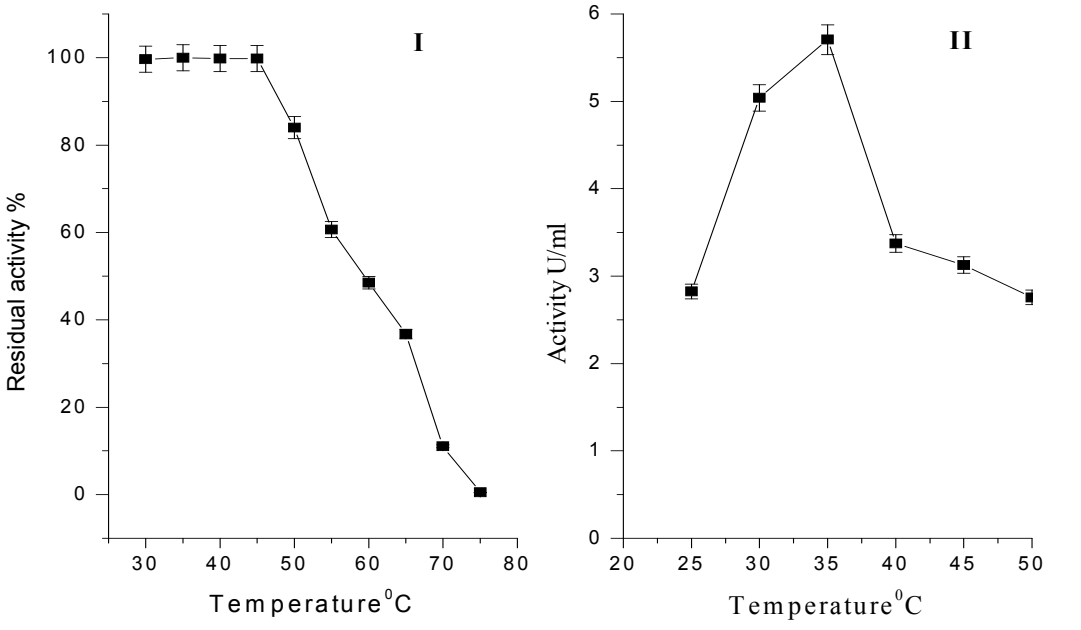


Fig. 5.4 Temperature stability (I) and temperature optimum (II) of *A. allahabadi* tannase

A. allahabadi tannase was incubated at different temperatures indicated for 30 min and residual activity was assayed under standard assay conditions for studying temperature stability of the enzyme.

Verticillium sp. was reported to have optimum temperature at 20^0C (Kasieczka-Burnecka *et al*., 2007). Tannase isolated from *Fusarium* was reported to have optimum temperature at 40^0C and relatively wider range of temperature stability (0^0 – 70^0C) (Hamdy, 2008). Similarly *P. variable* tannase was found to be stable in the broad temperature range 20-80^0C (Sharma *et al*., 2008).

5.2.4 Carbohydrate content

The carbohydrate content of *A. allahabadi* tannase was determined as described in 2.21 and was found to be 25.54 %. Tannases were reported to be glycoprotein in nature and the carbohydrate content varies from species to species. Carbohydrate content in *Verticillium* tannase I and II were reported to be 11 % and 26 % respectively (Kasieczka-Burnecka *et al*., 2007). Mahapatra *et al*. (2005) have documented carbohydrate content of 14.2 % for *A. awamori* nakazawa tannase. The higher % of carbohydrate may be responsible for the hydrophilicity of the enzyme.

5.2.5 Effect of metal ions

Different metal ions (Ca^{2+}, K^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Mn^{2+}, in the form of chloride salts) were dissolved in citrate buffer (50 mM, pH 5.0) to achieve effective concentration of each at 2 mM. Purified tannase was incubated in buffered salt solution for 30 min at 25^0C and assay was carried out under standard assay conditions to check the effect of metal ions.

Fig. 5.5 shows the effect of metal ions on tannase activity of *A. allahabadi*. This tannase certainly demonstrated metal ion effect different from other tannases wherein there was no loss of activity in presence of metal ions except mercury. *A. allahabadi* tannase was found to be considerably inactivated by Hg^{2+}. Similar observations were reported for *Lactobacillus plantarum* tannase. The reaction of mercury ions to protein thiol group and also with histidine and tryptophan groups were discussed by the authors as the probable reasons of considerable inactivation of the tannase in presence of mercury (Rodriguez *et al*., 2008a). However, Kar *et al.* (2003) showed 1 mM Hg^{+1} activated the tannase activity. Addition of magnesium and zinc ions increased the *A. allahabadi* tannase activity by 45 % and 27 % respectively as compared with that of the control in the present study. Similarly addition of magnesium increased tannase activity of *R. oryzae* enzyme by 38 % than that of the control (Kar *et al*., 2003). Authors have attributed the

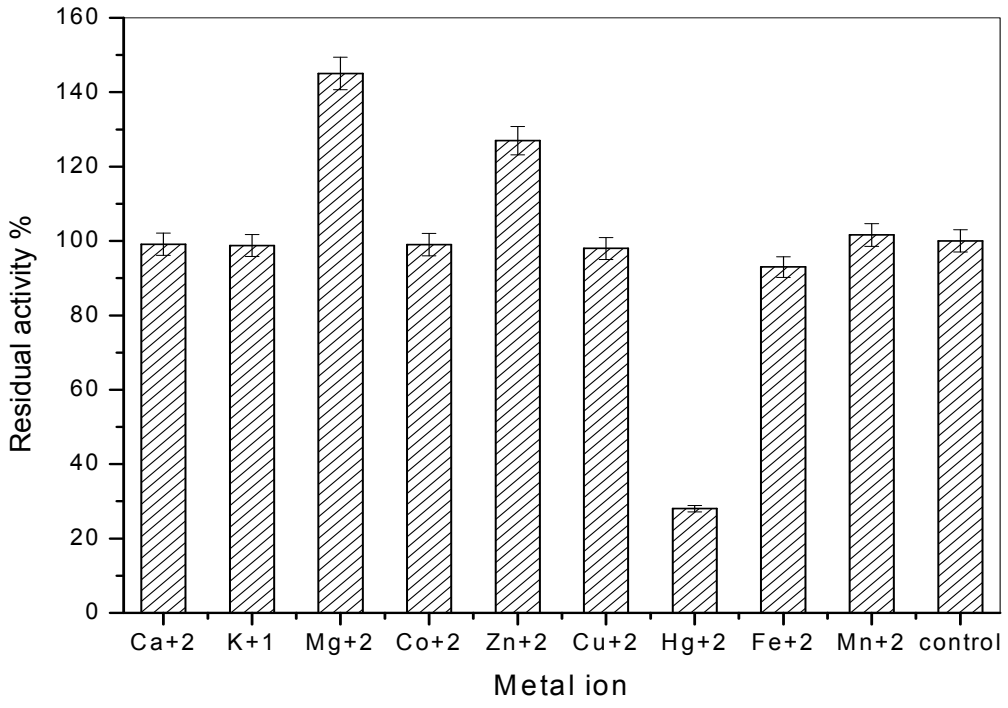


Fig.5.5 Effect of metal ion on tannase activity of *A. allahabadi*

The enzyme was incubated with metal ions (2 mM) for 30 min and the residual activity was determined using standard assay conditions.

increase or decrease in activity due to non specific ionic effects such as binding or aggregation of the enzyme. On the contrary, *A. niger* tannase was inhibited by addition of zinc (Kar *et al*., 2003; Sabu *et al*., 2005). Similarly Rajakumar and Nandy (1983) have reported the inhibition of *P. chrysogenum* tannase by addition of zinc. Metal ions such as Ca^{2+}, K^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+} did not affect the activity of *A. allahabadi* tannase while Fe^{2+} marginally decreased (93 %) the activity. The *R. oryzae* tannase was found to be partially inhibited by addition of ions such as Ca^{2+}, Hg^{2+}, Zn^{2+} and Ag^{2+} while Fe^{2+} and Co^{2+} were completely inhibited the tannase activity at 1 mM concentration (Kar *et al*., 2003). *Verticillium* tannase was also found to be partially inhibited by 5 mM concentration of Ca^{2+}, K^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+} while Co^{2+}, Pb^{2+}, Ag^{+} and Fe^{3+} completely inhibited the activity. Mg^{2+} was the only metal ion which stimulated the tannase activity by 23 % than that of control (Kasieczka-Burnecka *et al*., 2007).

5.2.6 Effect of EDTA

The effect of chelator, EDTA on *A. allahabadi* tannase was studied at concentrations ranging from 1mM to 5 mM. The tannase (20 U) was incubated in the EDTA for 30 min. The residual tannase activity was checked under standard assay conditions. The tannase activity was found to be partially inhibited (36 %) at 1 mM EDTA while there was complete inhibition of tannase activity at concentration 2 mM and above. Kar *et al.* (2003) reported 29.68 % inhibition of *R. oryzae* tannase by 1 mM EDTA. *A. niger* tannase activity was reduced by 20 % in presence of 0.1 % EDTA (Mata-Gomez *et al*., 2009). Loss of activity in presence of EDTA may be due to change in pH of the buffer used for the assay or due to conformational change in the enzyme due to chelating of the metal ion. This could be an interesting future work.

5.2.7 Effect of surfactants

Tannase activity in presence of different ionic / anionic surfactants was studied by incubating purified *A. allahabadi* tannase in different surfactants of concentrations 0.05 % and 0.5 % (w/v) at 25^0C for 30 min. As it is seen from the residual activity in Table 5.2 tannase activity was observed to be unaffected by anionic surfactants such as Tween 60 and Tween 80 at 0.05 % concentration. Tween 80 is predominantly composed of oleic acid (70%) while Tween 60 consists of stearic acid (50%) balanced primarily with palmitic acid. Though both the surfactants have different compositions, they did not affect the activity of *A. allahabadi* tannase. Another anionic surfactant, SDS at 0.05% caused inhibition of tannase activity. This inhibition may be the result the combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and the direct interactions with the protein molecule (Kar *et al*., 2003). Tween 20 is a polysorbate surfactant, a polyoxyethylene derivative of sorbitan monolaurate and is distinguished from the other members in the Tween range by the length of the polyoxyethylene chain and the fatty acid ester moiety. This surfactant also did not affect the activity of *A. allahabadi* tannase. At 0.05 % concentration a non ionic surfactant Triton X-100 was found to inhibit the activity partially. All surfactants inhibited tannase activity when concentration was at 0.5 %. The inhibition by Triton X 100 was an interesting observation because the inhibition by non ionic surfactant may not be unusual but surely not common. That may be due to expansion in size of the enzyme, which may be a part of future work. Similar findings

were documented where *A. awamori* tannase activity was found to be inhibited with increasing concentration of SDS (1-5 %) where at 5 % it was completed inhibited (Mahapatra *et al*., 2005). On the contrary Tween 80, Tween 60 and Tritron X-100 were found to be partially inhibitory at 0.05 % concentration for *Verticillium* tannase (Kasieczka-Burnecka *et al*., 2007). Mata-Gomez *et al.* (2009) have reported no significant effect on tannase activity of *A. niger* when enzyme was incubated with 0.1 % of Tween 20, Tween 80, Triton X-100 and SDS for 60 min.

Table 5.2 Effect of surfactants on *A. allahabadi* tannase

Surfactant	Concentration, % (v/v)	Residual tannase activity, %
Control	--	100 ± 1.1
Tween 20	0.05	99.01 ± 1.98
	0.5	15 ± 0.27
Tween 60	0.05	100 ± 1.78
	0.5	8.14 ± 0.15
Tween 80	0.05	100 ± 1.9
	0.5	22.50 ± 0.42
Triton- X 100	0.05	74 ± 1.55
	0.5	11 ± 0.24
SDS	0.05	82 ± 1.52
	0.5	19.54 ± 0.44

Control was without addition of surfactant

5.2.8 Effect of organic solvents

In living systems enzymes function in aqueous medium, so most of the enzyme studies are conducted in the aqueous buffered systems. In case of application of enzymes in biotechnology, there are many advantages of conducting enzymatic conversions in organic solvents over that of in water, viz. high solubility of most of the organic compounds in organic media, ability to carry out new reactions impossible in aqueous medium because of kinetic or thermodynamic restrictions, greater stability of enzymes, relative ease of product recovery from organic solvents as compared to water and insolubility of enzymes in non aqueous media (Zaks and Klibanov, 1985). Tannase has many potential applications in biotechnology and considering this potential, effect of organic solvents on *A. allahabadi* tannase was studied.

Effect of organic solvents on tannase activity was studied by incubating purified *A. allahabadi* tannase at 25^0C for 30 min in organic solvents such as acetone, methanol, ethanol, propanol, tetrahydrofuran (THF) and formaldehyde. Proportion of 1 ml of *A. allahabadi* tannase having 19 U activity in 1 ml of solvent (1:1) was used. The residual activity was determined under standard assay conditions. The compatibility of tannase with solvents was relevant for the preparation of gallates using gallic acid and alcohols such as ethanol, methanol and propanol in organic medium having low water content (Sharma and Gupta, 2004).

Table 5.3 Effect of organic solvents on *A. allahabadi* tannase

Solvent	**Residual activity, %**
Control	100 ± 1.2
Acetone	100 ± 2.2
Methanol	25.23 ± 0.58
Ethanol	75.12 ± 2.70
Propanol	85.0 ± 1.69
THF	36.0 ± 0.13
Formaldehyde	30.5 ± 0.70

In the present study, acetone did not affect the activity of *A. allahabadi* tannase. The acetone was used in 1:1 proportion and at this concentration no precipitation was observed. In presence of propanol, the recovery of tannase was around 80 % of the initial activity. Methanol, tetrahydrofurane (THF) and formaldehyde were observed to inhibit the tannase activity significantly (Table 5.3). On the contrary, Chhokar *et al.* (2010) reported increase in *A. awamori* tannase activity by butanol and benzene where the activity was increased by 27 – 99 % on incubating enzyme with these solvents. In another study on *A. niger*, heptane and petroleum ether were observed to activate the enzyme activity on incubating enzyme with 20 % (1:5) and 60 % (3:2) solvent concentration for 60 min whereas acetone, ethanol, tetrahydrofurane and formaldehyde found to be partially inhibitory (Mata-Gomez *et al*., 2009). The effect of the organic solvents alters the native structure of the proteins by disturbing the hydrophobic interactions between the non polar side chains of the amino acids (Asakura *et al*., 1978). This effect may be different for different solvents on the enzymes and hence the results

observed were varied with the source of the enzyme. The present investigation showed the compatibility of *A. allahabadi* tannase with solvents viz. acetone, propanol and ethanol.

5.2.9 Kinetic analysis of tannase by *A. allahabadi*

The kinetic properties of *A. allahabadi* tannase were determined using tannic acid and methyl gallate substrates (section 2.21). The enzyme showed higher affinity towards tannic acid with K_m value of 2.96 $x10^{-3}$ mM while that of methyl gallate was 1.21 mM (Figs. 5.6 and 5.7). The K_{cat} values for tannic acid and methyl gallate were 1.21 $x10^5$/min and 5.4 x 10^2/min respectively (Table 5.4).

Table 5.4 Kinetic constants of *A. allahabadi* tannase using tannic acid and methyl gallate

Substrate	K_m (mM)	V_{max} (μM/ml/min)	K_{cat} (min^{-1})	K_{cat}/K_m ($min^{-1}mM^{-1}$)
Tannic acid	2.96 x 10^{-3}	1.21	1.21 $x10^5$	4.08 $x10^7$
Methyl gallate	1.21	5.49 $x10^{-3}$	5.4 $x10^2$	4.46 $x10^2$

The K_{cat} (catalytic constant) defines the rate constant of the enzyme substrate complex once formed, to form product. It is also called as turnover number. It defines the number of catalytic cycles (turnovers), the enzyme can undergo in unit time or the number of molecule of substrate that one molecule of enzyme can convert into product in one unit time (Cornish-Bowden, 2003). The Turnover number can be calculated as V_{max} / $[E]_t$ where $[E]_t$ is micromoles of enzymes per ml. The specificities of the enzyme towards the substrate were calculated by the ratio of K_{cat}/Km which was 4.08 $x10^7$ $min^{-1}mM^{-1}$ for tannic acid and 4.46 $x10^2$ $min^{-1}mM^{-1}$ for methyl gallate. The K_{cat}/K_m values showed that tannic acid was the preferred substrate over methyl gallate. Higher Kcat implies very low catalytic time. Catalytic time in case of tannase catalyzed reaction signifies time required for hydrolysis reaction of one acyl bond in substrate. In case of tannic acid, catalytic time was calculated as 8.26 $x10^{-6}$ min and that in case of methyl gallate was 1.8 $x10^{-3}$ min. In the present study, utilization of higher tannic acid concentration by *A. allahabadi* in shorter period (50 % tannin was utilized in first 16 h) can be explained on the basis of high turnover number of tannase.

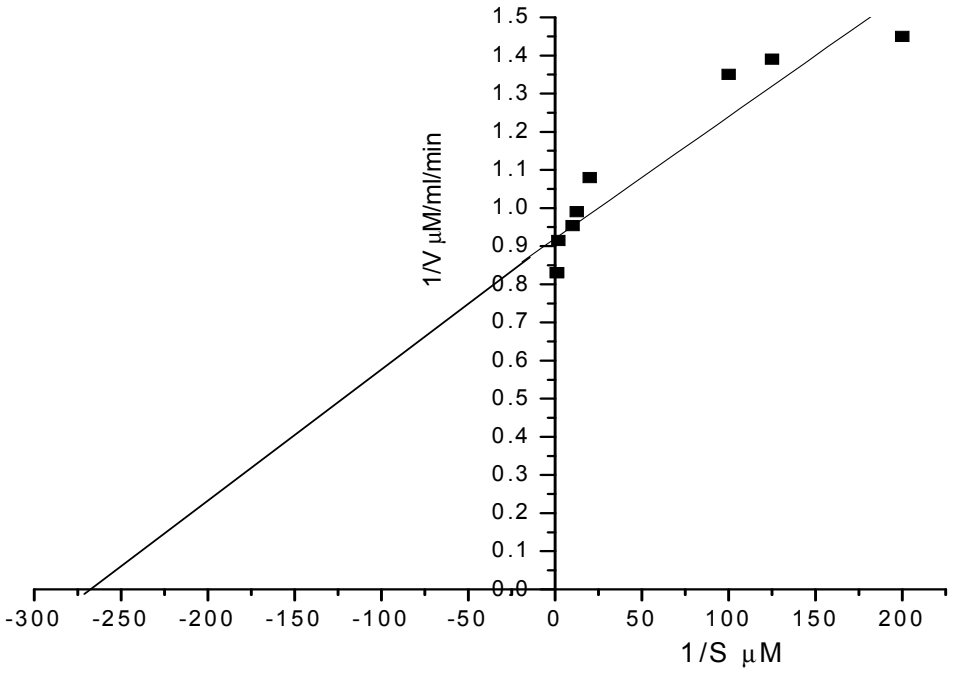


Fig. 5.6 Kinetic constants of *A.allahabdi* tannase using tannic acid (Lineweaver-Burk plot)

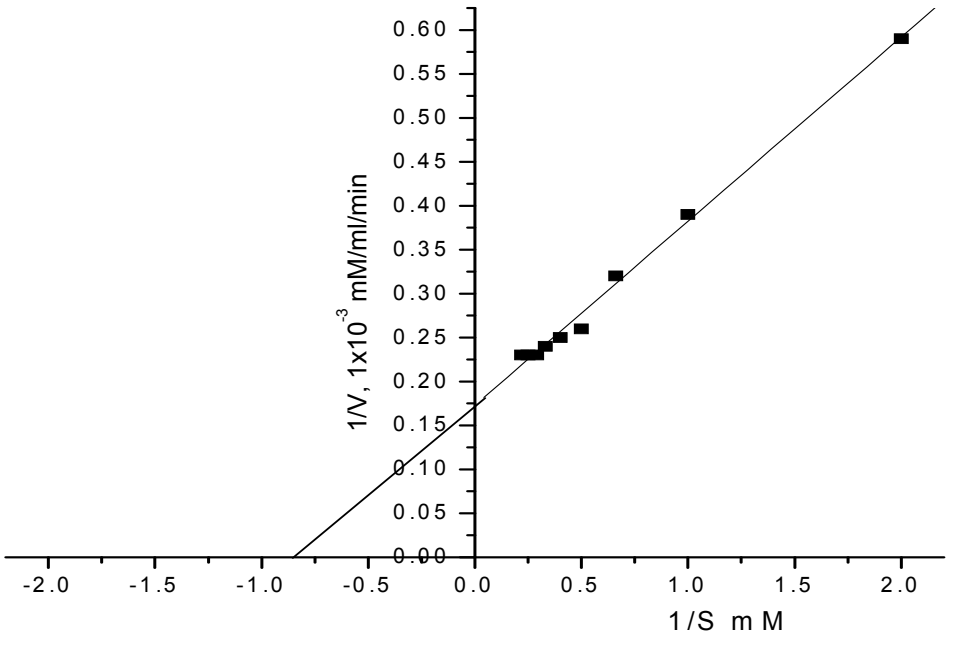


Fig. 5.7 Kinetic constants of *A.allahabdi* tannase using methyl gallate (Lineweaver-Burk plot)

Table 5.5 Summary of yield of *A. allahabadi* tannase immobilization

Immobilization	Enzyme added (A)	Activity of immobilized enzyme (B)	Specific activity U/mg	% yield (B/A x 100)
Entrapment				
Ca alginate	100 U	15.39 U/g	56.19	63.12
κ carageenan	100 U	6.77 U/g	29.38	24.72
Covalent binding				
Chitosan	100 U	12.6 U/g	26.64	12.6

Similarly *Verticillium* tannase showed temperature dependant (5^0C-30^0C) kinetic parameters for tannic acid and methyl gallate. It showed lower K_m for tannic acid (0.45 mM) than that of methyl gallate (1.44 mM) and the K_{cat}/K_m (efficiency of hydrolysis) was higher (1.31 x10^5 $min^{-1}mM^{-1}$) for tannic acid than that of methyl gallate (3.1 x10^4 $min^{-1}mM^{-1}$) (Kasieczka-Burnecka, 2007). In the study of *Fusarium* tananse, the kinetic constants were shown to have the values of V_{max}, K_m and K_{cat} for tannic acid as 0.116 μM/ml/min, 3.57 mM and 1.16 min^{-1} respectively (Hamdy, 2008). Higher values of V_{max} were also found for tannic acid in case of *P. variable* (1.11 uM/ml/min) and *P. variotii* (0.55 uM/ml/min) (Battestin and Macedo, 2007a; Sharma *et al*., 2008).

5.3 Immobilization of tannase

Immobilization of purified *A. allahabadi* tannase was carried out by two methods viz. entrapment using Ca alginate and k carageenan and covalent binding using chitosan. The immobilization was carried out as described under material and methods section 2.22. Entrapment of tannase in Ca alginate and in κ carageenan resulted in 3-4 mm compact beads. The beads were washed with citrate buffer (50 mM, pH 5.0) and kept at 4^0C till further use. Concentration of sodium alginate in the initial solution was observed to affect the bead properties. Though the higher polymer concentration in beads may give higher stability, it causes less diffusivity and hence low rate of reaction. The beads prepared with 3 % sodium alginate were fragile and loosely formed while that with 4, 5 and 6 % sodium alginate were non fragile and compact beads. There was no significant difference in the tannase activity of beads prepared with 3 % and 4 % sodium alginate concentration while beads with 5 % and 6 % sodium alginate showed less tannase activity (18 % and 23 % respectively) than that at 3 and 4 % sodium alginate. Table 5.5

shows yield of immobilized tannase by different methods. In this study, all these aspects were considered and the better immobilization system was finalized based on stability with respect to reusability and better rate of catalysis. Similarly, immobilization of *R.oryzae* tannase was done by using calcium alginate entrapment technique. The immobilized system was used for seven cycles and the efficiency dropped down to 70% after that (Hota *et al*., 2007).

A. oryzae tannase was immobilized by using micro encapsulation using Na alginate cross linked with glutaraldehyde. Immobilized tannase retained hydrolytic activity through successive batch cycles for total period of 39 h of processing (Boadi and Neufeld, 2001). Highest immobilized *A. allahabadi* tannase activity (15.39 U/g) and yield (63.12 %) was obtained when tannase was entrapped in Ca alginate. Thus Ca alginate immobilized tannase was used for further studies.

5.3.1 Characterization of immobilized tannase

Characters of immobilized *A. allahabadi* tannase viz. pH and temperature were studied. Effect of pH was studied by incubating immobilized tannase in buffers of different pH values at 25^0C for 30 min and that of temperature was studied by incubating immobilized tannase at different temperatures 20^0C. The Ca alginate beads of immobilized *A. allahabadi* tannase were not stable at lower pH. With the lowering of the pH below 3.5, there was apparent loss in the enzyme activity due to disintegration of the Ca alginate beads. This was checked by estimating the residual activity in the supernatant. The optimum pH of for free *A. allahabadi* tannase was at 5.0. Immobilized tannase did not show any change in optimum pH and was same as that of free tannase (pH 5.0). The stability of immobilized enzyme was observed over a slightly broader pH range (4.0 – 6.5) as compared to that of free enzyme (4.5-6.0). The optimum temperature of free tannase was at 35^0C whereas that of immobilized tannase was shifted to 40^0C. The increase in optimum temperature could be due to increased thermal stability. The thermal stability of immobilized tannase was observed to be over a broad temperature range from 20^0C – 60^0C. It could also retain around 80 % activity at 70^0C (Figs 5.8 and 5.9).

Su *et al.* (2009) immobilized *A. ficuum* tannase in calcium alginate which showed that optimum temperature of tannase was reduced by 10^0C to 35^0C. The immobilized tannase was stable from 20 to 40^0C and the activity was lost thereafter. pH stability of immobilized enzyme was 5.0. *R. oryzae* tannase immobilized in calcium alginate beads

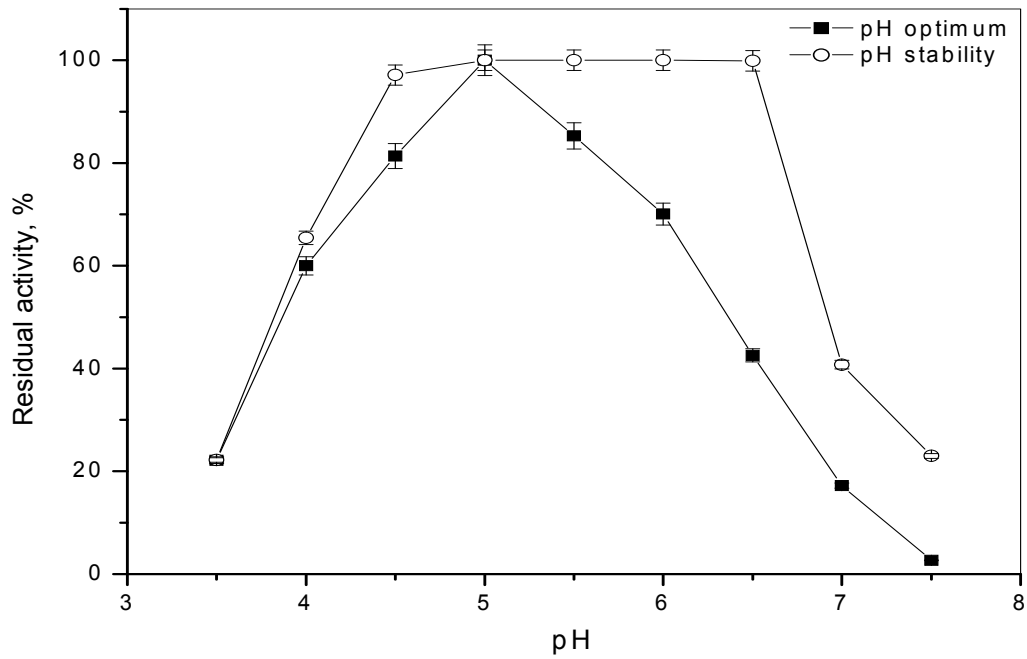


Fig. 5.8 Optimum pH and pH stability of immobilized tannase

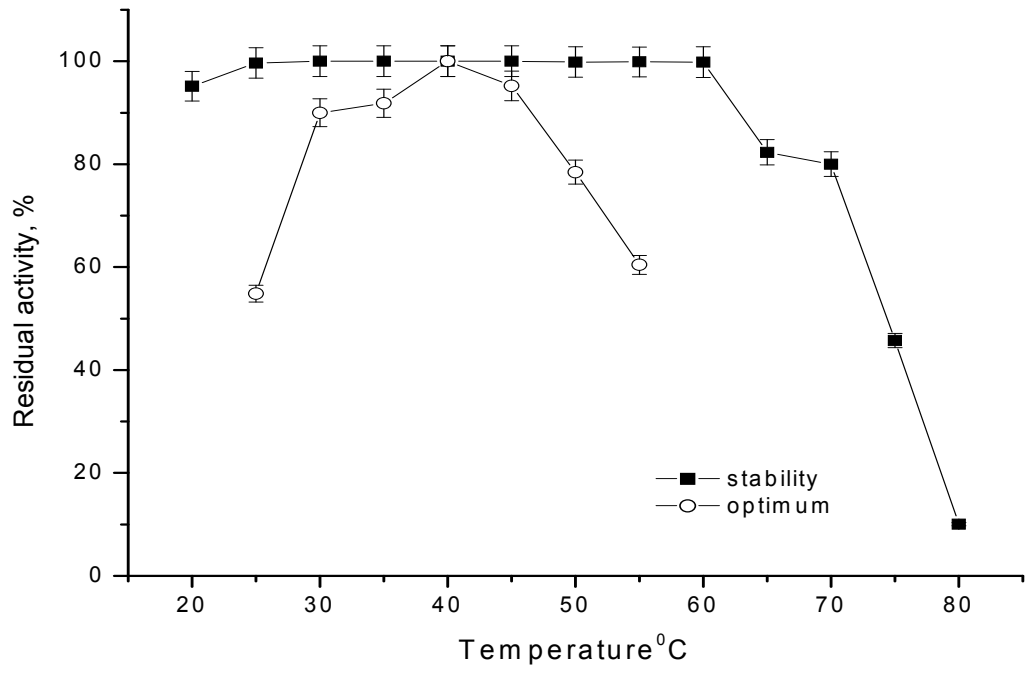


Fig. 5.9 Optimum temperature and temperature stability of immobilized tannase

showed shift in optimum temperature from 40^0C to 50^0C and the immobilized tannase was stable over temperature range of 20 to 60^0C. The optimum pH of the immobilized

tannase shifted to pH 4.5 from pH 5 and the tannase was stable over the narrow range of pH from 4 to 5 (Hota *et al.*, 2007).

5.4 Bioconversion of tannic acid using immobilized tannase

Immobilized tannase was studied for its efficiency of conversion of tannic acid to gallic acid. The reaction was carried out for 12 h at two different temperatures viz. 30^0C and 40^0C under shaking conditions with periodic sampling at every 2 h. Samples were checked for residual tannic acid and gallic acid production. The residual enzyme activity was determined after each cycle. The immobilized tannase retained the shape of beads when incubated in tannic acid of various concentrations ranging from 50 g/L to 400 g/L. The bioconversion efficiency was higher at 40^0C than that at 30^0C. Higher efficiency achieved is due to higher conversion in given incubation time. The rate constant of a reaction is higher at higher temperature as postulated by Arrehenius equation. Efficiency of bioconversion (93.70 %) was maintained till 5th cycle after which it was reduced to 60 % by 9th cycle (Fig 5.10). In first 5 cycles, highest bioconversion was observed in 6 – 8 h after which no significant tannic acid was left unreacted. The disappearance of tannic acid was monitored by HPLC. The tannase retained 86 % of its initial activity at the end of 9th cycle. Around 80 % gallic acid accumulation was observed till 5th cycle. In an

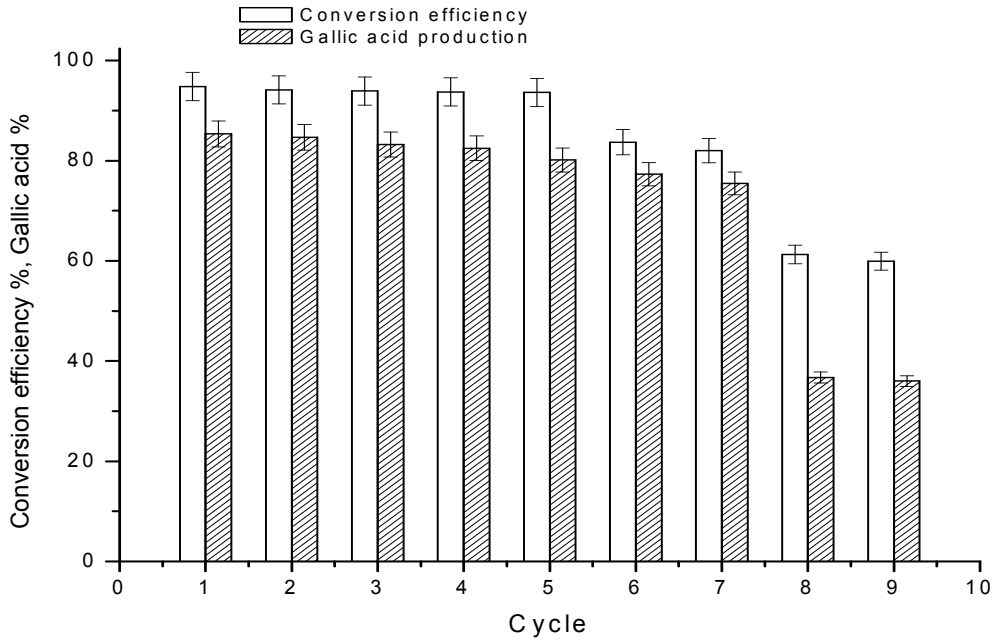


Fig. 5.10 Bioconversion of tannic acid (400 g/L) by immobilized tannase

experiment carried out using free *A. allahabadi* tannase, there was significant activity loss in first 60 min. This indicated that the enzyme stability was increased due to immobilization and it also was able to hydrolyze the tannic acid efficiently at high concentrations.

There are no reports available on the operational stability of immobilized tannase in high tannin (above 50 g/L) concentrations. Many researchers have carried out studies on immobilization of tannases using various techniques. Entrapment was reported to be most preferred technique for tannase immobilization (Hota *et al*., 2007; Su *et al*., 2009). Guo and Yang (2000) entrapped tannase in calcium alginate beads and used the immobilized preparation for the hydrolysis of chinese gallotannin to gallic acid. Under optimized conditions, the immobilized enzyme yielded 61% gallic acid. Abdel-Naby *et al*. (1999) immobilized *A. oryzae* tannase by several methods viz. adsorption, ionic binding, covalent binding and entrapment and evaluated the properties of the immobilized enzymes. Among the immobilized preparations, tannase bound covalently to chitosan exhibited highest efficiency (26.6%). Chitosan- tannase adduct showed a decrease in its optimum pH towards acid side (4.5) as compared to that of the soluble enzyme (pH 5.5) and this was attributed to the cationic nature of the matrix. Chitosan-tannase-conjugate exhibited good stability to repeated use in 20 g/L tannic acid and retained 85% of its initial activity after 17 cycles. *P.variable* tannase when adsorbed onto Amberlite IR-1204 showed highest efficiency (69%) than with XAD-7, DEAE-cellulose, celite and silica gel as immobilization matrices. The bound enzyme showed good stability to repeated use and retained 85% of its initial activity after 9 cycles (Sharma et al., 2008). *A. niger* tannase microencapsulated in liquid core alginate gels showed an efficiency of 36.8%. Encapsulation brought about an increase in the optimum pH and temperature to 6.0 and 40^{o}C respectively, from that of the soluble enzyme (pH 5.0 and 30^{o}C) (Yu *et al.,* 2004). Mahendran *et al.* (2006) entrapped *P. variotii* tannase in calcium alginate beads and used the immobilized system to study the hydrolysis of tannic acid. Time course of tannic acid hydrolysis showed an increase in the rate of hydrolysis with increase in the incubation time. Optimum hydrolysis (98%) was observed after 6 h of incubation. The bound enzyme was stable and retained approximately 85% of its initial activity after 8 cycles.

The results obtained with *A. allahabadi* showed significantly improved performance in terms of parameters viz. conversion efficiency of high tannin concentrations, reusability, stability towards both pH and temperature.

5.5 Bioconversion using immobilized whole mycelia of *A. allahabadi*

Immobilization of whole cells was carried out as described in 2.23. The obtained beads were of 4-5 mm in size. The yield of immobilized mycelia was 41.57 % (of 140 U initial activity) and the activity was 13.47 U/g. The immobilized mycelia were further studied for reusability for hydrolysis of tannic acid. The immobilized mycelia were incubated in various concentrations of tannic acid (20 g/L – 400 g/L) for 12 h. Periodic samples at every 2 h were drawn and checked for residual tannic acid and gallic acid accumulation. The residual enzyme activity was determined after each cycle. The tannic acid conversion efficiency was reduced to 50 % in 1st cycle when the tannic acid concentration was 400 g/L while it was 92 % at the end of the 1st cycle in 200 g/L (Fig. 5.11). This may be due to limitations to diffusion of substrate and in turn mass transfer. Hence operational stability was carried out using 200 g/L tannic acid at 30^{0}C under shaking conditions. Though free lyophilized mycelia were capable of conversion of

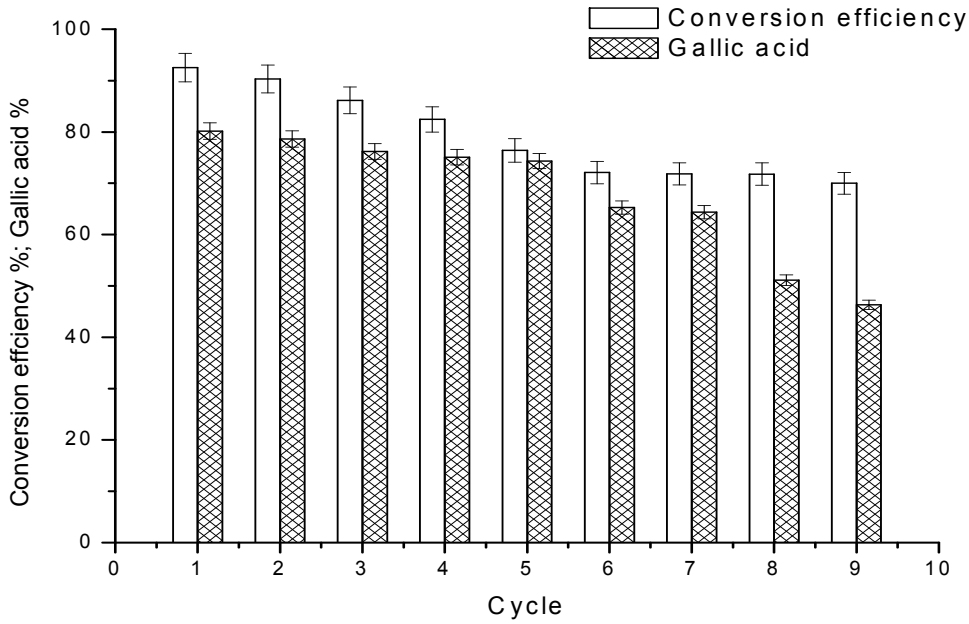


Fig. 5.11 Bioconversion of tannic acid (200 g/L) using immobilized mycelia.

The lyophilized mycelia were immobilized by entrapment in Ca alginate and then tested for the bioconversion of tannic acid to gallic acid for ten cycles (each of 12 h).

tannic acid (200 g/L) to gallic acid, the efficiency was observed to be 78.5 % at the end of 12 h in the first cycle and further incubation showed growth of mycelia resulting into increase in biomass by the end of 24 h. Efficiency of tannic acid bioconversion by immobilized mycelia slowly decreased to 76 % from 1st to 5th cycle and did not reduce significantly further. Gallic acid accumulation was highest (80 %) up to 2nd cycle and was around 74-76 % till 5th cycle while less gallic acid accumulated from 6th (65 %) to 9th cycle (46 %). The volumetric productivity of gallic acid using entrapped mycelia was 3.33 g/L/h which were lesser than the productivity obtained from the lyophilized free mycelia which was 13 g/L/h however the entrapped mycelia showed excellent reusability and storage stability which offers advantage over lyophilized free mycelia.

Very few researchers have carried out studies on immobilization of whole mycelia mostly using Ca alginate entrapment technique. Bajpai *et al.* (1999) have reported data obtained with immobilized mycelia of *Aspergilli, Fusarium* and *Trichoderma*. The immobilization was carried out by entrapping spores in Ca alginate beads. The beads were then incubated on shaker at 30^{0}C for 48 h to obtain entrapped mycelia. The maximum operational stability study was observed in immobilized mycelia of *A. niger* with no loss of activity and *A. fischerii* with 7.5 % loss in activity after 8 cycles. However highest hydrolysis of gallotannin (10 g/L, pH 5.0) was observed in *F. solani* (50.4 %) and *T. viridae* (51.1 %) at 35^{0}C and 45^{0}C after 3 h and 1 h respectively. Immobilized cells (2x10^5 spores) of *R. oryzae* in calcium alginate were reported to show maximum gallic acid formation in 20 g/L tannic acid. The conversion of tannic acid by immobilized cells was 78.5 % while it was higher (83.5 %) in free cells when incubated for 4 d. The immobilized cells were successfully used for 3 cycles (Misro *et al.*, 1997).

5.6 Bioconversion using recycled mycelia of *A. allahabadi*

The results obtained with immobilization of whole mycelia indicated efficient conversion high tannic acid concentration (80 % in first 5 cycles) and greater reusability. In another experiment at shake flask, 36 h old (late exponential growth phase) *A. allahabadi* biomass was harvested and washed thrice with sterile distilled water to remove any traces of tannic acid. The wet biomass (0.5 g/L dry weight) was inoculated in fresh tannic acid (200 g/L). The flask was incubated at 28^{0}C at 150 rpm for 48 h. Periodic samples were checked for residual tannic acid, gallic acid content, tannase activity and dry cell weight. For each cycle, biomass from previous cycle was harvested, washed and inoculated in

fresh tannic acid (200 g/L). Enzyme activity was found to be highest at 36 h and conversion of tannic acid was up to 90 % in 32 h in first two cycles. Moreover the biomass was increased by 18 %. Though biomass was observed to be increased in 3rd and 4th cycle, bioconversion efficiency was 74 % and 69 % respectively. The dry cell weight decreased after 5th cycle along with significant decrease in bioconversion efficiency. The recycling of mycelial biomass could be a good alternative for bioconversion of tannic acid at large scale to make the fermentation cost effective. With the recycling of the biomass the productivity increased three fold. The biomass of one batch can be used for three cycles and this may save the significant time of seed preparation and power input for aeration.

There are no reports in published literature seen on biomass recycle for tannic acid hydrolysis and gallic acid production. However, there are very few reports available of the recycling of the fungal biomass for the production of industrially important products. For instance, Schrickx *et al.* (1993) reported recycling of biomass of glucoamylase producing *A. niger* N 402 wild type as well as a glucoamylase overproducing transformant. After first recycling and about 150 h thereafter, the total of glucoamylase produced was increased. After this time no increase in the amount of biomass was observed. Authors suggested possibility of autolysis and cryptic growth taking place in a recycling fermenter where cell death rate equaling cell growth rate. Sampaio *et al.* (2005) reported recycling of *Debaramyces hansenii* biomass for four times for converting D xylose to xylitol without addition of additional nitrogen. The culture was reported to become unstable after four recycles because of lack of nitrogen. Whereas reduction in xylitol formation from 78% in first recycle to 18% in the fourth recycle was reported. It was postulated that recycling of fungal biomass promoted persistence and a recycling was relevant for those fungi that reside in a resource of patchy and limited environment (Falconer *et al*., 2007).

CHAPTER 6

CONCLUSIONS

Tannins are polyphenolic secondary metabolites of higher plants (such as chestnut, Oak wood, Trillo, Valonea, Myrobalan, Divi-Divi), classified as hydrolysable and non hydrolysable tannins (condensed tannins) (Khanbabee and Van Ree, 2001). They are considered as the classic defense compounds of plants against herbivores and have a broad spectrum of distribution in the plant kingdom which includes pteridophytes, gymnosperms and angiosperms and generally accumulate in large amounts in roots, bark, leaves and fruits (Frutos *et al*., 2004; Scalbert, 1991). The biosynthesis of hydrolysable tannins in higher plants was reported by coupling of a polyphenolic building block - gallic acid with diverse polyols such as D-glucopyranose (Khanbabee and Van Ree, 2001). Tannins are widely used in leather tanning and also have application in textile dyes, ink production, antioxidants in foods and coagulants in rubber production. Tannins thus constitute a major constituent of waste of these industries.

Complete enzymatic degradation of hydrolysable tannin involves hydrolysis by tannin acyl hydrolase, commonly known as tannase to gallic acid and/ or ellagic acid. Gallic acid is an industrially important molecule having wide applications from healthcare and food to dyes, inks, paints and photography. Recent studies showed gallic acid as a potential drug molecule of therapeutic value as an antimicrobial, anticancer, antiviral and antitumor agent.

Microorganisms, especially filamentous fungi were reported to be the more potent producers of tannase. More than 60 fungal strains were documented for tannase production by various researchers till date. Therefore the initial part of the work included isolation and screening of fungal culture (s) which produced extracellular tannase and could withstand high tannin concentration under submerged fermentation conditions. Two fungal isolates SM 70 and SM 88 producing high tannase (5.20 U/ml and 4.70 U/ml on 20 g/L, respectively) and withstanding high tannin levels (400 g/L) were identified for further work. These were further identified as *Aspergillus allahabadi* and *Aspergillus parvulus* using microbiological and molecular characteristics. Further screening of both

the strains for the conversion efficiency of tannic acid showed *A. allahabadi* as relatively more efficient culture with 79 % conversion of tannic acid (200 g/L) and tannase activity of 11 U/ml in 48 h under submerged fermentation conditions. To the best of our knowledge the species, *A. allahabadi* used in this study has not been reported before for tannase production and the utilization of high tannin concentrations under submerged conditions.

The plants containing gallotannins (*Caesalpinia spinosa*, *Caesalonia dignya* and *Quercus infectoria*) and ellagitannins (*Terminalia chebula* and *Terminalia bellerica*) were reported to contain hydrolysable tannins around 25 – 80 % of dry weight. The *C. spinosa* tannin was found to be the best source for the tannase production by *A. allahabadi*. With *A. allahabadi* tannase, the conversion efficiency of *C. spinosa* plant tannin (pentagalloyl quinic acid) (at 200 g/L) was comparable (78 %) with that of equal concentration of commercial tannic acid (pentagalloyl glucose). The results indicated that *A. allahabadi* tannase was able to utilize high concentration of two different tannin substrates (tannic acid and *C. spinosa* tannin) at equal efficiency suggesting the possibility of economical conversion of tannin at larger scale.

The optimization of nutritional (carbon, inorganic nitrogen, minerals etc) and process parameters (medium volume and shaking speed) for tannase production and tannin conversion to gallic acid by *A. allahabadi* at shake flask level was carried out. The tannase production with spore inoculum was found to be highest at 54 h while with mycelial inoculum it was at 48 h. Supplementation of NH_4NO_3, $ZnSO_4$ and $CaSO_4$ increased the tannase activity *C. spinosa* tannin as a sole carbon source.

Further the process parameters (agitation and aeration) were first optimized during scale up studied in fermenter (up to 100 L). The tip speed at 2 m/s and aeration at 0.6 vvm were useful to obtain maximum tannase activity. The productivity of tannase production at fermenter level increased to 655 U/L/h from that of 503U/L/h at shake flask. The tannase production in 100 L fermenter showed 95 % conversion efficiency of tannin to gallic acid with gallic acid yield of 89 %.

Morphology of filamentous fungi under submerged fermentation was reported to influence the end product significantly (Sven Kelly et al, 2004). *A. allahabadi* exhibited four different types of morphology under submerged fermentation during tannase production. Free dispersed mycelial morphology of *A. allahabadi* was seen when

maximum tannase activity (26 U/ml) was obtained. The viscosity results of fermentation broth showed the non Newtonian pseudo plastic behavior of broth. Higher concentration of tannic acid fermentation was observed to affect volume of the fermenter significantly. For instance, one kg production of gallic acid was obtained in 12.5 L fermenter volume if the initial tannin concentration was 50 g/L while the volume was reduced to 3.12 5L when 200 g/L was used.

The improvement studies for tannin utilization efficiency were attempted. The coculture of *A. allahabadi* and *A. parvulus* showed promising results compared to their respective monocultures. However further investigation at fermeneter scale is required to study the behavior and interaction of both the cultures in STR.

During purification and characterization of *A. allahabadi* tannase a single band was observed in SDS-PAGE and its minimum molecular weight was found to be 100 kDa. The optimum temperature and pH for activity of purified tannase were 35^0C and 5.0, respectively; whereas the temperature and pH stability of tannase were observed in the range of 25 – 45^0C and 4.5 – 6.0, respectively. Significant inhibition of the enzyme activity was observed with Hg^{2+} while there was no inhibition with metal ions like Mg^{2+}, Mn^{2+}, Ca^{2+} and Cu^{2+}. The K_m of the purified tannase for tannic acid and methyl gallate were 2.0 x 10^{-3} mM and 1.21 mM, respectively. The ratio K_{cat}/K_m 4.08 x10^7 min^{-1}/mM^{-1} for tannic acid and 4.46 x10^2 min^{-1}/mM^{-1} for methyl gallate indicated tannic acid as a preferred substrate over methyl gallate. In case of tannic acid, catalytic time (time required for hydrolysis reaction of one acyl bond in substrate) for *A. allahabadi* tannase was 8.26 x10^{-6} min and that in case of methyl gallate was 1.8 x10^{-3} min. The high turnover number (i.e. high K_{cat} value) of tannase suggested the utilization of high tannin concentration by *A. allahabadi* in shorter period (50 % of tannin (100 g/L) was utilized in first 16 h).

The immobilized purified tannase retained 85 % of initial activity in 9^{th} cycle of reuse. The conversion efficiency of tannic acid (200 g/L) was found to be 93 % till 5^{th} cycle and then decreased to 60 % at 9^{th} cycle. This is the first report on the operational stability of immobilized tannase in high tannin (>50g/L) concentrations. *A. allahabadi* mycelia were also immobilized in Ca alginate beads. The immobilized mycelia showed comparable results (92 % tannin conversion efficiency when 200 g/L tannin was used) as that with immobilized tannase.

In conclusion, *A. allahabadi* tannase was one of the efficient enzymes reported which converted high levels of tannin to gallic acid in a commercially viable technology. A technology was developed based on the work presented in Chapter 4 (Studies and optimization of tannase and gallic acid production by fermentation) and transferred to Spice Biotech India Pvt. Ltd., India.

ANNEXURE I

Optimization of extraction for tannins from plant sources

A.1 Effect of different extraction conditions on total extractable tannins

Plant parts from different plant sources (*C. spinosa, C. dignya, Q. infectoria, T. chebula, T. belerica and T. arjuna*) were processed and used for optimization of tannin extraction in water as described in section 2.9. The focus of the present study was on the gallic acid production from tannins by using tannase under submerged conditions. Plant tannins are available abundantly. It is prerequisite for maximum yield and purity of gallic acid as well as for efficiency of tannase that the tannins should be available without interference of non tannin substances or other polyphenolic compounds. The study was carried out to extract maximum tannins from the plant source.

Water was the obvious choice of solvent because of the application of extract in the fermentation. Extraction of tannins has number of limitations due to characteristics of tannins. Tannins are susceptible to high temperatures and thus lead to extraction of non tannin contents along with tannins and to hydrolysis (Zalacain *et al*., 2003). Longer exposure to air leads to oxidation of tannins which makes them dark in colour. Zalacain *et al.* (2003) demonstrated the optimum conditions for extraction optimization of sumac tannins (*Rhus coriaria*) at 45^0C for 60 min without agitation and discussed in detail about tannins and non tannins. The enhanced tannin extraction achieved in fine mesh plant material was due to the availability of maximum surface area for interaction with water.

A.2 Effect of static and stirring conditions at ambient and at 40^0C

Extraction by leaching at room temperature (30^0C) for 12 – 16 h without stirring showed 25 – 50 % (w/w) extraction of available tannins in plant material. Stirring at 90 rpm and increased temperature (40^0C) significantly increased the total extractable tannins by 20 – 40 % in all plant materials.

Tannin extraction was observed to be more effective (10 - 25 %) in case of fine powder of 80 – 100 mesh than coarse powder (Tables 1 and 2).

Table 1 Extraction of tannins at static conditions

Plant source	Coarse powder	Fine powder
C. spinosa	25.68	28.65
Q. infectoria	12.03	13.24
C. dignya	12.05	12.91
T. chebula	9.15	11.24
T. bellerica	2.13	3.50

Extractable tannins are expressed in w/w of plant material. Extraction was carried out at 30^0C for 12 – 16 h

Table 2 Extraction of tannins at static and stirring conditions

Plant source	Coarse powder		Fine powder	
	Static	Stirring	Static	Stirring
C. spinosa	26.87	30.26	30.72	38.67
Q. infectoria	12.89	13.45	17.35	20.14
C. dignya	14.26	16.51	18.39	21.58
T. chebula	9.83	12.16	12.32	14.11
T. bellerica	2.07	2.54	3.74	4.48

Extractable tannins are expressed in w/w of plant material. Extraction of coarse and fine plant material was carried out at 40^0C for 60 min

A.3 Effect of extraction time and extraction temperature

Effect of extraction time was studied using different ratio of plant material and water viz. 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8 under stirring conditions at 40^0C. The proportion of plant material : water at 1:2 and 1: 3 resulted in the thick slurry and hence did not use further. *T. bellerica* formed sticky mass at 1:4 proporation and could not be extracted further. The extract after filtration / centrifugation was analyzed for total soluble (total solids) and total tannins. All plant materials showed increase in extractable tannins and total soluble

solids as the extraction time increased from 30 min to 90 min. The yield of total solids and tannins were observed to be fairly constant from 90 min to 120 min at all material:water ratio. *Q. infectoria* and *T. bellerica* extracts turned dark brown at 120 min. Effect of extraction time at optimum material:water ratio is shown in Table 3.

Table 3 Effect of extraction time on total extractable tannins

Plant source	**Extraction time (min)**			
	30	60	90	120
C. spinosa	36.96	40.0	40.02	40.01
Q. infectoria	18.72	20.02	20.26	19.83
C. dignya	22.03	20.90	21.26	21.54
T. chebula	12.46	13.88	13.93	13.93
T. bellerica	4.62	5.29	5.23	5.2

Extractable tannins are expressed in w/w of powdered plant material. Extraction of plant material was carried out at 40^0C using fine powder with stirring

Table 4 Effect of temperature on total extractable tannins

Plant source	**Temperature, ^{0}C**		
	50	60	70
C. spinosa	41.92	44.45	47.73
Q. infectoria	24.76	26.30	27.59
C. dignya	30.55	32.73	33.93
T. chebula	14.93	15.78	16.57
T. bellerica	5.48	5.72	6.03

Extractable tannins are expressed in w/w of powdered plant material. Extraction was carried out using fine mesh plant powder with stirring at respective proportion of plant:water and extraction time

Total solids and total extractable tannins were observed to be increased by increase in temperature and were highest at 70^0C in all plant sources (Table 4). The increase in temperature did not affect the color of extract and also did not form sticky mass. The physicochemical analysis of extracts had shown that the extraction conditions were able to extract the desired characteristics of the original plant material into the extracts (Table 5). Based on the total extractable tannins and physicochemical properties, *C. spinosa* was found to be the best tannin source among all under investigations followed by *Q. infectoria*.

Table 5 Physicochemical characterization of plant extract (w/v)

Tests	***Caesalpinia spinosa***	***Caesalpinia dignya***	***Quercus infectoria***	***Terminalia chebula***	***Terminalia bellerica***	***Terminalia arjuna***
Ash content, %	3.152	3.58	1.61	2.42	5.65	4.80
Acid insoluble ash, %	0.069	0.056	0.0124	0.011	0.0054	0.093
Total nitrogen, %	0.399	0.147	0.012	0.509	0.893	0.374
Total carbohydrates, %	66.08	43.90	53.22	49.48	48.81	46.67
Total reducing sugars, %	5.168	5.59	5.08	12.04	15.73	10.13
Total extractable tannins, %	9	4.81	4.47	3.13	0.98	1.25
Residual Gallic acid, %	1.95	3.41	1.06	4.19	3.57	0.22
Elemental analysis (µg/100ml)						
Na	6.12	1.83	2.66	65.6	40.29	3.96
K	262.5	192	514.8	837.2	1219.0	348.6
Ca	1382.4	565.5	162	577.2	653.6	676.4
Mg	1320.2	585	302.6	823.2	768.5	721.5
Fe	20.7	2.67	4.68	17.85	5.81	12.18
Zn	0.54	0.45	0.36	0.55	0.99	0.17

The present study demonstrated 1:6 - 1:8 as the optimum plant material: water ratio for the recovery of maximum extractable tannins for 60 min at 70^0C. The extraction of fine powder with stirring at low rpm enhanced extraction of tannins without interference of non tannin substances and darkening of the extract. The residual gallic acid levels were also remained low (0.7 g/L). The most commonly used method was extraction by water at boiling temperature (90- 100^0C for 1 hour) (Pourrat *et al*., 1985; Pourrat *et al*., 1987; Barthomeuf *et al*., 1994a; Sariozlu and Kivanc, 2009) but the total extractable tannins, interference of other non tannin molecules and residual gallic acid were not discussed in the reports. In the present study, the physicochemical characterization of original plant material and extract revealed 80 – 90 % extraction of nitrogen, reducing sugars, and essential minerals in extract. There are no reports available on the physicochemical properties species of *Caesalpinia, Quercus* and *Terminalia* in the literature. However, there were a few reports about the physicochemical characterization of other plant tannin sources such as cashew testa (Lokeswari *et al*., 2007b), gobernadora bush (Trevino-Cueto *et al*., 2007) and creosote and tar bush (Belmares *et al*., 2009).

CHAPTER 7

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List of publications/ patents/ presentations/ technology transfer

Publications

Purification and characterization of tannase by *A. allahabadi* (manuscript under preparation)

Presentations

A part of work on isolation and screening of fungal isolates for tannase production and studies and optimization of tannase and gallic acid production by fermentation was presented at TIFAC, DST, Govt.of India in 2002 and 2007.

Patent

A novel process for preparation of gallic acid (3,4,5 trihydroxy benzoic acid) from high concentration of tannic acid by a submerged fermentation and subsequent conversion of the gallic acid to trimethoxybenzoic acid. (File no. 1141/MUM/2002, December 2002).

Technology Transfer

A technology was developed based on the work presented in **Chapter 4** (Studies and optimization of tannase and gallic acid production by fermentation) and transferred to Splice Biotech India Pvt. Ltd.