PRODUCTION OF TANNASE BY *ASPERGILLUS NIGER*

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ABSTRACT

PRODUCTION OF TANNASE BY *ASPERGILLUS NIGER*

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In this study, a filamentous fungus, *Aspergillus niger* was evaluated in terms of extracellular tannase production. The effect of tannic acid, glucose and nitrogen sources on tannase and biomass productions was investigated and their concentrations were optimized. The highest enzyme activity was recorded as 316 U/ml in the optimized medium containing 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl in a shake-flask bioreactor at 35^oC and 175 rpm.

The bioreaction profile including tannic acid, gallic acid, pyrogallol, glucose concentrations, pH, biomass and extracellular tannase production were determined under the optimized conditions. The maximum extracellular tannase activity (316 U/ml) was observed on the 4^{th} day of cultivation. However, biomass continued to increase up to the 9^{th} day of fermentation. Increase in biomass concentration during the first two days and after the 7^{th} day was high. The microorganism used tannic acid and glucose during the first two days by considering the sharp decrease in tannic acid and glucose concentrations. The increase in biomass concentration after the 7^{th} day was directly proportional to the decrease in pyrogallol concentration in this period of time. The pH of the cultivation medium decreased from 5.5 to 2.3 owing to the assimilation of glucose and the production of gallic acid.

Keywords: Tannase, *Aspergillus niger*, Enzyme production, Cultivation profile, Tannic acid.

ÖZ

*ASPERGILLUS NIGER´*DAN TANNAZ ÜRETİMİ

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Bu çalışmada ipliksi bir küf olan *Aspergillus niger*′dan tannaz üretimi incelenmiştir. Tannik asit, glikoz ve azot kaynaklarının, tannaz üretimi ve biyokütle artışı üzerindeki etkisi araştırılmış ve optimum derişimleri belirlenmiştir. En yüksek enzim aktivitesi (316 U/ml), çalkalamalı bioreaktörde 35^{o}C ve 175 rpm de yapılan 8% Tannik asit, 1 % Glikoz, 0,4% $(NH_4)_2HPO_4$, 0,1% K_2HPO_4, 0,1% $MgSO_4.7H_2O$, 0,01% $ZnSO_4.7H_2O$, 0,0005% NaCl içeren ortamda görülmüştür.

Optimize edilen ortam koşullarında, tannik asit, gallik asit, pyrogallol, glikoz konsantrasyonlarının, pH, biyokütle ve hücre dışı tannaz enzimi üretiminin fermentasyon profili çıkarılmıştır. En yüksek enzim aktivitesi (316 U/ml), 4. günde görülmüştür. Bunun yanı sıra, biyokütledeki artış 9 gün boyunca olmuştur. 2. güne kadar ve 7. günden sonra biyokütle konsantrasyonunda büyük oranda artış olduğu görülmüştür. İlk iki günde tannik asit ve glikoz konsantrasyonundaki düşüş göz önüne alınarak, mikroorganizmanın bu sürede tannik asiti ve glikozu kullandığı tahmin edilmektedir. 7. günden sonra biyokütle konsantrasyonundaki artışın ise mikroorganizma tarafından üretilen pirogallol konsantrasyonundaki düşüş ile orantılı olduğu gözlenmektedir. Başlangıçta 5,5 olan üretim ortamının pH′sı, 9. günün sonunda 2,3′e düşmüştür. Bu düşüşün, glikozun kullanılması sonucu açığa çıkan asitlerden ve gallik asit üretiminden kaynaklandığı düşünülmektedir.

Anahtar kelimeler: Tannaz, *Aspergillus niger*, Enzim üretimi, Üretim ortamının profili, Tannik asit.

To my mother and father

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Da: Dalton

d_f: Dilution factor

DNS: Dinitrosalisilic acid

HPLC: High Performance Liquid chromatography

LSC: Liquid surface culture

MSSC: Modified solid state culture

nm: Nanometer

PDA: Potato Dextrose Agar

SmC: Submerged culture

SSC: Solid state culture

Tannase: Tannin acyl hydrolase

TLC: Thin Layer Chromatography

U: Enzyme activity unit

UV: Ultra violet

CHAPTER 1

INTRODUCTION

1.1 Tannins

Tannins are naturally occurring water-soluble polyphenols with a molecular weight of 300 Da to 3000 Da, even up to 20 000 Da. These compounds are common in nearly every part of plants, namely in leaves, fruits, bark, wood, root and seed, and can accumulate in large amounts in particular organs and tissues of the plant. They are also present in common foodstuffs such as tea, strawberry, raspberry, blackberry, grape, mango, hazelnut and walnut, agricultural wastes and agro-industrial byproducts (Mingshu *et al*., 2006).

After lignins, tannins are the second most abundant group of plant phenolics. They are considered as plant secondary substances because they are not involved in metabolic pathways (Bhat *et al*., 1998). Although they have a function in wound healing and act structurally as pigments, they have no direct function at the intracellular level of metabolism. Tannins accumulated in plants protect the defenseless parts of the plants from microbial attack by inactivating the viruses and invasive extracellular enzymes of microorganisms via direct tanning action. Enzymes secreted by attacking microorganisms are wholly or partially inactivated by complex formation between microbial substrates such as polysaccharides and nonenzyme proteins in the plant, and tannin molecule (Lekha and Lonsane, 1997). Tannins also contribute to the chemical defenses that reduce damages to plants by insect and mammalian herbivores (Hartzefelt *et al*., 2002).

Tannins have a range of effects on animals, microorganisms and environment. A large number of phenolic hydroxyl groups allow tannins to form large insoluble and indigestible complexes, mainly with proteins and to a lesser extent with starch, cellulose, pectin and digestive enzymes. Therefore, they cause reduction in nutritional values of food and feed intake in ruminant animals. They also inhibit the growth of a number of microorganisms and resist microbial attack by inhibiting their enzyme activity via disrupting the membrane. Despite these toxic and antimicrobial properties of tannins, some filamentous fungi, bacteria and yeast are quite resistant to tannins and can degrade them (Goel *et al*., 2005). Moreover, the tannins in agricultural wastes cause serious pollution in the environment; thus, they have negative effects on living organisms (Kumar *et al*., 1999).

Tannins can be divided into three major groups on the basis of their structure and properties.

1.1 .1 Hydrolysable Tannins

Hydrolysable tannins have a glucose molecule as a central core that is partially or totally esterified with gallic acid and derivatives of gallic acid. The major hydrolysable tannins are extracted from Chinese gall (*Rhus semialata*), sumac (*Rhus coriara*), Turkish gall (*Quercus infectoria*), tara (*Caesalpina spinosa*), myrobalan nuts (*Terminalia chebula*), and chestnut (*Castanea sativa*). Hydrolysable tannins are toxic and cause poisoning if large amounts of high tannin containing plant material are consumed (Bhat *et al*., 1998).

The hydrolysable tannins are sub-classified into gallotannins and ellagitannins.

1.1.1.1 Gallotannins

Gallotannins are composed of esters of gallic acid and glucose. They are easily hydrolysed by acids, bases or certain enzymes into monomeric products (Mingshu *et al*., 2006). They are commonly found in tara pods (*Caesalpina spinosa*), gall nuts (pathological excrescences) from *Quercus infectoria* (Turkish gall) & *Rhus semialata* (Chinese gall) and sumac leaves (*Rhus coriara*) (Bhat *et al.*, 1998).

1.1.1.1.1 Tannic Acid

Tannic acid, the commercial form of gallotannins, is not a true acid but an acid like substance called a polyphenol. Its structure is based mainly on glucose esters of gallic acid. It is produced from the secondary metabolism of the plant and commonly found in the bark and fruits of the plants, especially in gallnuts (Andrade Jr. *et al*., 2005).

Tannic acid has various toxic properties to vivo. It precipitates proteins, inhibits the growth of microorganisms (Nakamura *et al*., 2003), inhibits some intestinal microbial enzymes of the bovine rumen (Makkar *et al*., 1988), inhibits protein absorption from intestinal tract (Nakamura *et al*., 2003), increases water, fat and nitrogen contents of faeces**,** and the amount of faecal weight (Bravo *et al*., 1994), induces metabolic acidosis and methohemoglobinemia in sheep and is very toxic to mice when given orally (Zhu and Filippich, 1992) and damages the abomasum, liver and kidney in sheep when administered by intra-abomasal dosing (Zhu and Filippich, 1995). It is reported as harmful to animals if large amounts of tannic acid is consumed or injected in blood stream (Khan *et al*., 2000). However, tannic acid has various beneficial effects on human health. It has antimutagenic, anticancer and antioxidant properties (Srivastava *et al*., 2000). It reduces total cholesterol and triglycerides in rats (Yugarani *et al*., 1992), suppresses lipogenesis

induced by insulin in rats (Ong *et al*., 1995), reduces the tumourigenicity in the skin and other organs when added to the diet or drinking water, cures burns, diarrhoea and chemical antidotes in poisoning and is used as a local astringent (Khan *et al*., 2000).

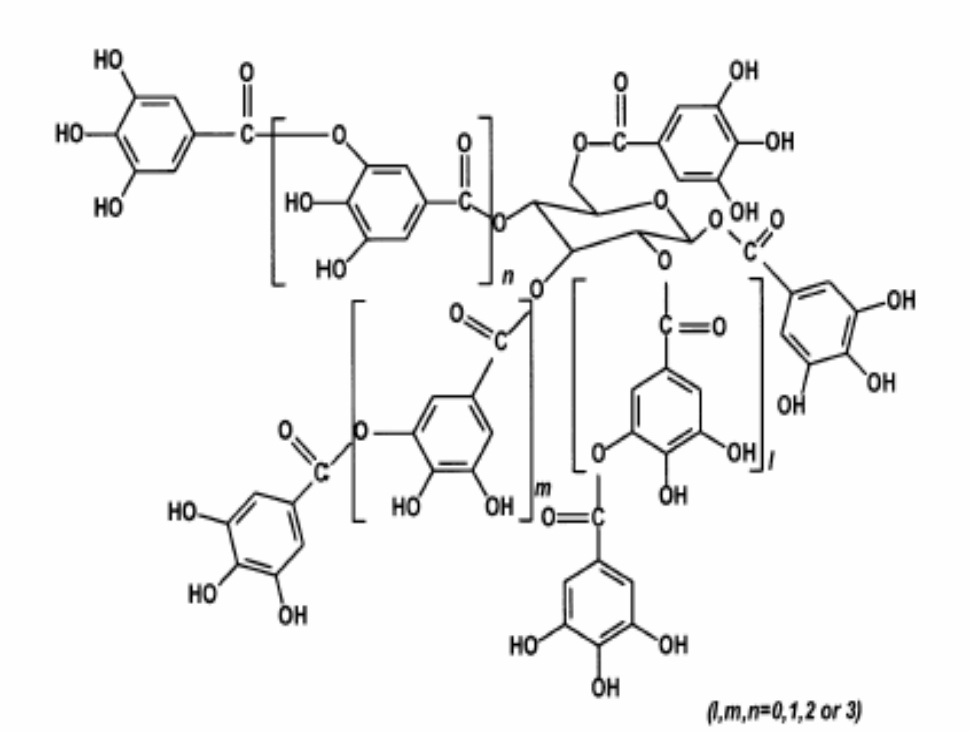

Figure 1.1. Tannic acid (Nakamura *et al*., 2003)

Tannic acid is used in many industrial, pharmaceutical and food additive applications. In pharmaceutical industry, tannic acid is used as a raw material in medicinal products for human. It is used as a clarifying agent in wine and beer industries and as a flavoring agent in baked food, candy and meat products (Khan *et al*., 2000). It is also used in gallic acid production, the tanning of leather, wood dyeing, industrial effluent treatment and ink-making industry.

1.1.1.2 Ellagitannins

Ellagitannins are composed of esters of ellagic acid and glucose. Ellagitannins are not easily hydrolyzed because of their complex structure including C-C bonds. Although they are not hydrolysable, they are classified as hydrolysable tannins for historical reasons (Khanbabaee and Ree, 2001). They are commonly found in the wood of oak (*Quercus spp*.), chestnut (*Castanea spp*.) and myrobalan (*Terminalia chebula*) (Bhat *et al*., 1998).

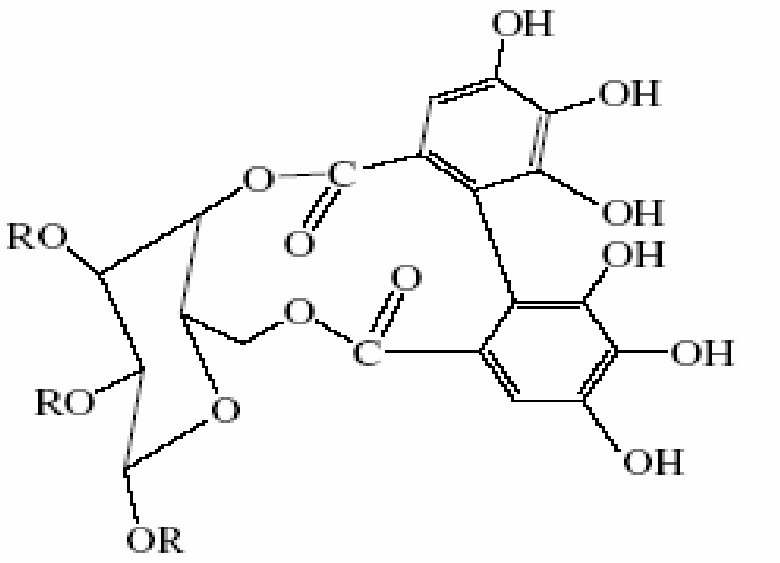

Figure 1.2 Ellagitannin (Mingshu *et al*., 2006)

1.1.2 Condensed Tannins

Condensed tannins are also known as proanthocyanidins, and consist of flavonoid groups frequently linked with either C4-C6 or C4-C8 bonds which are considered not to be hydrolysable (Romani *et al*., 2006). They are usually more abundant in tree barks and woods than their hydrolysable counterparts. A very

interesting difference between condensed tannins and hydrolysable tannins is the fact that condensed tannins do not contain any sugar moieties.

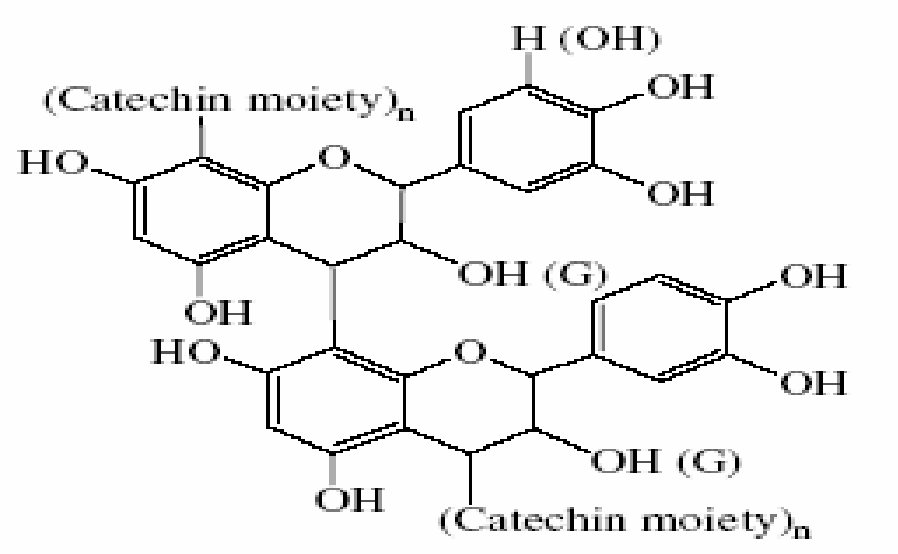

Figure 1.3. Condensed tannin (Mingshu *et al*., 2006)

The most important commercial condensed tannins are extracted from wattle (*Acacia mollissima* and *A. mearnsii*), quebracho (*Schinopsis lorentzii* and *S. balansae*) and tree barks (Bhat *et al*., 1998).

1.1.3 Complex Tannins

Complex tannins are an intermediate group that shares the characteristics of hydrolysable tannins and condensed tannins. They are composed of catechin or epicatechin unit linked glycosidically to a gallotannin or an ellagitannins unit. They yield catechin or epicatechin and gallic acid or ellagic acid on hydrolysis (Mingshu *et al*., 2006). The catechin tannins are quite common in tropical shrub legumes and tea leaves (Bhat *et al*., 1998).

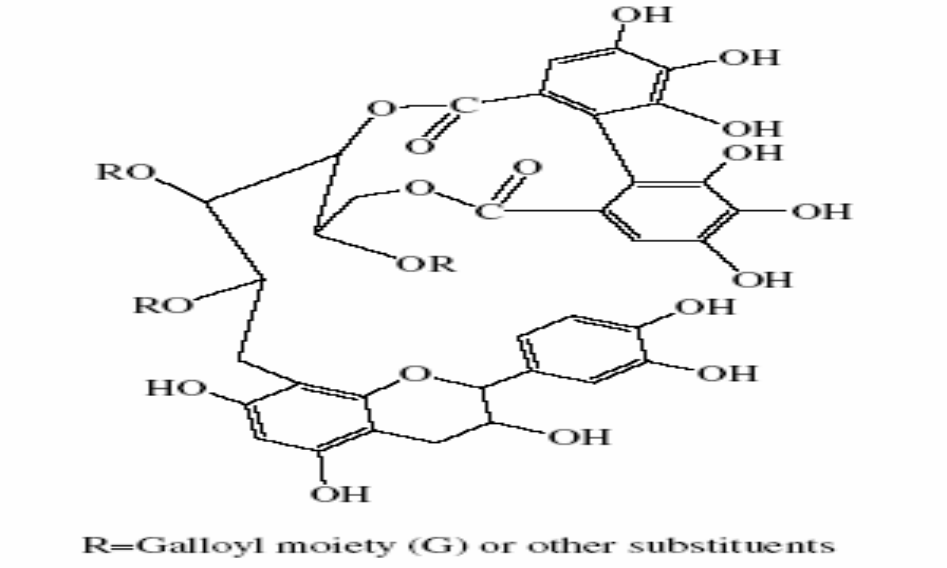

Figure 1.4 Catechin tannins (Mingshu et al., 2006)

1.2 Gallic Acid

Gallic acid (3,4,5-trihydroxy benzoic acid, $C_6H_2(OH)_3COOH$), naturally occurring polyhydroxyphenolic compound, is a colorless crystalline organic acid. It has two functional groups in the same molecule, hydroxyl groups and a carboxylic acid group.

Gallic acid is found in gallnuts, sumach, tea leaves, oak bark, green tea, apple-peels, grapes, strawberries, pineapples, bananas, lemons and many other plants, both in its free state and as part of the tannin molecule. It is slightly soluble in warm water (60^{o}C); besides, it is soluble in organic solvents such as ether, chloroform and alcohol. It can be obtained by the hydrolysis of tannic acid with sulfuric acid or hydrolysis of tannic acid with tannase. Enzymatic synthesis is preferred due to product purity and less energy usage (Banerjee, 2004).

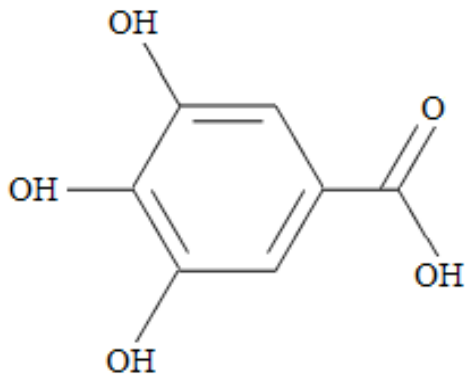

Figure 1.5 Gallic acid (Bhat *et al*., 1998)

Gallic acid finds applications in pharmaceutical, cosmetic, food and chemical industries. The major usage of gallic acid is in pharmaceutical industries, particularly in the manufacturing of trimethoprim (TMP), an antibacterial agent, used in combination with sulfonamide. In addition, it has various potential therapeutic properties including anti-cancer, antimicrobial, antimutagenic and anti-inflammatory properties (Soong and Barlow, 2006). Gallic acid is used in food industries in the enzymatic synthesis of gallic acid esters, e.g. propyl gallate, which is used as an antioxidant in fats, oils and beverages. It is used in the manufacture of pyrogallol used in staining fur, leather and hair and as a photographic developer. It is also used as a raw material for inks, paints and colour developers (Kar and Banerjee, 2000) and as a photosensitive resin in semiconductor production (Mondal and Pati, 2000).

1.3 Tannase

Tannin Acyl Hydrolase (E.C. 3.1.1.20), accidentally discovered by Teighem in 1867, is commonly referred to as tannase. Tannase are esterases able to hydrolyze the 'ester' bond (galloyl ester of an alcohol moiety) and the 'depside' bond (galloyl ester of gallic acid) in hydrolysable tannins and gallic acid esters, thereby releasing gallic acid and glucose (Figure 1.6) (Yu *et al*., 2004). Tannase hydrolyze only substrates that contain at least two phenolic OH groups in the acid component. The esterified COOH group must be on the oxidized benzene ring and must not be ortho to one of the OH groups (Lekha and Lonsane, 1997).

Tannase is a ubiquitous enzyme of the microbial world and present in plants, animals and microorganisms. Since it is an inducible enzyme, it can only be produced in the presence of its substrate, tannic acid, or substrate analogue, e.g. methyl gallate, ethyl gallate, n-propylgallate, m-Digallic acid (Belmares *et al*., 2004; Lekha and Lonsane, 1997). It is known to be produced both membrane bound and extracellular forms based on the fermentation type (Lekha and Lonsane, 1997).

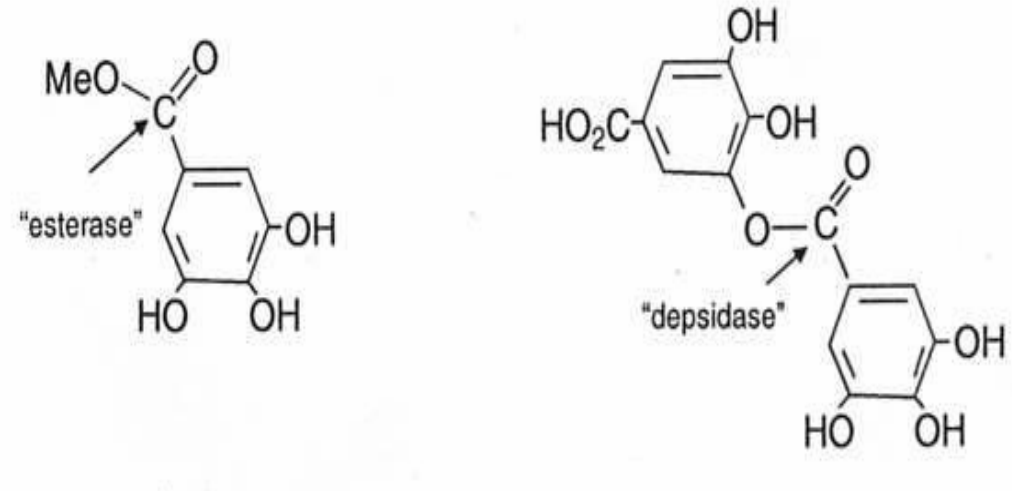

Figure 1.6 Esterase and depsidase activities of tannase (Banerjee,2004)

1.3.1 Sources of Tannase

1.3.1.1 Plants

Many hydrolysable tannin-rich plant materials such as myrobolan (*Terminalia chebula*) fruits, divi-divi (*Caesalpinia coriaria*) and dhawa (*Anogeissus latifolia*) leaves, and many condensed tannin-rich plant materials such as avaram (*Cassia auriculata*), babul (*Acacia arabica*), and konnam (*Cassia fistula*) bark are the sources of tannase. Hydrolysable and condensed tannins may be produced in the same plant, but generally in separate tissues (Lekha and Lonsane, 1997).

1.3.1.2 Animals

It is reported that low levels of tannase was found in the rumen mucosa of cattle. Tannase is also reported to have been obtained from bovine mucosa membrane of the rumen and small intestine. Moreover, it is produced by the gall larvae developed in the plant galls in order to hydrolase tannic acid present in plant galls (Lekha and Lonsane, 1997).

1.3.1.3 Microorganisms

Although many enzymes are obtained from animal and plant sources, microorganisms have been the most important source for the production of industrial enzymes due to their biochemical diversity and their technical and economic advantages. Microbial enzymes are also known to be more stable than analogous proteins obtained from plant or animal sources. Moreover, microbes can be subjected to genetic manipulation more readily than animals and plants. Another

important advantage of microorganisms is that they can produce huge amounts of enzymes in a short time by fermentation.

It is known that tannins inhibit the growth of microorganisms, resist microbial attack and biodegradation. Although these antimicrobial properties of tannins, many filamentous fungi, bacteria and yeast can resist to tannins and are able to grow on them. Table 1.1 summarizes the microorganisms producing tannase.

Not all tannases are equally active against different tannin substrates. Fungal tannases efficiently degrade different types of hydrolysable tannins. Bacterial tannases can degrade tannic acid and natural tannins such as chestnut, tara, oak and mayrobalan tannins. Yeast tannases can degrade tannin acid, whereas natural tannins can be degraded weakly (Bhat *et al*., 1998).

1.3.1.3.1 Fungal Tannases

Of all the microorganisms, the fungi are known as the most potent tannase producers. In literature, there is extensive research on fungal tannases.

Bradoo *et al*. (1996) screened fifty fungi belonging to various groups for extracellular tannase production. It was reported that the enzyme was produced after 48 hours of growth in a medium containing tannic acid as the sole source of carbon, and *Aspergillus niger* and *Aspergillus japonicus* were the best producers (29.13 and 25.6 U/ml, respectively).

Tannase activity of Aspergillus and Penicillum with Fusarium and Trichoderma were compared by Bajpai and Patil (1996). *Fusarium solanii* and *Trichoderma viride* exhibited higher tannase activity as compared to other species tested. 88 and 84 mole percent conversions were obtained after 24 hours incubation, respectively.

Table1.1 Microorganisms producing tannin acyl hydrolase (Bhat et al., 1998)

Bacteria		
*	*Achromobacter* sp.	(Lewis & Starkey, 1969)
□	*Bacillus pumilis*	(Deschamps *et al.,* 1983)
□	*Bacillus polymyxa*	(Deschamps *et al.,* 1983)
□	*Corynebacterium* sp.	(Deschamps *et al.,* 1983)
*	*Pseudomonas solanacearum*	(Deschamps 1989)
*	*Selenomonas ruminatium*	(Skene & Brooker 1995)
Fungi		
♦	*Aspergillus niger*	(Bradoo *et al.,* 1996)
♦	*Aspergillus oryzae*	(Bradoo *et al.,* 1996)
*	*Aspergillus flavus*	(Yamada *et al.,* 1968)
♦	*Aspergillus japonicus*	(Bradoo *et al.,* 1996)
*	*Aspergillus aureus*	(Bajpai & Patil, 1996)
♦	*Aspergillus awamori*	(Bradoo *et al.,* 1996)
*	*Aspergillus fischeri*	(Bajpai & Patil, 1996)
*	*Aspergillus rugulosus*	(Bradoo *et al.,* 1996)
*	*Aspergillus parasiticus*	(Bajpai & Patil, 1996)
*	*Aspergillus terreus*	(Bajpai & Patil, 1996)
*	*Penicillium chrysogenum*	(Bradoo *et al.,* 1996)
*	*Penicillium notatum*	(Ganga *et al.,* 1977)
*	*Penicillium islandicum*	(Ganga *et al.,* 1977)
*	*Penicillium digitatum*	(Bradoo *et al.,* 1996)
*	*Penicillium acrellanum*	(Bradoo *et al.,* 1996)
*	*Penicillium carylophilum*	(Bradoo *et al.,* 1996)
*	*Penicillium charlesii*	(Bradoo *et al.,* 1996)
*	*Penicillium citrinium*	(Bradoo *et al.,* 1996)
□	*Cryphonectria parasitica*	(Farias *et al.,* 1992)
□	*Fusarium solani*	(Bradoo *et al.,* 1996)
*	*Fusarium oxysporium*	(Bradoo *et al.,* 1996)
□	*Rhizopus oryzae*	(Hadi *et al.,* 1994)
□	*Trichoderma viride*	(Bradoo *et al.,* 1996)
*	*Trichoderma hamatum*	(Bradoo *et al.,* 1996)

Table 1.1 (continued)

*	*Trichoderma harzianum*	(Bradoo *et al.,* 1996)
*	*Helicostylum* sp.	(Bradoo *et al.,* 1996)
*	*Cunnighamella* sp.	(Bradoo *et al.,* 1996)
*	*Syncephalastrum racemosum*	(Bradoo *et al.,* 1996)
♠	*Neurospora crassa*	(Bradoo *et al.,* 1996)
Yeasts		
□	*Candida* sp.	(Aoki *et al.,* 1976)
□	*Pichia* spp.	(Deschamps 1989)
*	*Debaryomyces hansenii*	(Deschamps 1989)
♠ Poor producer * Moderate producer □ Good producer ♦ Best Producer		

Pinto *et al*. (2001) screened a number of *Aspergillus niger* strains for tannase production in solid state cultivation. Among the different strains, *Aspergillus niger* 11T25A5 was found as the best tannase producer (67.5 $U.g^{-1}$ / 72 hours of fermentation).

In another study done by Mukherjee and Banerjee (2004), the production of tannase by *Rhizopus oryzae* and *Aspergillus foetidus* in modified solid state cultivation were studied. It is reported that *Aspergilus foetidus* exhibit higher tannase activity (36.4 U/ml) than *Rhizopus oryzae* (31.8 U/ml) although maximal production in *Aspergilus foetidus* was reached after a longer incubation period.

35 Aspergilli and 25 Penicilli were studied both qualitatively on tannic acid agar plates and quantitatively in broth for the ability of tannase production by Batra and Saxena (2005). Among Aspergilli tested, *Aspergillus fumigatus* (8.3 IU/ml), *Aspergillus versicolor* (7.0 IU/ml), *Aspergillus flavus* (4.95 IU/ml) and *Aspergillus caespitosum* (4.47 IU/ml) and among Penicillli, *Penicillium charlesii* (4.82 IU/ml), *Penicillium variable* (4.70 IU/ml), *Penicillium crustosum* (4.7 IU/ml) and *Penicillium restrictum* (4.47 IU/ml) were reported as the potent tannase producers.

Tannin degradation by yeasts has not been studied a lot. Aoki *et al*. (1976) isolated and reported that yeast species belonging to *Candida* were able to produce tannase in order to hydrolyse the ester and depside linkages in tannic acid to liberate gallic acid and glucose.

1.3.1.3.2 Bacterial Tannases

Some bacterial cultures have developed the ability to express extracellular tannase to degrade tannins, thus releasing gallic acid and glucose. Deschamps *et al.* (1983) showed that strains of *Bacillus pumilus, B. polymyxa, Corynebacterium sp.,* and *Klebsiella pneumoniae* were able to produce extracellular tannase with chestnut bark extract as the sole source of carbon. Among these strains, best tannase production (0,064 units/ml) was obtained by *Corynebacterium sp.* Q 40 after 5 h of cultivation.

Kumar *et al*. (1999) reported that *Citrobacter freundi*, isolated from tannery effluent, could grow at concentrations as high as 5% (w/v) of tannic acid and produced extracellular tannase in order to hydrolase the substrate, tannic acid.

A bacterial strain named *Bacillus licheniformis* KBR 6 that were able to produce extracellular tannase in the medium containing only tannic acid was isolated by Mondal and Pati (2000). Studies show that addition of basal salts and glucose to the medium containing only tannic acid increased the enzyme production 2.36 fold.

Ayed and Hamdi (2002) reported that *Lactobacillus plantarum* produced extracellular tannase after 24 hours of growth and the enzyme production (6 U/ml) was maximum in the medium containing 2 g glucose l^{-1} and 7 g tannic acid l^{-1} .

Vaquero *et al.* (2004) conducted screening of oenological lactic acid bacteria species of the genus *Lactobacillus, Leuconostoc, Oenococcus* or

Pediococcus for tannase production. *Lactobacilluarum plantorum* was found as the only species showing tannase activity.

1.3.2 Physiological Significance of Tannase in Plants, Animals and Microorganisms

1.3.2.1 Plants

The tannin content in the plant material may serve as a defense mechanism against microbial invasion and attacks from herbivores. Thus, it supplies the durability of certain long-lived trees. When the plant leaves are under attack from herbivores, the cells lose compartmentation, which brings the tannase into contact with the tannin substrate in the leaves. The substrate is then hydrolyzed into harmful low molecular weight phenolic compounds, which can be precursors for toxic substances in higher plants and are toxic to animals. When the plants are under attack from microbial invasion, enzymes secreted by microorganisms are inhibited by tannins accumulated in plants (Lekha and Lonsane, 1997).

It was hypothesized that plants synthesize chebulinic acid, gallic acid, hexahydroxyphenic acid and high amounts of sugars during growth. When the fruit ripens, these acids synthesized by plant during the growth are esterified with glucose by the help of tannase in order to form complex tannins. After abscission of the fruit, the esterase hydrolyzes the preformed tannins. It was also recorded that the condensed tannins are formed as intermediates or precursors that will later be transformed into complex tannin molecules (Lekha and Lonsane, 1997)

1.3.2.2 Animals

Tannins are widespread in plants used as feed. They form complex with proteins, starch, etc.; therefore, they reduce the nutritional value of the animal feed. Tannins also form complexes with digestive enzymes and inhibit the digestion and availability of proteins. They react with the mucoproteins secreted from the digestive tract. Any tannin that escapes from this reaction can form complexes with the proteins of the outer cellular layer of the gut, thereby reducing the passage of the nutrients through the gut. Low levels of tannase found in rumen mucosa hydrolyze tannic acid present in the diet to phenols and sugar, and reduce the toxicity resulted from these compounds (Lekha and Lonsane, 1997).

1.3.2.3 Microorganisms

Tannins accumulate in large amounts in particular organs and tissues of the plant and serve as a defense mechanism for microbial attack. Tannin accumulation prevents the germination of spores of the attacking fungi and penetration of fungal hyphae and bacteria. Tannase produced by microorganisms helps to invade the host plant by hydrolyzing the tannin content accumulated in the bark of the plants. Tannase also have an important role in decomposing and recycling the tannin containing materials in soil (Lekha and Lonsane, 1997).

1.3.3 The Physicochemical Properties of Tannase

1.3.3.1 pH Optimum and Stability

Tannase is known to be an acidic protein with an optimum pH generally between 5 and 6. Libuchi *et al.* (1968) reported that tannase from *Aspergillus oryzae* was shown to be stable at a pH range of 3 to 7.5 for 12h and at a pH range

of 4.5 to 6.0 for 25 hours. It was also confirmed that the optimum pH for reaction was pH of 5.5.

Barthomeuf *et al.* (1994) showed that the tannase from *Aspergillus niger* LCF 8 contained both esterase and depsidase activity with the esterase and tannase (esterase+depsidase) activities peaking at a pH of 5.0 and 6.0, respectively. The stability was also good over a wide pH range between a pH of 3.5 and 8.0. Similarly, Lekha *et al.* (1994) studied the tannase from *A. niger* PKL 104 in submerged (SmC), liquid surface (LSC) and solid state (SSC) cultures. The optimum pH values were found as 5.5 for extracellular tannase and 5.0 for the intracellular tannase. It was also confirmed that extracellular tannase produced in SSC was completely stable over a wide pH range of 2-8 compared with 4-7 and 4.5-5.5 in the case of intra- and extracellular tannase from the SmC.

Tannase from *Aspergillus aculeatus* DBF 9 showed the optimum activity at pH 5.0, and stability at pH 4-6 (Banerjee *et al.*, 2001).

According to Batra and Saxena (2005), tannase from *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus flavus*, *Penicillium crustosum* and *Penicillium restrictum* showed optimum tannase activity at pH 5.0, whereas *Aspergillus caespitosum, Penicillium variable, Penicillium crustosum* showed pH optima at 6.0.

Deschamps *et al.* (1983) showed that extracellular tannase from *Bacillus pumilus, Bacillus polymyxia, Corynebacterium sp. and Klebsiella pneumonia* had an optimum activity at pH 5.5.

According to the Mondal and Pati (2000), extracellular tannase produced by newly isolated *Bacillus licheniformis* KBR 6 in submerged fermentation was active over a pH range 3.5 to 6 and showed the optimum enzyme activity at pH 5.75.

The tannase from *Candida* sp. K-1 showed an optimum activity at a pH value of 6.0. It is also mentioned that the enzyme was stable over a wide pH range, from a pH of 3.5 to 7.5 (Aoki *et al.,* 1976).

The plant tannase isolated from Penduculate oak has a pH optimum of approximately 5.0 (Niehaus and Gross, 1997).

1.3.3.2 Temperature Optimum and Heat Stability

Libuchi *et al*. (1968) reported that optimum temperature for tannase from *Aspergillus oryzae* was 30-40^{o}C and tannase was stable under 30^{o}C.

Barthomeuf *et al.* (1994) showed that the tannase from *Aspergillus niger* LCF 8 has an optimum activity at 35^{o}C and it was stable below 50^{o}C. According to Lekha and Lonsane (1994), intracellular tannase from *Aspergillus niger* PKL 104 produced in SmC had a temperature optimum at 70^{o}C compared to 60^{o}C for the extracellular enzyme from the SmC and SSC.

Tannase from *Aspergillus aculeatus* DBF 9 showed the optimum activity at a range of 50-60^{o}C (Banerjee *et al*., 2001).

According to Batra and Saxena (2005), tannase from *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus flavus* and *Penicillium variable* showed optimum tannase activity at 60^{o}C, whereas *Aspergillus caespitosum, Penicillium restrictum, Penicillium crustosum, Penicillium charlesii* showed temperature optima at 40^{o}C

Extracellular tannase produced by newly isolated *Bacillus licheniformis* KBR 6 in submerged culture was active over a temperature range 20 to 70^{o}C and showed the optimum activity at 60^{o}C (Mondal and Pati, 2000).

The tannase from *Candida* sp. K-1 showed an optimum activity at 50^{o}C. It is also mentioned that the enzyme was stable below 50^{o}C (Aoki *et al.,* 1976).

The plant tannase isolated from Penduculate oak has a temperature optimum of 35-40^{o}C (Niehaus and Gross 1997).

1.3.3.3 Molecular Mass

Tannase is a high molecular weight protein whose molecular weight varies between 186 KDa and 300 KDa, depending on the strain of the microorganism. Barthomeuf *et al*. (1994) reported that the tannase from *Aspergillus niger* LCF 8 is a glycoprotein of about 186 KDa containing 43% sugars with a pI value of 4.3.

In case of *A. niger* MTCC 2425, purified tannase protein made up of two polypeptides of molecular weight 102 and 83 KDa and total molecular weight of approximately 185 KDa (Bhardwaj *et al*., 2003).

According to Hatamoto *et al*. (1996) the native tannase from *A. oryzae* consisted of four pairs of the two subunits, forming a hetero-octamer with a molecular mass of about 300 KDa.

Native tannase from *Candida* sp. K-1 had a total molecular weight of 250 KDa (Aoki *et al.,* 1976).

Niehaus and Gross, (1997) showed that native enzyme from the *Penduculate oak* preferentially exists as a tetramer of apparently four identical subunits and has a total molecular weight of 300 KDa.

1.3.4 Reaction Catalyzed by Tannase

Lagemaat and Pyle (2005) studied the enzymatic conversion of tannic acid to gallic acid and glucose by tannase. In stage 1, the microorganism produces a basic level or constitutive amount of tannase that hydrolyses tannic acid to glucose and gallic acid**.** They mentioned that the production rate can be enhanced by the addition of small amounts of glucose in the initial growth medium since glucose can act as a catabolic inducer which enters the citric acid cycle via glycolysis and presumably metabolized faster than gallic acid. Microorganisms can also break the gallic acid and use it in the citric acid cycle for energy production. In the 2nd step, the gallic acid and glucose produced were used for the formation of biomass. In the 3rd stage, tannase which catalyze the reaction was synthesized. Spores were reported to form in stage 4.

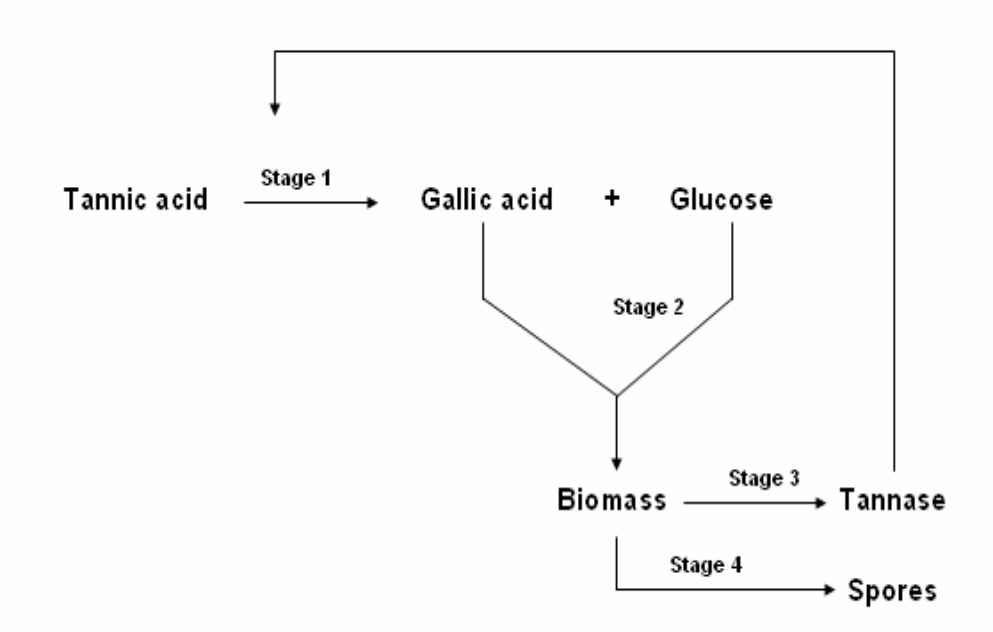

Figure 1.7 Major biochemical processes representing the growth of *Penicillium glabrum* in the static batch PUF (Lagemaat and Pyle, 2005)

Lekha and Lonsane (1997) studied the hydrolyzing pathway of tannase from *Aspergillus oryzae*. According to this research, tannase was shown to hydrolyse tannic acid (I) completely to gallic acid and glucose through 2,3,4,6-tetragalloyl glucose (III) and two kinds of monogalloyl glucose (IV). This is supported by the facts that the same products were detected in the hydrolysate of 1,2,3,4,6-, pentagalloyl glucose, and tannase produced depsidic gallic acid of methyl.m.digallate first. R_1 and R_2 are gallate and digallate, respectively.

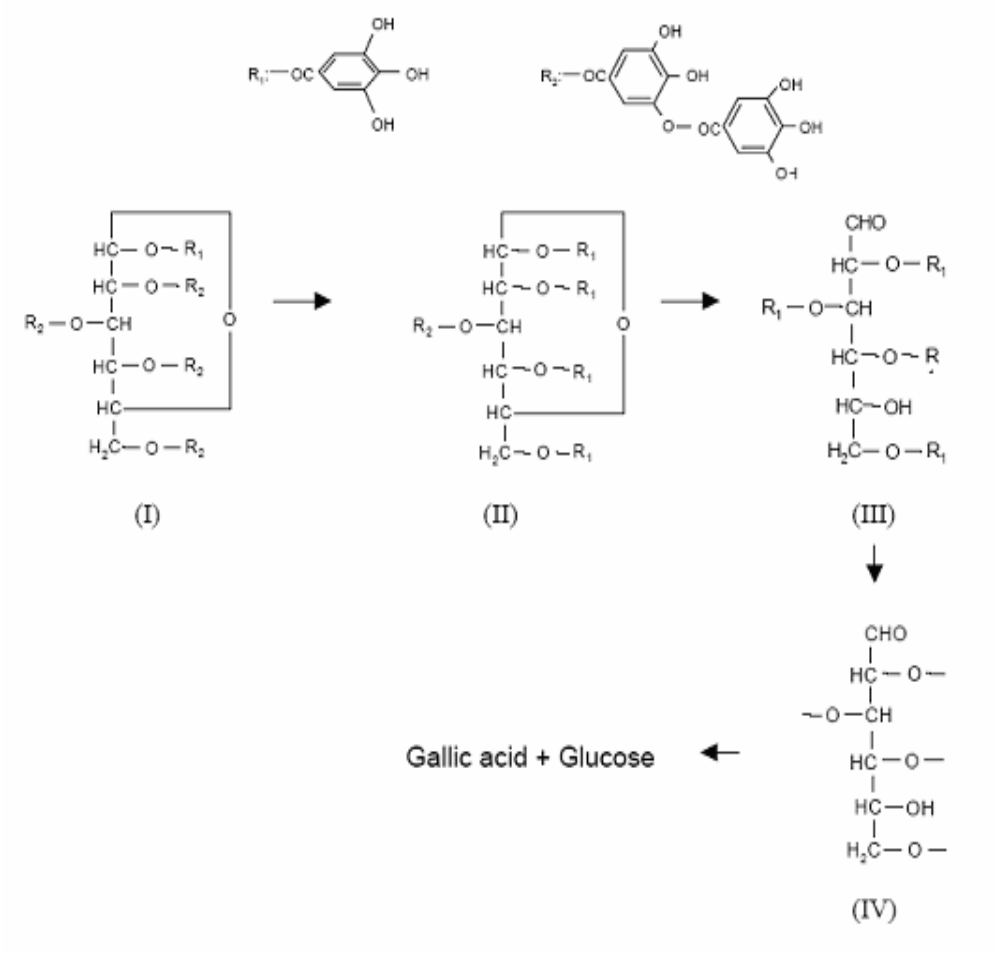

Figure 1.8 Hydrolyzing pathway of tannic acid by tannase (Lekha and Lonsane, 1997)

1.3.5 Applications of Tannase

Tannase has been considered to be a very versatile enzyme. It has wide applications in food, beverage, brewing, cosmetic, chemical and pharmaceutical industries. These applications can be explained in detail in the following sections.

1.3.5.1 Instant Tea

Consumers prefer clear products. The main problem in the manufacture of cold tea drinks such as iced tea is cold water solubility. Polymeric black tea polyphenols form hydrogen bonds with caffeine, which leads to orangy brown precipitate, named tea cream. Tea cream is formed when the tea is allowed to stand for a few hours at or below temperatures of 40^0C (Powell *et al.,* 1993).

Chemical and enzymatic methods are used for solubilizing the tea cream. In the enzymatic method, tannase converts green tea to black tea of high quality and good color that yields a good milk reaction. Tannase catalyzes the hydrolysis of the ester linkages between galloyl groups and various compounds found in unconverted tea leaves (Figure 1.9).

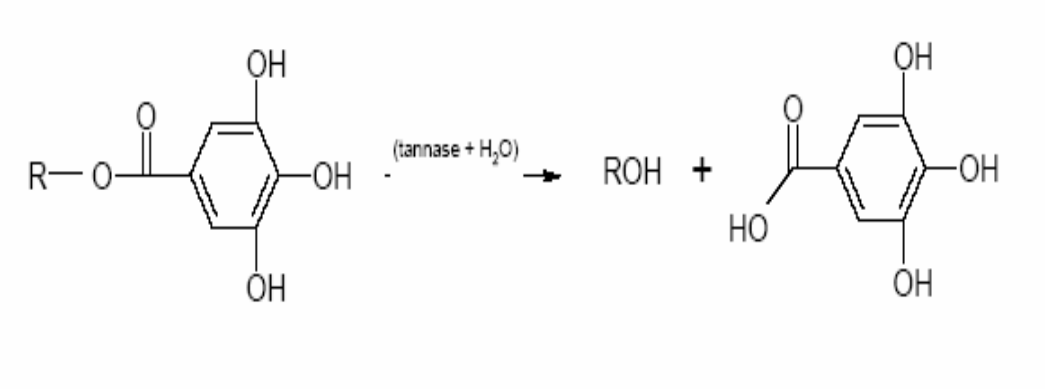

Figure 1.9 Deesterification of tea phenols by tannase (Lekha and Lonsane, 1997)

The deesterification reaction enhances the natural levels of epicatechin and gallic acid in green tea and forms large amounts of epitheaflavic acid, which is responsible for the bright reddish-black tea-like colour of tea and has very good cold-water solubility (Lekha and Lonsane, 1997).

1.3.5.2 Beer and Wine Production

Discoloration and haze development occurs during beer storage due to the complex formation between worth phenolics and other chemicals in beer mixture. Tannase has been reported to be used along with laccase to reduce the chill haze formation in beer (Lekha and Lonsane, 1997).

In wine making, tannins cause turbidity and severe quality problems. Tannase can be used to solve these problems (Belmares *et al*., 2004). Moreover, fruit juices are treated with a mixture of lactase and tannase to stabilize and clarify the product (Lekha and Lonsane, 1997).

1.3.5.3 Production of Gallic acid

Although gallic acid can be synthesized either by chemically or enzymatically, enzymatic synthesis is preferred due to its selectivity and purity. Tannase is used to hydrolase tannin containing substances to produce gallic acid, which is mainly used as an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry. Studies have shown that gallic acid production and tannase synthesis are directly proportional and as more tannase is synthesized, more gallic acid is produced (Banerjee, 2004).

1.3.5.4 Animal Feed Additives

The use of a number of enzymes in animal feed has attracted attention because tannin containing plant materials are used as food and animal feed, although tannins has antinutritional effects. It is well known that tannins form an insoluble complex with proteins. This formation of tannins with dietary and endogenous proteins, as well as with digestive enzymes, plays an important role in nonruminants. Tannins interfere with normal digestion and lead to a drain of high quality proteins from the body. They are also known to interfere with iron. Other negative effects of tannins include damage to the mucosal lining of the gastrointestinal tract, alteration in the excretion of certain cations, and increased the excretion of certain proteins and essential amino acids. In order to improve the digestibility of the food and animal feed, tannase is used to remove these undesirable effects of tannins (Lekha and Lonsane, 1997).

1.3.5.5 Miscellaneous

Tannase can be used in the treatment of wastewater contaminated with phenolic compounds (Aguilar *et al*., 2001.b). It is also used in cosmetology to eliminate the turbidity of plant extracts and in the leather industry to homogenize tannin preparation for high-grade leather tannins (Lekha and Lonsane, 1997). Moreover, it can be used as a sensitive analytical probe for determining the structures of naturally occurring gallic acid esters (Seth and Chand, 2000).

1.4 Production of Tannase

1.4.1 *Aspergillus niger*

Aspergillus species are a ubiquitous group of filamentous fungi with a large number of species that are commonly isolated from soil, plant debris, and indoor air environments. Most of the aspergilli are harmless saprophytes, but some of them can cause serious diseases in humans and animals. They can be invasive and cause mycotoxicoses and allergic reactions.

One of the most common and easily identifiable species of the genus Aspergillus is *Aspergillus niger* which is also used in this study for the production of tannase. *A. niger* is a saprophytic mold which grows rapidly on a variety of organic substrates and produce colonies that consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. It is very important for industrial applications due to its good fermentation capabilities, high amounts of protein secretion, a wide range of cell wall degrading as well as phenolic acid esterase enzymes (Hegde *et al*., 2006).

A. niger is a well known contaminant and often causes the deterioration of stored food material, archive reserves and museum collection. It is less likely to cause disease than some other Aspergillus species, but if large amounts of the spores are breathed in, the serious lung disease aspergillosis can occur. It is also an opportunistic fungus causing otomycosis and implicating in nosocomial infections, especially in immunocompromised and transplanted patients (Rasooli *et al*., 2006).

1.4.2 Optimization of Tannase Production

Microorganisms are highly dependent on the environmental conditions for their growth, just as the product yield depends on the enzyme synthesis. There are several factors affecting tannase production, such as substrates inducing tannase activity, carbon source, nitrogen source, additives of metal ions, temperature, pH, shaking rate and aeration.

Bradoo *et al*. (1997) investigated the effect of different carbon and nitrogen sources on the production of extracellular tannase from *Aspergillus japonicus*. Tannic acid was used as a carbon source and it was revealed that an increase in the substrate concentration up to 3% tannic acid causes an increase in the enzyme production although there was a little change in biomass. It was also reported that addition of sugars did not have a important effect on enzyme production with the exception of glucose and 0.2% glucose addition was found to favor both enzyme production and growth. Among the different nitrogen sources tested, $NaNO_3$ was recorded as the best nitrogen source for both enzyme production and growth.

Bajpai and Patil (1997) studied the ability of gallotannin, methyl gallate, gallic acid and pyrogallol to enhance the tannase activity in some members of fungi. *Fusarium solari* showed the maximum basal tannase activity and *Aspergillus niger* was devoid of it. The maximum induction ratios were recorded when *Aspergillus fischerii* (26.7), *Fusarium solari* (26.1) and *Trichoderma viride* (40.7) were induced with gallic acid, gallotannin and methyl gallate, respectively. The gallotannin tolerance limits were also investigated. Maximum gallotannin concentrations tolerated was found to be 20, 4, 3 and 3% for *A. niger, A. Fischerii, F. Solari* and *T. Viride*.

A study done by Bhat *et al*. (1997) showed that *Aspergillus niger* van Tieghem MTCC 2425 could tolerate tannic acid and quebracho tannin up to 150 g/L without its growth being inhibited. Maximum growth was obtained with 30 g/L tannin in quebracho medium and 110 g/L tannin in tannic acid medium, respectively.

In another study, the tannase activity produced by *Rhizopus oryzae* in modified solid state fermentation was maximum when 20 g of teri pod cover powder was used (Kar and Banerjee, 1999).

Seth and Chand (2000) studied the effect of tannic acid concentration on growth and intracellular enzyme synthesis by *Aspergillus awamori*. Studies showed that the initial substrate concentration has a positive interaction with the agitation speed. Therefore, an increase in the substrate concentration from 2.5% to 4.5% at a higher agitation speed (above 300 rpm) enhanced enzyme synthesis, whereas an increase in the substrate concentration at a lower agitation speed decreased enzyme activity due to the precipitation of gallic acid on the cell surface. Moreover, an increase in agitation speed beyond 400 rpm at low levels of substrate concentration decreased the enzyme activity due to the increased shearing effect exerted on the cells.

The effect of different medium composition on extracellular tannase from the newly isolated *Bacillus licheniformis* KBR 6 was examined. Studies showed that the enzyme production in M_2 medium (tannic acid + basal salts) and M_3 medium (tannic acid + basal salts + glucose) is 1.8 and 2.36 fold higher than M_1 medium (only tannic acid). Maximum enzyme production and growth of the organism was obtained at 18-21 h and 30-36 h, respectively (Mondal and Pati, 2000).

The effect of Cesalpinia digyna concentration, a tannin rich forest residue which is used as a substrate for the production of tannase from *R. Oryzae,* was studied by Kar and Banerjee (2000). The tannase activity reached a peak of 20 g/L substrate content in SSC and modified solid state culture (MSSC), and 10 g/L substrate content in SmC. Following the maximum, the activity declines in SmC, SSF and MSSC due to catabolite repression, substrate scarcity and the excretion of toxic substances. The effect of the incubation period on tannase production was studied. The optimum incubation period under SSC and MSSC was found 72 h whereas for the SmC it was 48 h.

Tannase from newly isolated *Aspergillus aculeatus* DBF 9 were examined with different tannic acid and glucose concentrations. Maximum intracellular and extracellular tannase activities occurred in the medium containing 1-2% (w/v) tannic acid and 0.05-0.1% (w/v) glucose (Banerjee *et al*., 2001).

Aguilar *et al.* (2001.a) studied the induction and repression patterns of tannase production by *A. niger* Aa-20 in the solid state and the submerged cultures in which tannic acid and glucose were used as carbon sources. Studies in which tannic acid was used as a carbon source showed that an increase in tannic acid up to 150 g/L enhanced the expression of the tannase activity in SSC, whereas it repressed the expression of the tannase activity in SmC after reaching its optimum value with 25 g/l tannic acid. Experiments were also carried out by using different glucose concentrations in addition to tannic acid at 25 g/l. These experiments showed that the maximum tannase activity was reached with 12.5 g/l glucose in both SSC and SmC, and higher amounts of glucose addition caused a strong catabolite repression in SmC, but a small repression effect on SSC. The effects of gallic acid, tannic acid and glucose as a carbon source in SmC were also investigated in this study. Results showed that tannase was not induced by gallic acid, although tannic acid was the best inducer.

In another study done by Aguilar *et al.* (2001.b), the effects of different tannic acid and glucose concentrations on the production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state culture were studied. It was reported that addition of high tannic acid concentrations led to an increase in total tannase activity in SSC. In the case of SmC, the maximum tannase activity was obtained with 50 g/L tannic acid, and the activity decreased due to the protease production up to 50 g/L tannic acid. Aguilar *et al*. (2001.b) also showed that an increase in glucose concentration from 6.25 to 200 g/L caused a decrease in the tannase activity in SSC. In the case of SmC, the tannase activity increased when the glucose concentration increased from 6.25 to 25 g/L followed by a sharp decrease up to 50 g/L.

Mukherjee and Banerjee (2004) investigated the production of tannase and gallic acid from tannin rich mixed substrates by *Rhizopus oryzae* and *Aspergillus foetidus* in modified solid state fermentation. It was confirmed that optimum tannase activity and gallic acid yield was obtained with a substrate ratio of (4:6) of *Terminalia chebula* powder and *Cesalpinia digya* powder and 30 g of powdered substrate resulted in the maximum tannase activity.

Tamarind seed powder and palm kernel cake, two novel agro residues, were examined to determine if these residues could be used as substrates for the production of *Aspergillus niger* ATCC 16620 in SSC. The studies revealed that the most efficient carbon source was found to be tannic acid in PKC medium and glycerol in TSP medium. Different nitrogen sources were also investigated. It was reported that PKC had enough nitrogen required for the production of enzyme and the enzyme activity was maximum when the PKC was used as nitrogen source as well as carbon source. In the case of TSP medium, potassium nitrate was the most efficient nitrogen source (Sabu *et al*., 2005).

1.5 Aim of the study

The aim of this study was to analyze extracellular tannase production by *Aspergillus niger* in shake-flask bioreactors. The effect of aeration, tannic acid, glucose and nitrogen sources on tannase and biomass productions was investigated and their concentrations were optimized. Moreover, the fermentation profile including tannic acid, gallic acid, glucose, pyrogallol, pH, biomass and extracellular tannase activity was determined under optimized conditions, which is not present in the literature.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Tannic acid (Merck), gallic acid (Sigma), potato dextrose agar (Merck) and all other chemicals were of analytical grade, either from Merck Ltd. (Germany) or Sigma-Aldrich Ltd. (USA). Thin layer plates were obtained from Merck (Germany).

2.1.2 Microorganism

The strain of *Aspergillus niger* was kindly provided by Dr. Ceyda Pembeci from Food Science and Technology Research Institute, Marmara Research Center, TÜBİTAK, from the TÜBİTAK MRC Culture Collection.

2.2 Methods

2.2.1 Maintenance and Development of *Aspergillus niger*

A. niger was grown on standard PDA slants at 35°C until sporulation and the slants were stored at 4°C. Stock cultures were subcultured every month. The composition of PDA medium is given in Appendix A.

Spores of *A. niger* were counted in order to ensure uniformity between inoculum suspensions used to start cultivation. For inoculum preparation, 10 ml of sterile 0.01% Tween 80 solution in distilled water was added to a fully sporulated 1-week old agar slant culture. The spores were scraped by using an inoculation needle under strict aseptic conditions. The number of spores in the suspension was then counted with an improved Thoma counting chamber by using a microscope (Leica) and was used as inoculum (Lekha and Lonsane, 1997).

2.2.2 Cultivation

Spore suspension was added to inoculate (to supply $1x10^6$ spores/ml growth medium) 500 ml shake flasks containing 50 ml of growth medium. The growth medium composition is glucose, 1%; $(NH_4)_2HPO_4$, 0.5%; K_2HPO_4, 0.1%; $MgSO_4.7H_2O$, 0.1%; $ZnSO_4.7H_2O$, 0.01%; NaCl, 0.0005% in 1L distilled water. All the above mentioned chemicals were dissolved in distilled water and autoclaved. For the induction of tannase, tannic acid was added to the medium after filter sterilization with 0.02 µm filter. The cultures were incubated in a shaker incubator (Type: AI 70, Minitron, Infors AG) at 175 rpm and 35°C.

Enzyme activity measurements were carried out every 24 hour starting from the 2nd till the 9th day of cultivations. Samples collected at 24 hour intervals were centrifuged at 14,000xg for 1 minute. The cell-free culture broth obtained was used for the measurement of extracellular tannase activity and concentrations of tannic acid, gallic acid, glucose, pyrogallol and protein while pellet was used for fungal biomass determination.

2.2.3 Fungal Biomass Determination

Fungal biomass was determined by filtrating 50 ml growth medium through Whatman No. 1 filter paper and washed with distilled water. The resulting filtrate was dried at 80°C overnight until constant weight was reached. The dry filtrate was weighed.

2.2.4 Enzyme Assays

2.2.4.1. Determination of Extracellular Tannase Activity

The modified UV-spectrophotometric method of Libuchi *et al*. (1967) was adopted here to estimate extracellular tannase activity. The method determines the degree of hydrolysis of ester bonds in tannic acid by measuring the decrease in absorbance at 310 nm.

The substrate solution was 0.35% (w/v) tannic acid in citrate buffer (0.05M, pH 5.5) and prepared freshly just before use to prevent turbidity formation. Culture supernatant was used as crude enzyme source. Both substrate and enzyme solutions were preincubated for 5 minutes at 40°C before the activity measurement. To 10 ml of substrate solution, 2.5 ml of the enzyme sample was added and incubated at 40°C

for 15 minutes. 200 µl of reaction samples were taken in a time range of 2.5 minutes for 15 minutes. After 50 times dilution in 90% ethanol, the absorbances were measured at 310 nm. The substrate blank contained buffer solution instead of enzyme solution and the enzyme blank contained buffer solution instead of substrate solution.

The initial straight part of the progress curve was used to calculate the initial reaction rate and express tannase activity. One unit of tannase activity was defined as the amount of enzyme which hydrolyzes 1 µmol of the ester bond of tannic acid per minute at 40°C and pH 5.5 under the conditions described above (Libuchi *et al*., 1967). The following equation was used to calculate extracellular tannase activity.

$$\text{Tannase activity (U/ml)} = \frac{\text{Initial reaction rate (OD/min)} \times 20.3 \times 1.0 \text{ (ml)} \times 1.04 \times d_f}{0.71 \times 0.25 \text{ (ml)}}$$

20.3: Micromoles of tannic acid in 1.0 ml of substrate solution

0.71: Change in absorbance after complete hydrolysis of 20.3 µmol of tannic acid under the assay conditions

1.04: A factor for correction between the method of Libuchi *et al*. (1967) and the method of TAH (www.kikkoman.co.jp/bio/j/rinsyou/pdf/63_TAH.pdf).

d_f : Dilution factor

2.2.4.2. Protein Analysis

Protein concentration was determined using Bradford Method (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard protein. Composition of reagents, procedure and standard curve are given in Appendices B, C and D, sequentially.

2.2.4.3 Monitoring Product (Gallic acid and Pyrogallol) Formation and Substrate (Tannic acid) Utilization by Thin Layer Chromatography (TLC)

Tannic acid, gallic acid and pyrogallol in the cultivation medium were analyzed according to the method of Sharma *et al*. (1998) with some modifications.

TLC was carried out on silica gel F_{254} gel plate (Merck). After 3 times dilution in metanol, samples (4 µl) were loaded onto TLC plates. The mobile phase (the running solvent) was cloroform-ethyl acetate-acetic acid(50-50-1 [vol:vol:vol]). After running in the solvent system was completed, thin layer plates were left for drying for 1 day. The plates were sprayed with ethanol-sulfuric acid reagent (90-10 [vol:vol]) and heated at 120^{o}C for 20 min. Tannic acid consumption and product formation were monitored under day light.

TLC plates were analyzed by UVP BioImaging Systems (Ultra-Violet Products Ltd., UK) in order to quantify tannic acid during the cultivation. Labworks Image Acquisition and Analysis Software Ver. 4.6 was used in the system. Quantification was based on the darkness and the area of the samples loaded.

2.2.4.4 High Performance Liquid Chromatography (HPLC) Analyses of Tannic acid, Gallic acid, Pyrogallol and Glucose

Samples were withdrawn from the production media were kept at 4^{0}C before analyzed by Varian ProStar HPLC system.

Tannic acid, gallic acid and pyrogallol concentrations in the tannase production media were analyzed according to the method of Zhu *et al*., (1992) with some modifications.

The separation was achieved by using a reversed-phase Microsorb-MV 100 C18 column (150mm x 4.6, 5µm) (Catalog no: R0086200D5, Varian) and a gradient elution with 0.025% phosphoric acid in water (A) and 0.025% phosphoric acid in methanol (B) at a flow rate of 0.6 ml/min. The gradient of elution follows: 10% B gradually increased to 15% B at 5 minutes, to 24% B at 15 minutes, to 64% B at 18 minutes, to 100% B at 28 minutes and returned to 10% B at 38 minutes. Column oven temperature was 25^{o}C. Prostar 330 PDA detector was used at 260 nm.

Glucose analysis was carried out using Carbohydrates Ca (300 x 6.5mm) (Catalog no: CP28351) column. The solvent system was distilled water at a flow rate of 0.6 ml/min. Column oven temperature was 80^{o}C. RI detector (Varian ProStar Model 350) was used.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of Tannase Activity Assay Parameters

Extracellular tannase activity was assayed UV-spectrophotometrically according to the method of Libuchi *et al.* (1967) and TAH procedure (www.kikkoman.co.jp/bio/j/rinsyou/pdf/63_TAH.pdf). This method determines tannase activity by measuring the hydrolysis of ester bonds in tannic acid at 310 nm. To calculate tannase activity, absorbance versus time graphs were drawn and the initial reaction rates were determined. As shown in Figure 3.1.a there were large deviations in absorbance versus time graphs when this method was applied directly.

In order to minimize the deviations, some modifications were done on the method of Libuchi *et al*. (1967) and TAH procedure. The first modification was the addition of the spectrophotometric assay decribed by Bajpai and Patil (1996) to the enzymatic reaction described by Libuchi *et al.* (1967). According to the spectrophotometric assay decribed by Bajpai and Patil (1996), the absorbances were measured at 254 nm and 290 nm where the maximum difference in absorbance of gallic acid and tannic acid was observed. However, the results showed that the sensitivity could not be increased. So, it was decided to continue with the previous method using absorbance at 310 nm, since the later had a very time-consuming and complex procedure.

Afterwards, reaction conditions were modified. According to the TAH procedure, reaction took place in different test tubes and for each data, one tube was used. In this procedure, it was hard to keep the substrate-enzyme ratio constant in each test tube. To maintain consistency, the reaction was started to carry out in a 50 ml erlenmayer flask instead of various test tubes. Samples were withdrawn from the same reaction medium and added to 90% ethanol solution. The dilution factor was also optimized. Libuchi *et al.* (1967) diluted the reaction mixture 100 times with 90% ethanol to stop the reaction. Different dilution ratios, such as 1/100, 1/80, 1/50 and 1/20 were tried and it was seen that 1/50 dilution ratio gave reasonable values. Finally, a reaction period to observe the initial reaction rate was determined. The reactions were followed in a time range of 10 minutes to 90 minutes. After these modifications, a linear initial reaction rate region was obtained up to 15 minutes; therefore, the reaction was followed for a 15 minutes period (Figure 3.1.b).

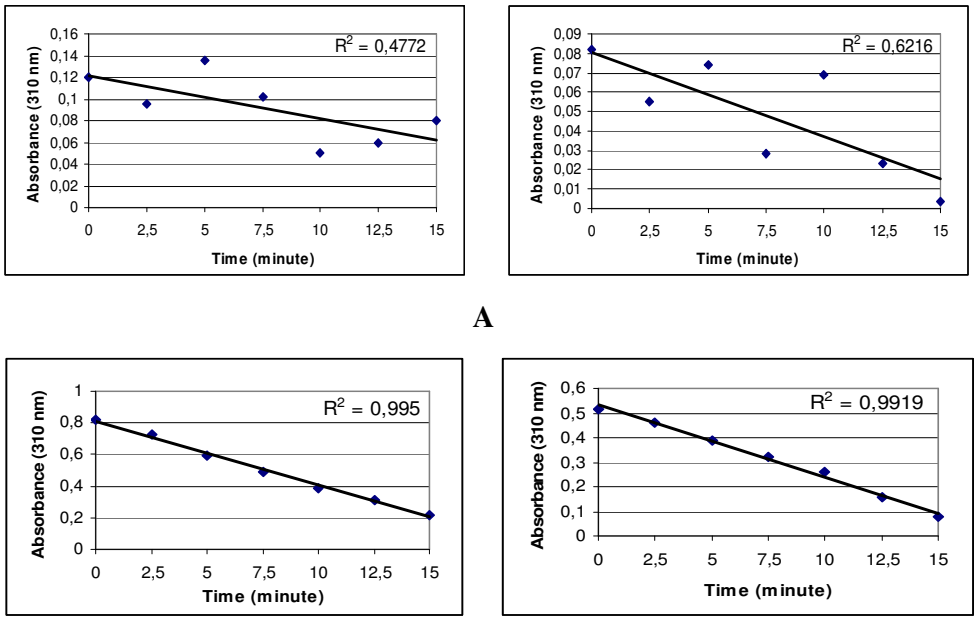

Figure 3.1 Progress curves of tannic acid hydrolysis reactions by tannase
A) Original method of Libuchi et al. (1967)
B) After modification of reaction conditions

3.2 Production of tannase from *Aspergillus niger*

In literature, tannase from *A. niger* has been produced in submerged, solid state and liquid surface cultures. All of these cultivation systems have some advantages and disadvantages.

Tannase production has been mostly studied in SmC and SSC. Submerged cultivation is the preferred method in general, since sterilization and process control are easier to engineer in this system. SSC has advantages over SmC and LSC, especially regarding the economy and simplicity of the process. However, it is hard to control process parameters in SSC and it is under the risk of contamination.

Different growth media has been used for the production of tannase from *A. niger* in literature. Tables 3-1, 3-2 and 3-3 present some of the growth media used for tannase production in SSC, LSC and SmC, respectively. As indicated in Tables, there is not much difference between the cultivation medium components and their concentrations in SmC, SSC and LSC.

Table 3.1 Different growth media used for tannase production from *A. niger* in SSC

Cultivation medium	**Cultivation conditions**	**Location of tannase**	**Reference**
Tannic acid, 2% $CaCl_2.2H_2O$, 0.002% KH_2PO_4, 0.1% $MnCl_2.6H_2O$, 0.0004% $(NH_4)_2SO_4$, 0.2% $Na_2MoO_4.2H_2O$, 0.0002% $MgSO_4.7H_2O$, 0.02% $FeSO_4.7H_2O$, 0.00025%	30°C	Intracellular and extracellular	Aguilar et al., 1999
KH_2PO_4, 0. 1% $MnCl_2.6H_2O$, 0.0004% $(NH_4)_2SO_4$, 0.2% $Na_2MoO_4.2H_2O$, 0.0002% $MgSO_4.7H_2O$, 0.02% $FeSO_4.7H_2O$, 0.00025% $CaCl_2.2H_2O$, 0.002% Various concentrations of tannic acid (1.25-10%)and glucose (0.625-5%) were added to the medium.	30°C	Intracellular and extracellular	Aguilar et al., 2001

Table 3.2 Different growth media used for tannase production from *A. niger* in LSC

Cultivation medium		Cultivation conditions	Location of tannase	Reference
Tannic acid, 2%	$CaCl_2.2H_2O$, 0.002%	30°C carried out statically	Intracellular and extracellular	Lekha and Lonsane, (1994)
KH_2PO_4, 0.1%	$Na_2MoO_4.2H_2O$, 0.0002%			
NH_4NO_3, 0.2%	$FeSO_4.7H_2O$, 0.00025%			
$MgSO_4.7H_2O$, 0.02%	$MnCl_2.6H_2O$, 0.0004%			

Table 3.3 Different growth media used for tannase production from *A. niger* in SmC

Cultivation medium		Cultivation conditions	Location of tannase	Reference
Tannic acid, 2%	$CaCl_2.2H_2O$, 0.002%	30°C and 220rpm shake-flask bioreactor	Intracellular and extracellular	Lekha and Lonsane, (1994)
KH_2PO_4, 0.1%	$Na_2MoO_4.2H_2O$, 0.0002%			
NH_4NO_3, 0.2%	$FeSO_4.7H_2O$, 0.00025%			
$MgSO_4.7H_2O$, 0.02%	$MnCl_2.6H_2O$, 0.0004%			
Tannic acid, 1%	KH_2PO_4, 0.152%	37°C under static conditions	Extracellular	Bradoo *et al.,* 1996
$NaNO_3$, 0.6%	$Cu(NO_3)_2.3H_2O$, traces			
KCl, 0.052%	$ZnSO_4.7H_2O$, traces			
$MgSO_4.7H_2O$, 0.052%	$FeSO_4.7H_2O$, traces			
Tannic acid, 2%	$MgSO_4$, 0.05%	30°C and 120rpm Shake –flask bioreactor	Intracellular	Sharma *et al*., 2000
$NaNO_3$, 0.3%	KCl, 0.05%			
K_2HPO_4, 0.1%				
KH_2PO_4, 0. 1%	$MnCl_2.6H_2O$, 0.0004%	30°C 220 rpm shake -flask bioreactor	Intracellular and extracellular	Aguilar *et al*., 2001
$(NH_4)_2SO_4$, 0.2%	$Na_2MoO_4.2H_2O$, 0.0002%			
$MgSO_4.7H_2O$, 0.02%	$FeSO_4.7H_2O$, 0.00025%			
$CaCl_2.2H_2O$, 0.002%				
Various concentrations of tannic acid (1.25-10%) and glucose (0.625-5%) were added to the medium.				
Tannic acid, 2%	$MgSO_4$, 0.05%	30°C and 120rpm shake-flask bioreactor	-	Bhardwaj *et al*., 2003
$NaNO_3$, 0.3%	KCl, 0.05%			
K_2HPO_4, 0.1%				
Tannins, 5%	$MgSO_4.7H_2O$, 0.1%	30-33°C	Extracellluar and intracellular	Belmares *et al*., 2004
Glucose, 1%	$ZnSO_4.7H_2O$, 0.01%			
$(NH_4)_2HPO_4$, 0.5%	NaCl, 0.0005%			
KH_2PO_4, 0.1%				

After selection of SmC as the most suitable cultivation type, different media used for tannase production by *A. niger* in SmC were compared and the medium used by Belmares *et al*. (2004) was chosen to start with. Tannic acid ranged from 0.5% to 10% was used as the carbon source as well as inducer in all media, and the tannic acid concentration was 5% in the selected medium. Different from others, Belmares *et al*. (2004) added 1% glucose as an additional carbon source. They reported that addition of 1% glucose induced tannase production. Different nitrogen sources (NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$) were used in a range of 0.2-0.6%, and in the selected medium nitrogen source concentration was 0.5%. All media contained 0.1% KH_2PO_4 or K_2HPO_4 and 0.02-0.1% $MgSO_4.7H_2O$. The selected medium contained 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$. Besides low amounts of different salts, $Cu(NO_3)_2$, $FeSO_4$, $MnCl_2$, NaCl and KCl, were also added to the media in literature. The selected one contained 0.01% $ZnSO_4.7H_2O$ and 0.0005% NaCl.

A. niger was cultivated in the selected medium. Daily samples withdrawn from the cultivation medium were assayed spectrophotometrically for the production of extracellular tannase. The time cource of tannase production is shown in Figure 3.2.

Extracellular tannase production first appeared on the 2nd day of cultivation and the maximum extracellular tannase activity, 193 U/ml, was observed on the 5th day of cultivation. Thereafter, tannase activity decreased. The reason of the decrease in tannase activity after the 5th day may be the catabolic repression of gallic acid which is produced from tannic acid, tannic acid limitation, secretion of toxic substances, namely catechuic acid, 2,6-dihyroxy benzoic acid and pyrogallol which can cause cell lysis, low pH values at the end of the culture or proteolytic cleavage. Kar and Banerjee (2000) showed that extracellular tannase activity in SmC decreased at the end of the cultivation. Aguilar *et al*. (2001.a and b) also observed such decrease in the activity. They assumed that the decrease in tannase activity might be related to the protease production which is favoured by lack of substrate at the end of the cultivation and extracellular tannase might be broken down by proteases.

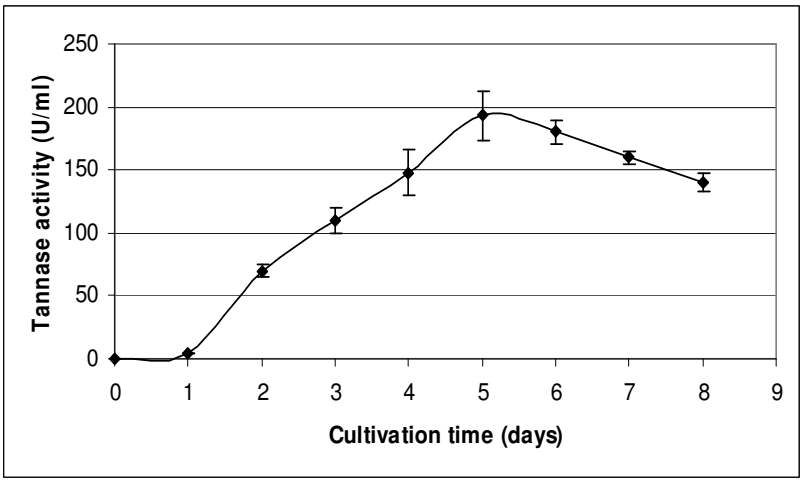

Figure 3.2 Time course of extracellular tannase production by *A. niger* in SmC. Cultivation medium: 5% Tannic acid, 1% Glucose, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35°C, 175 rpm.

Lekha and Lonsane (1994) also studied the extracellular tannase production from *A. niger* PKL 104 in a medium containing 2% tannic acid in submerged cultivation. Similar to our study, tannic acid was used as the carbon source and inducer. Tannase activity was assayed at 310 nm, pH 4.7 and 30°C. The maximum extracellular tannase activity (57 U/ml) was obtained on the 6th day of cultivation. In our case, tannase activity was assayed at 310 nm, pH 5.5 and 40°C and the maximum extracellular tannase activity (193 U/ml) was obtained on the 5th day of cultivation. The temperature difference of 10°C should increase activity, but it could not result in an increase of about 300%. The pH difference also might not cause such increase in activity. This increase might be related to the increase in tannic acid concentration from 2% to 8% and changes in medium composition.

As will be discussed in section 3.3.2.1, by using 2% tannic acid, the maximum extracellular tannase activity, 75 U/ml, was obtained on the 4th day. According to Lekha and Lonsane (1994), the extracellular tannase activity was

30 U/ml on the 4th day of cultivation. In addition to the increase that might be related from the differences in temperature and pH, another parameter must have contributed to 150% increase in tannase activity. This effective parameter must be the medium composition. Therefore, increase in $MgSO_4$ concentration and addition of different cations might be effective, however the most important parameter was probably the addition of glucose and change in nitrogen source and nitrogen source concentration. These results may show that the selected cultivation medium was better than the one used by Lekha and Lonsane (1994) for extracellular tannase production.

3.3 Effect of Culture Conditions on Tannase Production

In a typical cultivation process, microbial growth and enzyme production are affected by number of conditions. They are mainly temperature, pH, aeration, shaking rate, type and concentration of carbon source, inducer, nitrogen source and salts.

In literature, the optimum cultivation temperature and the pH for tannase production by *A. niger* were found to be 30-35^{o}C and 5-5.5, respectively. The shaking rate differs from 120 to 220 rpm. Besides, there was not any study about the effect of aeration on tannase production by *A. niger* in SmC. In our study, temperature, pH and shaking rate were selected as 35^{o}C, 5.5 and 175 rpm, respectively and the effect of bioreactor's gas volume/culture volume of the bioreactor was investigated.

There were studies about the effect of carbon source and inducer on tannase production by *A. niger* in SmC in literature, whereas there was not any study about the effect of nitrogen source. In this study, the most important parameters in tannase and biomass production, namely tannic acid and glucose concentrations, type and concentration of nitrogen source were investigated. The effects of salts on tannase production by *A. niger*, however, were beyond the scope of this study

despite the lack of studies reported in literature. These effects are certainly worth to investigate in future studies.

3.3.1 Effect of Bioreactor's Gas Volume / Culture Volume on Tannase Production

In literature, there is not any study about the effect of aeration on tannase production by *A. niger* in SmC. In this study, firstly the possible effect of bioreactor's gas volume / culture volume ratio in the bioreactor on tannase production was investigated.

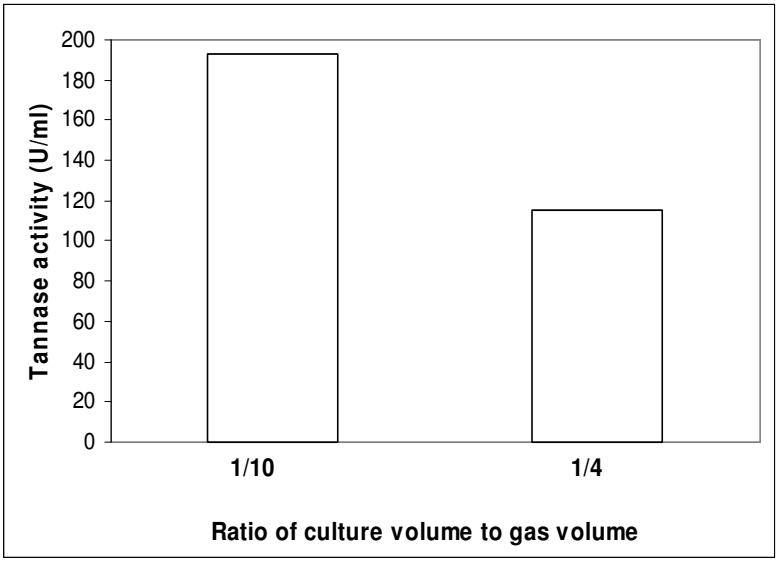

Figure 3.3 The effect of the ratio of the bioreactor's gas volume to culture volume on maximum tannase production by *A. niger* in SmC. Cultivation medium: 5% Tannic acid, 1% Glucose, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35°C, 175 rpm. 1/4; 7th day of the cultivation, 1/10; 5th day of the cultivation

Results showed that increase in culture volume/bioreactor's gas volume ratio from 1/10 to 1/4 caused 40% decrease in tannase production. Moreover, the maximum extracellular tannase activity was obtained in a longer time interval, 7 days instead of 5 days. Therefore, decreasing culture volume which increases O_2 concentration favored tannase production and culture volume/bioreactor volume ratio was selected as 1/10 in further experiments.

3.3.2 Effect of Carbon Source and Inducer on Tannase Production

3.3.2.1 Effect of Tannic Acid Concentration on Tannase Production

In literature, tannic acid, methyl gallate, m-digallic acid, ethyl gallate and n-propylgallate are used as a carbon source for tannase production (Belmares *et al*., 2004, Lekha and Lonsane, 1997). Phenolic compounds such as tannic acid, gallic acid and methyl gallate are known to induce tannase synthesis (Bajpai and Patil, 1997).

Aguilar *et al*. (2001.a and b) investigated the effect of different carbon sources such as tannic acid, gallic acid and glucose on tannase production from *A. niger* Aa-20 and tannic acid was found as the most efficient carbon souce as well as inducer. Similarly, Sabu *et al*. (2005) confirmed that tannic acid was the most efficient carbon source for tannase from *A. niger* ATCC 16620. Therefore, tannic acid was used as a carbon source as well as inducer in this study.

Lekha and Lonsane (1997) also reported that the initial tannic acid concentration is a crucial factor for tannase production. They mentioned that initial tannic acid concentration affected the levels of enzyme, and the cultivation period depends on both the microorganism and the tannic acid concentration.

In view of the importance of tannic acid on tannase production, different concentrations of tannic acid (2-12%) were added to the production medium.

The effects of tannic acid concentration on enzyme production are shown in Figure 3.4. During the cultivation, the highest activity was observed with the medium containing 8% tannic acid, whereas the lowest activity was observed with the medium containing 2% tannic acid. The maximum extracellular tannase activity was observed on the 7th day of cultivation with the medium containing 8% tannic acid.

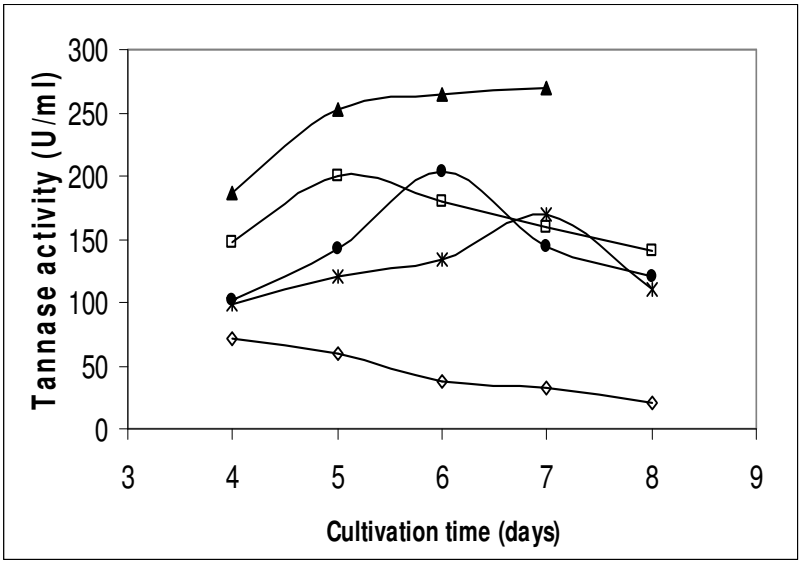

Figure 3.4 The effect of different tannic acid concentrations; 2% (◊), 5% (□), 8% (▲), 10% (●), 12% (x) on tannase production. Cultivation medium: 1% Glucose, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^{o}C and 175 rpm.

To evaluate the effects of tannic acid concentration on enzyme production and biomass, 7th day cultivation samples were compared as shown in Figure 3.5. Increases in tannic acid concentration up to 8% resulted in increase in both tannase activity and growth. Maximum tannase activity was obtained with 8% tannic acid whereas maximum growth was favoured with 10% tannic acid. Above 8% tannic acid, enzyme production was reduced significantly. A number of factors could be

responsible for the decline in tannase activity with increasing substrate quantity. It may be resulted from change in pH, catabolite repression caused by the substrate and/or end product, gallic acid. It may also be resulted from the precipitation of extracellular tannase produced in the cultivation medium by tannic acid or deposition of gallic acid on cell surface at tannic acid concentrations higher than 8%. Kar and Banerjee (1999) also observed such a decrease with increasing substrate concentration. They assumed that the intermediate hydrolysates, namely 1,2,3,4,6-pentagalloyl glucose, 2,3,4,6-tetragalloyl glucose, and monogalloyl glucose produced during hydrolysis of tannic acid could bind competitively or noncompetitively to substrate binding sites, thus caused a decrease with increasing substrate concentration. They also added that hydroxyl groups in gallic acid could form hydrogen bonds with the amino acids present in the active sites of the enzyme, which leaded conformational changes.

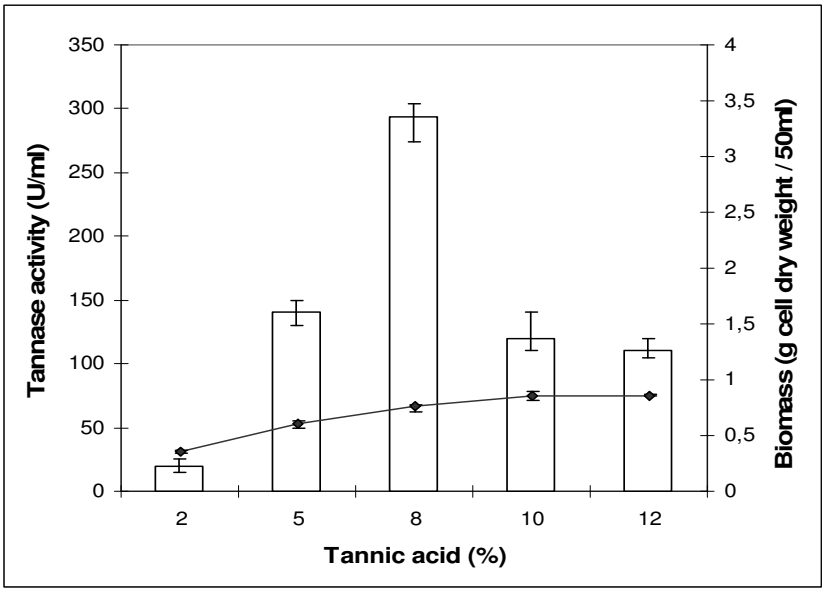

Figure 3.5 The effect of tannic acid concentration on tannase production; bars and biomass production; ♦. Cultivation medium: 1% Glucose, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^{o}C and 175 rpm. Cells were harvested on the 7th day of cultivation.

Aguilar *et al*. (2001.b) also studied the effect of tannic acid concentration on tannase production from *A. niger* Aa-20. Similar to our results, increase in tannic acid concentration from 1.25% to 5% resulted in an increase in tannase production, however, the maximum was reached at 5% tannic acid. They also showed that higher tannic acid concentrations (10%) caused a decrease in tannase production. According to the study done by Banerjee *et al.* (2001), maximum growth and extracellular tannase production from *Aspergillus aculeatus* DBF 9 occurred in the presence of 2% tannic acid.

Aguilar et al. (2001.a) studied the effect of glucose concentration on biomass production. Maximum biomass production (0.6g / 50ml) was obtained in a medium containing 1.25% glucose and 2.5% tannic acid. In our case, biomass production, 0.35g / 50ml, was obtained in a medium containing 1% glucose and 2% tannic acid. The increase in initial glucose concentration might cause increase in tannase production. The type and concentration of salts added to the cultivation medium might also have affected the tannase production.

Figure 3.6 presents the effect of tannic acid concentration on tannase/biomass yield coefficients. Addition of tannic acid up to 8% caused an increase in tannase/biomass yield. Maximum yield was obtained in the presence of 8% tannic acid. Above 8% tannic acid, yield decreased showing a possible repression of tannic acid on tannase production. This decrease may be resulted from the precipitation of tannic acid with proteins.

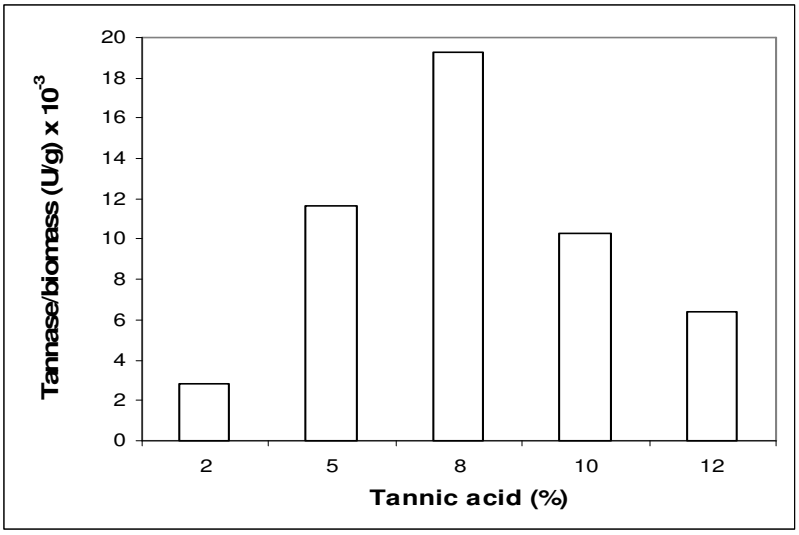

Figure 3.6 The effect of tannic acid concentration on tannase/biomass ratio. Cultivation medium: 1% Glucose, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation was performed at 35^{o}C and 175 rpm. Cells were harvested on the 7th day of cultivation.

3.3.2.2 Effect of Glucose Concentration on Tannase Production

In literature, there are a few studies showing the effect of sugars as additional carbon sources on tannase production. Belmares *et al*. (2004) reported that addition of carbon sources such as glucose, sucrose, maltose, arabinose to the culture medium at initial concentrations from 10 to 30 g/l improves tannase production by *A. niger*. On the other hand, Bradoo *et al*. (1997) found that the addition of sugars did not have a positive effect on tannase production from *Aspergillus japonicus* with the exception of glucose. Huang *et al.* (2005) also reported an increase in tannase production when sucrose or glucose was present in addition to tannic acid in the medium.

In this study, different concentrations of glucose as an additional carbon source were added to the medium to improve the tannase production. The influence of initial glucose concentration on extracellular tannase production is shown in Figure 3.7.

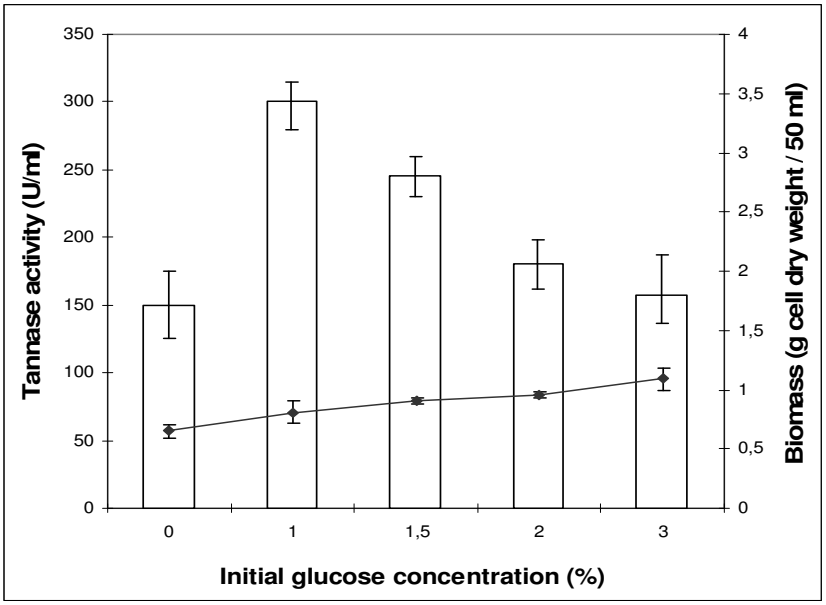

Figure 3.7 The effect of glucose concentration on tannase production; bars and biomass production; ♦. Cultivation medium: 8% Tannic acid, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^{o}C and 175 rpm. Cells were harvested on the 7th day of cultivation.

The results showed us that tannase production and growth were minimum in the absence of glucose. Lekha and Lonsane (1997) reported that large size of tannic acid prevents uptake through the cell membrane and therefore can not be an inducer. In the absence of glucose, microorganism produces a basic level or constitutive amount of tannase that hydrolyses tannic acid to glucose and gallic

acid, which can enter the microbial cell and function as an inducer. But, the quantities are insufficient to support growth of a strain that produces tannase. Similar to our results, Mondal and Pati (2000) also observed that the tannase production from *Bacillus licheniformis* reached its peak value when glucose was present (0.1%) in addition to tannic acid (1%) in the medium.

Maximum extracellular tannase production was obtained with 1% glucose in a medium containing 8% tannic acid. Addition of glucose above 1% caused slightly higher biomass but lower tannase production. Glucose concentration higher than 1% may cause catabolic repression. This decrease might be because the glucose is effectively used by the microorganism instead of tannic acid, since it can enter the microbiol cell and is presumably metabolised faster than the other carbon sources (Legamaat and Pyle, 2005). When the amount of glucose is high in the initial medium, microorganism does not prefer to use tannic acid, which reduces enzyme production.

This result shows us that the production rate can be enhanced by the addition of small amounts of glucose in the initial growth medium since glucose can act as a catabolic inducer. Similarly, Aguilar *et al.* (2001.b) found that increase in glucose concentration from 0.625% to 2.5% in addition to 2.5% tannic acid caused increase in tannase production by *A. niger* Aa-20. They also mentioned that glucose concentrations higher than 2.5% resulted in strong catabolic repression. According to the study done by Banerjee *et al.* (2001), the maximum extracellular tannase production from *A. aculeatus* DBF 9 occurred at 0.1% glucose in medium containing 2% tannic acid and further increase caused decrease in tannase production. They assumed that higher glucose concentrations repressed enzyme production due to the availability of readymade carbon source. They also reported that increase in glucose concentration caused increase in biomass production.

Figure 3.8 presents the effect of glucose concentrations on tannase/biomass yield coefficients. Maximum yield was obtained with 1% glucose.

These results support the suggestion by Aguilar *et al.* (2001.a) that maximum yield was obtained with 1.25% glucose. It was also confirmed that further increase caused decrease and 5% glucose led to complete repression of tannase activity.

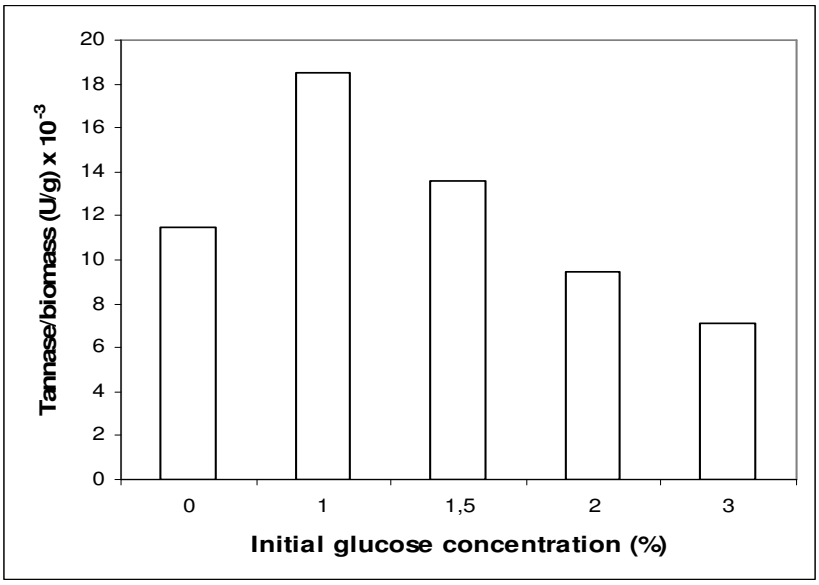

Figure 3.8 The effect of glucose concentration on tannase/biomass ratio. Cultivation medium: 8% Tannic acid, 0.5% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^oC and 175 rpm. Cells were harvested on the 7th day of cultivation.

3.3.2.3 Effect of Nitrogen Source on Tannase Production

The effect of different organic and inorganic nitrogen sources was studied to increase tannase production. In this respect, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4NO_3, $NaNO_3$, peptone and yeast extract were tested. Organic and inorganic sources were added at a concentration of 0.5%.

As shown in Figure 3.9, change in nitrogen source did not cause much change in biomass. However, maximum tannase production was obtained when $(NH_4)_2HPO_4$ was used as a nitrogen source. $NaNO_3$ was also found as good nitrogen source for tannase production. Results also showed us that $(NH_4)_2SO_4$ and NH_4NO_3 were inhibitory for tannase production. These results suggest that Na and PO_4 may enhance the extracellular tannase production. Yeast extract and peptone are known as very efficient nitrogen sources; however they were not found as efficient as $(NH_4)_2HPO_4$ for tannase production. This may be resulted from the precipitation of tannic acid with proteins found in yeast extract and peptone.

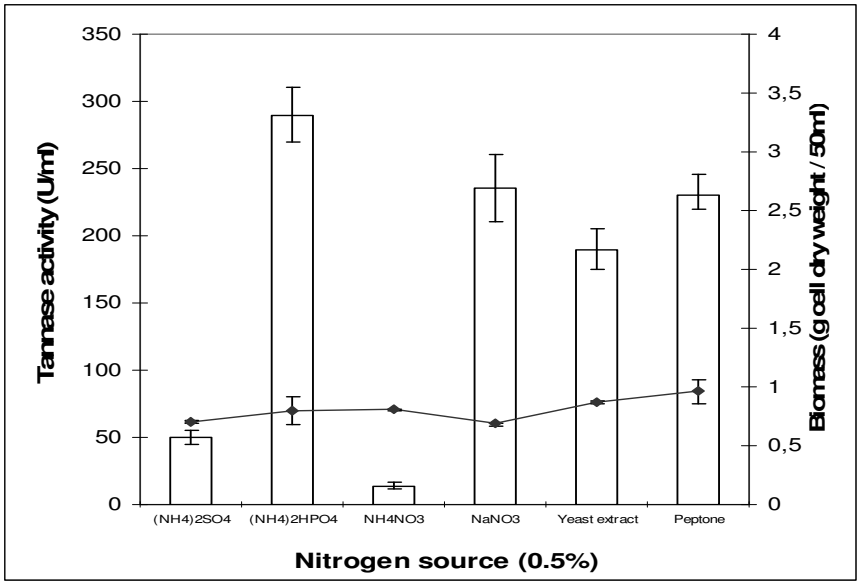

Figure 3.9 The effect of nitrogen source on tannase production; bars and biomass production; ♦. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.5% nitrogen source, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation was performed at 35^oC and 175 rpm. Cells were harvested on the 7th day of cultivation.

In literature, best nitrogen source differs from one fungus to another. For instance, Bradoo *et al.* (1997) also investigated the effect of different nitrogen souces on extracellular tannase production from *A. japonicus* and 0.2% $NaNO_3$ was found as the most efficient nitrogen source among NH_4NO_3, KNO_3, NH_4Cl and aspartic acid.

Figure 3.10 shows the effect of different nitrogen sources on tannase sythesis measured as tannase / biomass yield coefficients. Tannase / biomass yield was directly proportional with the tannase production because there is not much change in growth. So, maximum yield was obtained when $(NH_4)_2HPO_4$ used as a nitrogen source.

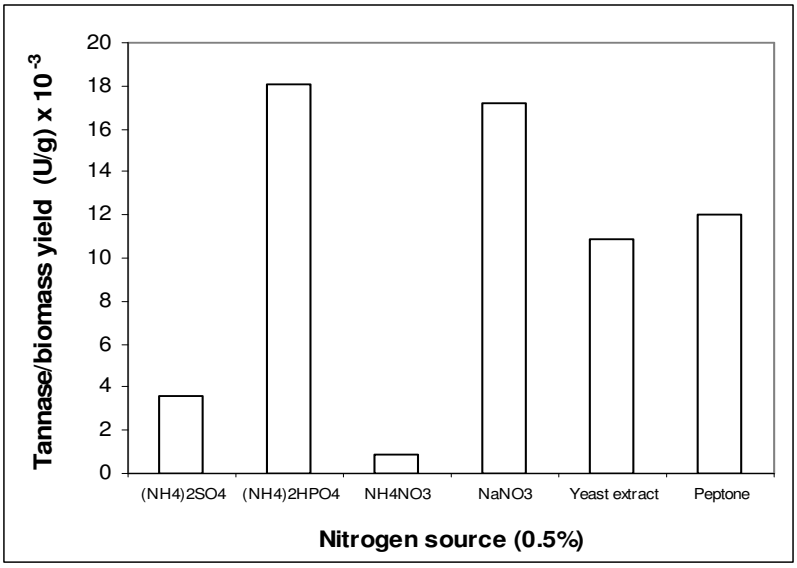

Figure 3.10 The effect of nitrogen source on tannase/ biomass ratio. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.5% nitrogen source, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^{o}C and 175 rpm. Cells were harvested on the 7th day of cultivation.

3.3.2.4 Effect of $(NH_4)_2HPO_4$ Concentration on Tannase Production

After determining $(NH_4)_2HPO_4$ as the most efficient nitrogen source, the effect of different concentrations of $(NH_4)_2HPO_4$, in the range of 0.2-0.8%, were investigated. The effect of $(NH_4)_2HPO_4$ concentration on tannase and biomass production is shown in Figure 3.11.

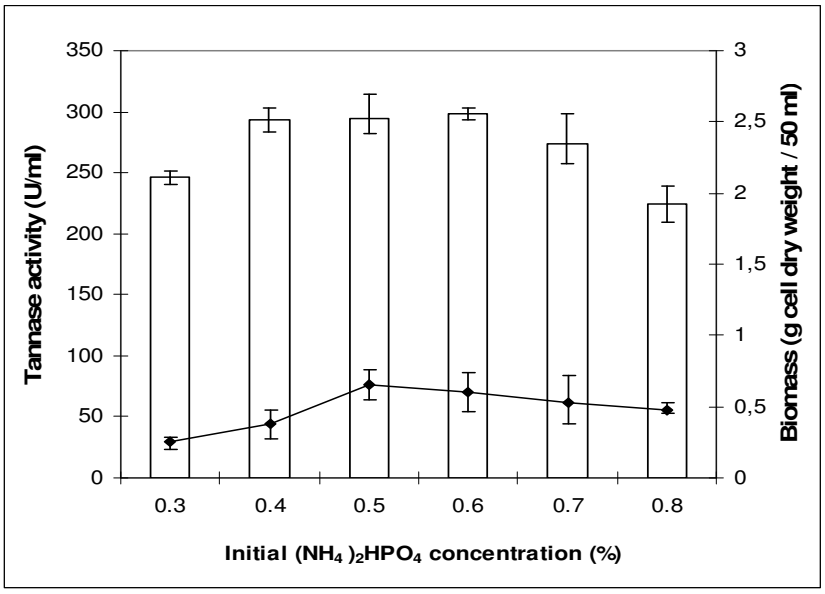

Figure 3.11 The effect of $(NH_4)_2HPO_4$ concentration on tannase production; bars and biomass production; ♦. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^oC and 175 rpm. Cells were harvested on the 7th day of cultivation.

Tannase production was nearly the same in a range of 0.4-0.6%. It was also shown that higher concentrations decreased tannase production. So, 0.4% $(NH_4)_2HPO_4$ was the preferred as nitrogen source concentration in order to

decrease the cost of enzyme production. It was also observed that increase in nitrogen source concentration up to 0.5% increased biomass production, reached a maximum with 0.5% $(NH_4)_2HPO_4$ and further increase decreased the biomass production.

Figure 3.12 shows the effect of different $(NH_4)_2HPO_4$ concentrations on tannase / biomass yield coefficients. Results showed that 0.3% $(NH_4)_2HPO_4$ was the most efficient among all and increase in $(NH_4)_2HPO_4$ concentration up to 3% decreased tannase/biomass yield.

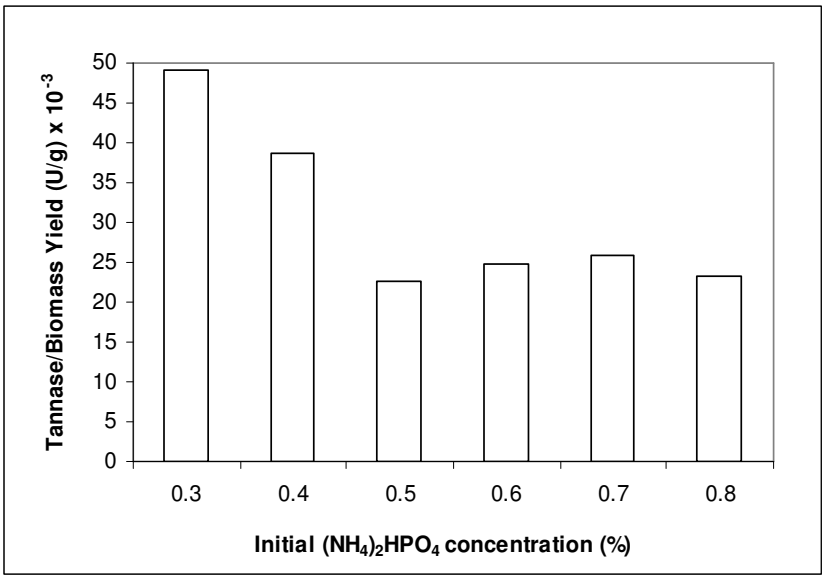

Figure 3.12 The effect of nitrogen source concentration on tannase/ biomass ratio. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed in submerged culture at 35^oC and 175 rpm. Cells were harvested on the 7th day of cultivation.

3.4 Determination of the Cultivation Profile

The profiles including tannic acid, gallic acid, glucose, pyrogallol and protein concentrations, pH, biomass production and extracellular tannase activities were determined under the optimised conditions.

In this respect, firstly, extracellular tannase production profile was determined between the 1st and the 9th days of production under the optimized conditions which were 8% tannic acid, 1% glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Time course of extracellular tannase activities is shown in Figure 3.13.

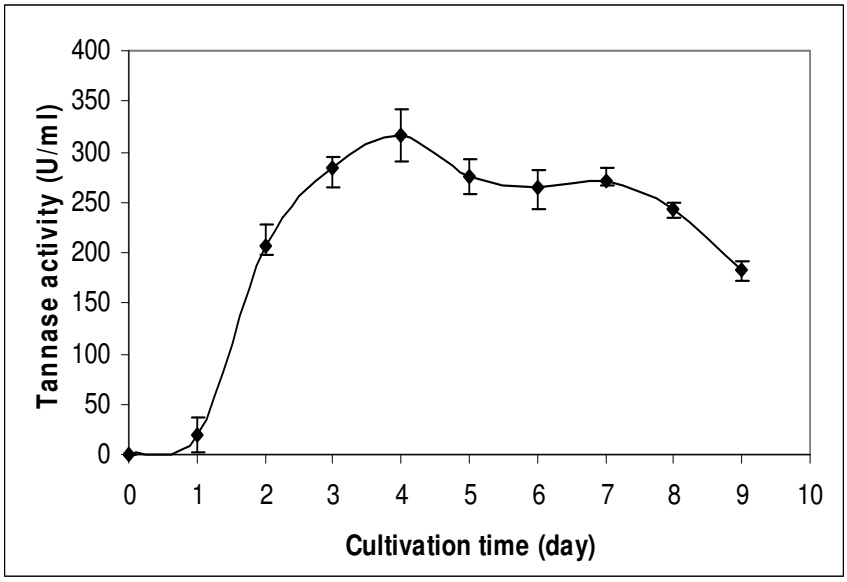

Figure 3.13 Time course of extracellular tannase production by *A. niger* in SmC. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35°C and 175 rpm.

Maximum extracellular tannase activity was found as 193 U/ml before the optimization. After the optimization, it increased 64%, and reached a value of 316 U/ml. Moreover, the maximum extracellular tannase activity was obtained in a shorter time interval, 4 days instead of 5 days (Figures 3.2 and 3.13).

Extracellular tannase production first appeared on the first day of cultivation; however the amount of extracellular tannase was very low. Tannase production increased up to 4^{th} day and the maximum extracellular tannase activity was observed on the 4^{th} day of cultivation. Thereafter, tannase activity decreased. Possible reasons of this decrease are proteolytic action; tannic acid-protein complex formation; the catabolic repression of gallic acid; tannic acid limitation; secretion of toxic substances, namely catechuic acid, 2,6-dihyroxy benzoic acid and pyrogallol; low pH values at the end of the bioreaction.

Lekha and Lonsane (1997) suggested that the microorganism produced a basic level of tannase at the beginning of the cultivation. This suggestion can be supported on the basis of our results. As shown in Figure 3.14, although the amount of extracellular tannase was low, hydrolysis of tannic acid started on the first day of cultivation. According to the study done by Lagemaat and Pyle (2005), although tannic acid hydrolysis occured at the start of the cultivation, the amount of extracellular tannase was quite low. They reported that the enzyme was mainly intracellular during the first day of the cultivation. Similar to Lagemaat's (2005), in this study, it is also possible that most of the enzyme was intracellular during the first day of cultivation. But, in this study, only extracellular tannase activity was determined.

Lekha and Lonsane (1994) mentioned that enzyme produced by *A. niger* PKL 104 was exclusively intracellular during the first 48h of cultivation. But in this study and in the study done by Lagemaat and Pyle (2005), the extracellular tannase production started on the first day. The difference may be resulted from the addition of free glucose to the growth medium. In this study and the study done by Lagemaat and Pyle (2005), free glucose was added to the initial growth medium as

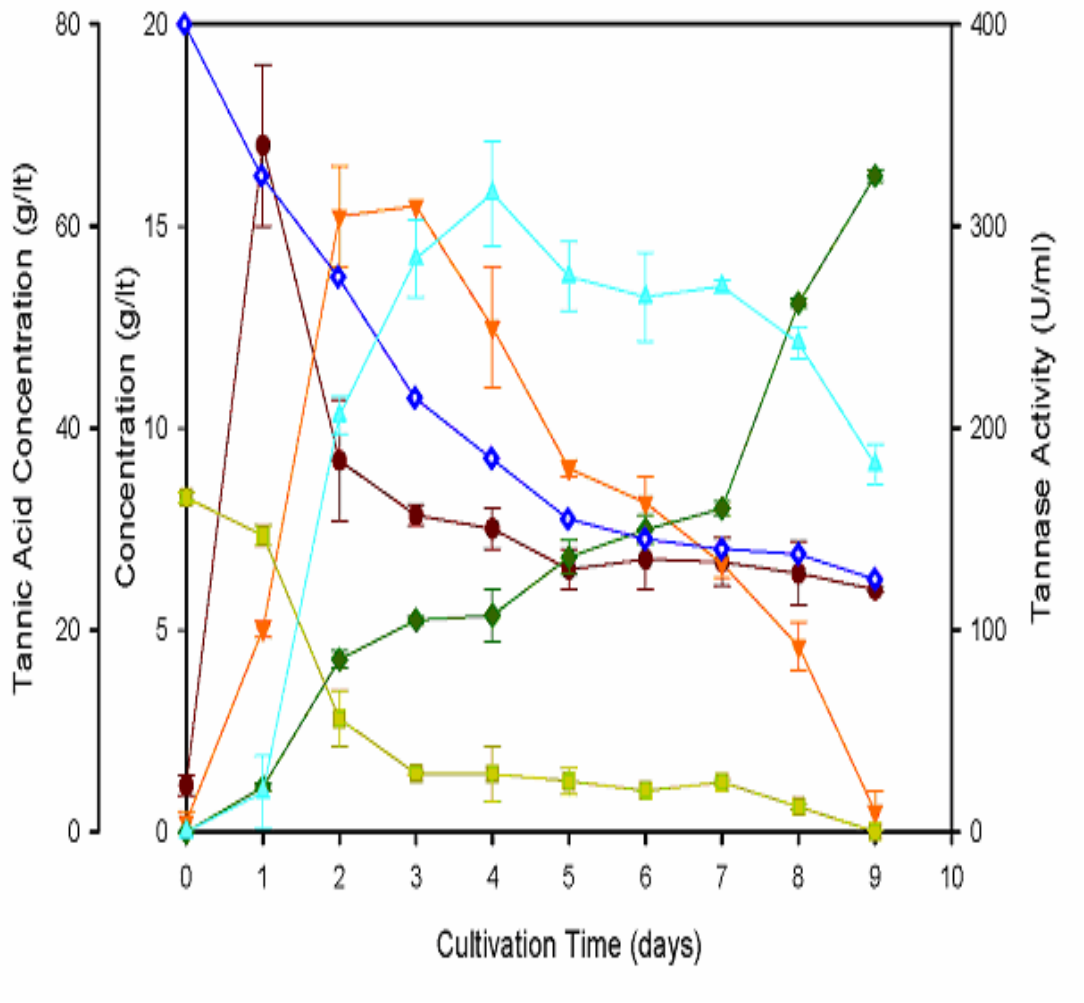

Figure 3.14 Time course of dry weight (♦), extracellular tannase production (▲), tannic acid (▫), gallic acid (●), glucose (■) and pyrogallol (▼) by A. niger in SmC. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed in submerged culture at 35°C, 175 rpm.

an additional carbon source, whereas in the study done by Lekha and Lonsane (1994) there was no glucose addition to the gowth medium initialy. So, as indicated by Legamaat and Pyle (2005), the exhaustion of the glucose increased the rate of extarcellular tannase formation. Lekha and Lonsane (1997) also added that addition of glucose to the growth medium containing tannic acid shortened the lag phase.

Figure 3.14 shows the bioreaction profile during tannase production by *A. niger*. The extracellular tannase production started with biomass production on the first day of cultivation. Therefore, it can be concluded that the tannase production was directly proportional to the growth of organism and the extracellular tannase production was immediately started during the initial phase of growth.

More than one log phase were observed in growth profile. In the first day, glucose and tannic acid concentrations decreased, whereas gallic acid concentration increased. In this period of time, the microorganism tried to adjust to the new environment and used glucose as a readily available carbon source and tannic acid as both carbon source and inducer. Whether the microorganism used gallic acid as a carbon source on the first day or not was unknown.

Lagemaat and Pyle (2005) found that the microorganism did not consume significant quantities of gallic acid until 18 hours of the cultivation and in this period, the microorganism must have utilized glucose and biomass increased. Moreover, they mentioned that the formation of biomass from glucose was more efficient than from gallic acid. Therefore, it can be concluded that glucose concentration is a very important parameter for biomass production.

Another product, pyrogallol, formation also started on the first day. This may show that the increase in gallic acid concentration favors the gallic acid decarboxylase production so gallic acid is converted to pyrogallol. Therefore, results indicated that both tannase and gallic acid decarboxylase were produced in the cultivation medium. Similar results were obtained by Sharma *et al.* (1998) in which *A. niger* van Tiegham utilized tannic acid and degraded it to gallic acid and

pyrogallol by tannase and gallic acid decarboxylase, respectively. Kumar *et al.* (1999) also mentioned that the metabolic intermediates in the tannic acid containing culture of *Citrobacter freundii* were gallic acid, glucose and pyrogallol.

On the second day, biomass production increased sharply. The amount of tannic acid and glucose concentrations decreased since the microorganism continued to utilize both tannic acid and glucose as a carbon source. Gallic acid concentration decreased, whereas pyrogallol concentration increased. Therefore, gallic acid convertion to pyrogallol also continued on the second day. The decrease in gallic acid concentration may be a result of the consumption of gallic acid by the microorganism.

On the third day, tannic acid and glucose concentrations continued to decrease, while biomass production has still increased. However, biomass production rate decreased. This may be resulted from the decrease in glucose concentration or production of toxic substances in the cultivation medium. The amount of gallic acid continued decreasing, whereas increase in pyrogallol concentration was very slight. Since the glucose concenration was low on the third day, the microorganism might have looked for another carbon source. It might utilize gallic acid as a carbon source in addition to glucose. So, the amount of gallic acid converted to pyrogallol decreased. The decrease in pyrogallol production rate might also be related to the utilization of pyrogallol by the microorganism as a carbon source on the third day. The reason why the microorganism looked for another carbon source although high amounts of tannic acid was found in the cultivation medium might be decreased utilizability of tannic acid because of tannic acid-protein complex formation.

On the 4^{th} day, biomass production and glucose concentrations did not change significantly. However, tannic acid, gallic acid and pyrogallol concentrations decreased. Results shows that the microorganism started to use pyrogallol in this period of time in addition to tannic acid and gallic acid.

On the 5th and the 6th days, biomass production increased, while tannic acid, gallic acid, glucose and pyrogallol concentrations continued to decrease. However, change in glucose and gallic acid concentrations was not significant. Therefore, it can be concluded from these results that the microorganism continued to utilize tannic acid and pyrogallol in this period of time.

The biomass production continued to increase on the 7th day, but not as much as the one through the 5th and the 6th days. This period of time, gallic acid and glucose concentrations did not change significantly whereas pyrogallol and tannic acid concentrations decreased. Therefore, the microorganism used tannic acid and pyrogallol as a carbon source. The decrease in pyrogallol concentration might be also related to the convertion of pyrogallol to pyruvate or another molecule. This suggestion was supported by the study done by Legamaat and Pyle (2005). According to their study, it is possible that pyrogallol was converted to 2-hydroxymuconic acid by pyrogallol 1,2 dioxygenase and 2-hydroxymuconic acid presumably was converted to pyruvic acid and propionic acid. The microorganism might started to utilize pyruvate or another carbon source produced in the cultivation medium on the 7th day of cultivation.

After 7th day, biomass production increased sharply. This period of time, microorganism utilized all the pyrogallol and glucose found in the cultivation medium, however tannic acid and gallic acid consumption were low. Hence, the microorganism must have supplied most of the energy needed from another carbon source. The microorganism might utilized pyruvate or another carbon source produced in the cultivation medium after the 7th day of bioreaction, which resulted in much increase in biomass production.

Besides the investigation of change of carbon source and product concentrations, biomass and extracellular tannase productions during the bioreaction, the pH profile was also evaluated.

As shown in Figure 3.15, initial pH of the medium is 5.5. During the first 3 days of bioreaction, the pH of the medium decreased to 2.8. This should be due to the production of gallic acid. After 3th day it reached a stationary phase, since in this time the change in glucose and gallic acid concentrations were not significant. Thereafter pH decreased in the 9th day. This decrease may be resulted from the utilization of residual glucose by the microorganism or production of 2-hydroxymuconic acid, pyruvic acid and propionic acid in the cultivation medium.

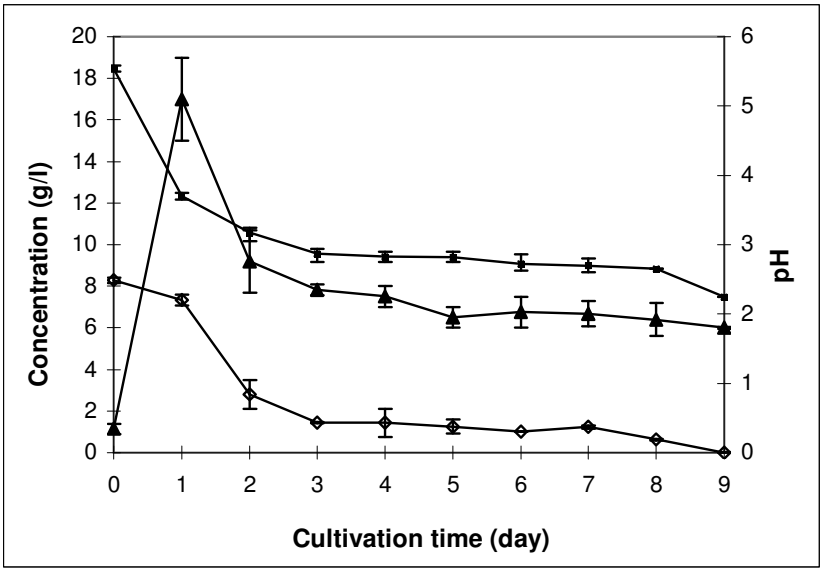

Figure 3.15 Time course of gallic acid (▲), glucose (◊) and pH (■) by *A.niger* in SmC. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35^{o}C and 175 rpm.

Change in protein concentration during the bioreaction was also investigated. Time course of extracellular tannase production and protein concentration were shown in Figure 3.16.

The extracellular tannase production continued from the 1st day till 9th day of bioreaction; however, protein concentration was found as zero during the cultivation, except the first day. This result supports the knowledge of Bhat *et al*. (1998) about the ability of tannic acid to precipitate proteins. Therefore, tannic acid bound to proteins and a complex formed.

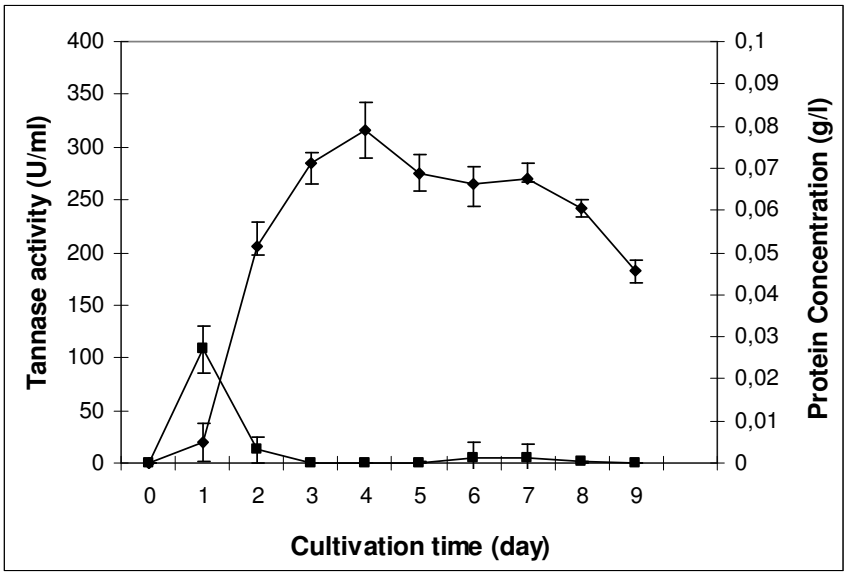

Figure 3.16 Time course of protein concentration (■) and extracellular tannase production (♦) by *A. niger* in SmC. Cultivation medium: 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35°C and 175 rpm.

The reason for the initial increase in protein concentration followed by a decrease may be related to the decrease in pH of the cultivation medium. As seen in Figure 3.17, the protein concentration increased on the 1st day in which extracellular tannase production started. Protein concentration decreased sharply on the 2nd day, and thereafter it was measured as zero during the cultivation. Similarly, pH of the bioreaction decreased sharply during the first three days. The

pH of the medium which was 5.5 initially decreased to 2.8 on the 3rd day. Thereafter, it did not change significantly during the bioreaction. Results indicated that complex formation between tannic acid and proteins was related to the pH of the cultivation and in our case, tannic acid might precipitate most of the proteins at or below at a pH value of 2.8.

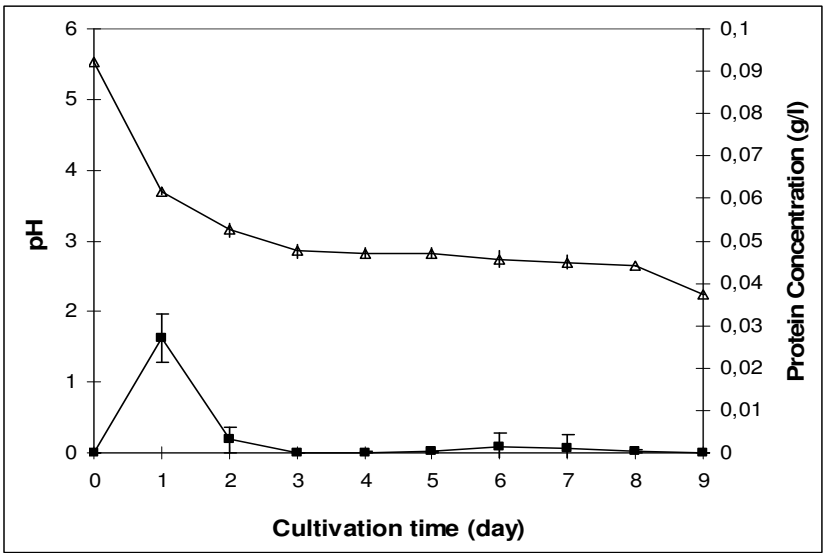

Figure 3.17 Time course of protein concentration (■) and pH (Δ) Cultivation medium: 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed at 35°C and 175 rpm.

These results support the suggestion of Giner-Chaves, (1996) that precipitation of proteins by tannins is maximum at pH values near the isoelectric point of the protein. He also added that at high pH, phenolic hydroxyls are ionized and proteins have net negative charges. Under these conditions, precipitation does not occur because proteins exhibit repulsive forces. According to the study done by Ramirez-Coronel *et al.* (2003), the pI value of tannase produced in SmC have an isoelectric point around 4.

The presence of tannic acid, gallic acid and pyrogallol in the bioreaction medium was also followed by thin layer chromatography analysis.

Thin layer chromatographic analysis of the cultivation broth reveals that tannase hydrolyzed the tannic acid into gallic acid. As seen in Figure 3.18, tannic acid concentration decreased during the bioreaction. After the second day, tannic acid concentration decreased sharply, but it was not consumed totally during the bioreaction. Gallic acid production started on the first day of cultivation. Gallic acid concentration was maximum on the first day, thereafter it decreased.

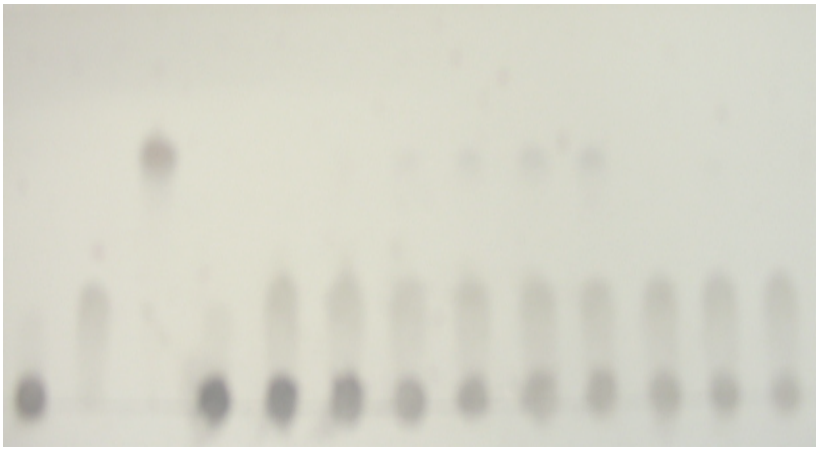

Figure 3.18 TLC results of biotransformation of tannic acid

Actually the microorganism degrades tannic acid by tannase into gallic acid and glucose. But glucose is not visible in the chromatograph, as the chromatographic strain $FeCl_3$ can detect only phenolic compounds.

Pyrogallol was also detected on TLC plate. This also shows the presence of gallic acid dexarboxylase in the cultivation medium. Gallic acid was converted to pyrogallol after the first day of cultivation, and maximum pyrogallol production

was obtained on the 6th day of the cultivation. The results contradicts the results obtained from HPLC (Figure 3.14). Considering the HPLC is more reliable method than TLC, it can be said that another product which has R_F value equal or very similar to pyrogallol might be seen on TLC plate.

Another distinction between the HPLC and TLC results was observed in tannic acid concentrations as seen in Figure 3.19. The presence of tannic acid could be easily detected on TLC plate and tannic acid concentration was quantified from TLC plate by using UVP Biolmaging Systems. Contrary to the results obtained from TLC, the tannic acid concentration was nearly zero after the 2nd day in HPLC resuls. These results show that the tannic acid forms complex with proteins which can not be detected by HPLC although it can be easily seen in TLC.

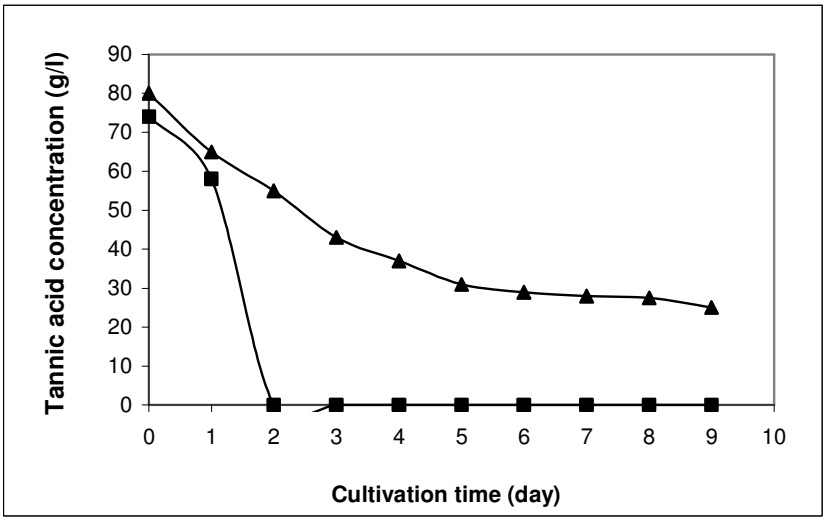

Figure 3.19 Time course of tannic acid concentration. HPLC analyses (■), TLC analysis (▲). Cultivation medium: 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl. Cultivation performed in submerged culture at 35°C, 175 rpm

To support this suggestion, different amounts of BSA (5%, 2,5%, 1%, 0.1%, 0.01% and 0.005%) was added to the 8% tannic acid solution and these samples were left at +4^{o}C for 2 days to induce complex precipitation. After 2 days, the precipitation could be easily seen by naked eye. After removing the precipitation, samples were analyzed by TLC in order to see whether all tannic acid precipitated with proteins or not.

As seen in Figure 3.20, tannic acid could be easily seen in all samples although it precipitated with proteins. It is seen that the amount of tannic acid was a little bit lower in the first sample containing 5% BSA, since the amount of protein was very high when compared to other samples. The amount of tannic acid was nearly equal in the other samples. This result supported our findings in TLC. Therefore, it can be concluded that not all tannic acid precipitated with proteins even if 5% BSA was added to the medium.

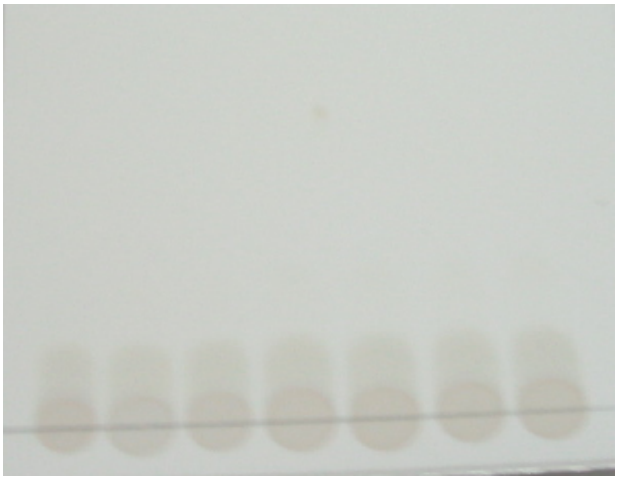

Figure 3.20 TLC results of tannic acid precipitation with BSA

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to analyze extracellular tannase production by *Aspergillus niger* in shake-flask bioreactors. The effect of aeration, tannic acid, glucose and nitrogen sources on tannase and biomass productions was investigated and their concentrations were optimized. The results given below were concluded from these studies.

• Aeration is an important parameter affecting tannase production from *A. niger*.

• Initial tannic acid concentration is a crucial factor for tannase and biomass production.

• Glucose addition favors extracellular tannase and biomass production from *A. niger*.

• Type and concentration of nitrogen source are effective parameters on tannase and biomass production.

• The optimum conditions are; 8% Tannic acid, 1% Glucose, 0.4% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.01% $ZnSO_4.7H_2O$, 0.0005% NaCl in a shake-flask bioreactor with a culture volume/bioreactor's gas volume ratio of 1/10 at 35^{o}C and 175 rpm.

The fermentation profile including tannic acid, gallic acid, glucose, pyrogallol, pH, biomass and extracellular tannase activity were determined under optimised conditions. The results given below were concluded from these studies.

• The highest tannase activity was found as 316 U/ml on the 4th day of cultivation.

• Biomass production increased during the cultivation. Increase in biomass concentration during the first two days and after the seventh day was high.

• Metabolic intermediates detected in the tannic acid containing culture of *A. niger* were gallic acid, glucose and pyrogallol.

• Pyrogallol was presumably converted to pyruvate in the cultivation medium.

• Tannic acid, gallic acid, glucose and pyrogallol were used as a carbon source during the cultivation.

• The pH of the cultivation medium decreased from 5.5 to 2.3 during the cultivation.

Future studies may involve the investigation of the parameters given below on tannase production.

• The effect of type and concentration of salts on tannase production

• pH control

• O_2 control

• Fed-batch experiments to keep tannic acid at the optimum level.

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APPENDIX A

MEDIUM COMPOSITION

Potato Dextrose Agar

Agar agar ..... 15 g/L
Dextrose..... 20 g/L
Potato extract ..... 4 g/L

39 g potato dextrose agar was dissolved in 1 L pure water.

Broth Medium for *Aspergillus niger* (Belmares et al., 2004)

Tannic acid ..... 50 g/L
Glucose..... 10 g/L
$(NH_4)_2HPO_4$..... 5 g/L
K_2HPO_4 ..... 1 g/L
$MgSO_4.7H_2O$ ..... 1 g/L
$ZnSO_4.7H_2O$ ..... 0.1 g/L
NaCl ..... 0.005 g/L

APPENDIX B

PREPARATION OF BRADFORD REAGENT

Chemicals used to prepare concentrated stock reagent solution (5x stock) were given in Table A.1.

Table B.1 Bradford reagent preparation procedure

Chemicals	Amount used
85% Ortho-phosphoric acid	500 ml
95% Ethanol	250 ml
Brillant Blue G-250 dye	250 ml

These chemicals were mixed and diluted to 1 L with distilled water to prepare 5x concentrated stock reagent solution.

The stock solution was stored at 4^{o}C. To prepare diluted (1x) reagent solution 1 volume concentrate was mixed with 4 volumes of distilled water. This solution was well mixed and filtrated.

Bradford reagent should wait at least 24 hours at room temperature before use.

APPENDIX C

PREPARATION OF PROTEIN STANDARD FOR BRADFORD METHOD

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

The assay was based on the observation that the absorbance maximum for an acidic solution of Coomaasie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay was useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

10 mg/ml Bovine serum albumin (BSA) was prepared as a stock solution. This stock solution was diluted at different ratios given in Table C.1.

Table C.1 BSA dilution ratios for Bradford method

Protein (µg/ml)	0	10	20	30	40
BSA stock (ml)	0	0.1	0.2	0.3	0.4
dH_2O (ml)	10	9.9	9.8	9.7	9.6

After preparation of diluted BSA samples, 0.5 ml BSA reagent and 5 ml of Bradford reagent were mixed in a glass test tube. Ten minutes later absorbance at 595 nm was measured by using a spectrophotometer.

APPENDIX D

STANDARD CURVE FOR BRADFORD METHOD

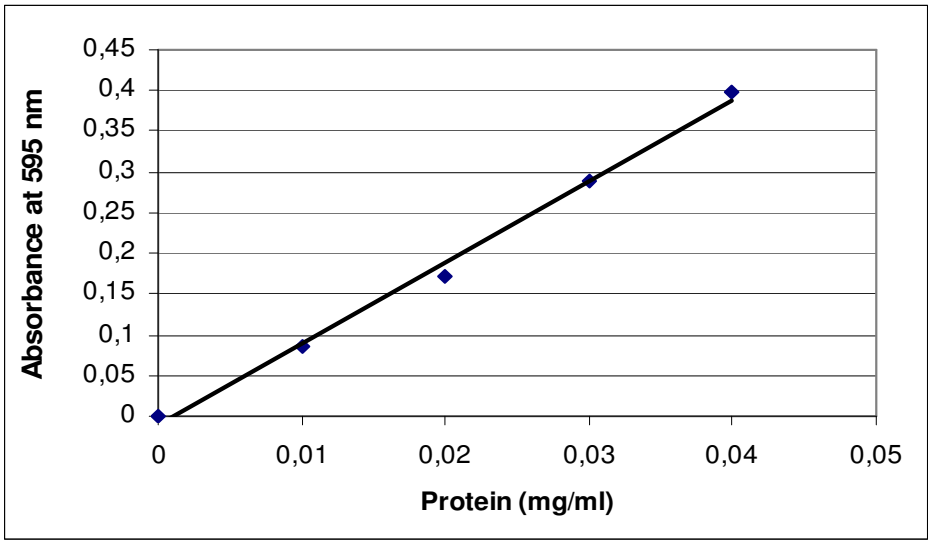

Figure D.1 BSA standard curve for Bradford Method

APPENDIX E

DATA

Time course of extracellular tannase production, tannic acid, gallic acid, pyrogallol, glucose and dry weight by A. niger in SmC (Figure 3.14).

Table E.1 Data for Figure 3.14.

Days	Extracellular tannase activity (U/ml)	Tannic acid (g/l)	Gallic acid (g/l)	Pyrogallol (g/l)	Glucose (g/l)	Dry weight (g/l)
0	0	80	1,2	0,3	8,3	0
1	19,6	65	17	5,0	7,3	1,1
2	206,3	55	9,2	15,2	2,8	4,3
3	283,9	43	7,8	15,5	1,5	5,2
4	316,2	37	7,5	12,5	1,4	5,4
5	275,2	31	6,5	9	1,3	6,8
6	265,0	29	6,8	8,2	1,0	7,5
7	270,3	28	6,7	6,6	1,2	8
8	242,2	27,5	6,4	4,6	0,6	13,1
9	182,0	25	6	0,5	0,0	16,3

APPENDIX F

COMPUTER OUTPUTS OF THE HPLC CHROMATOGRAMS

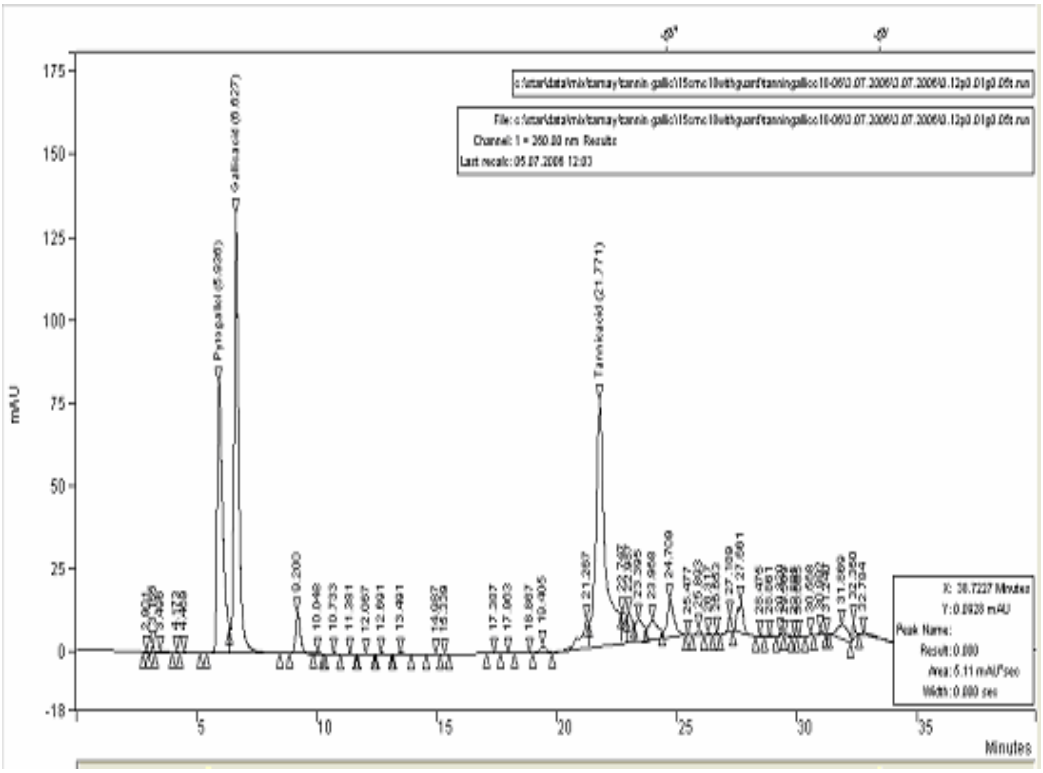

Figure F.1 HPLC results of the standards

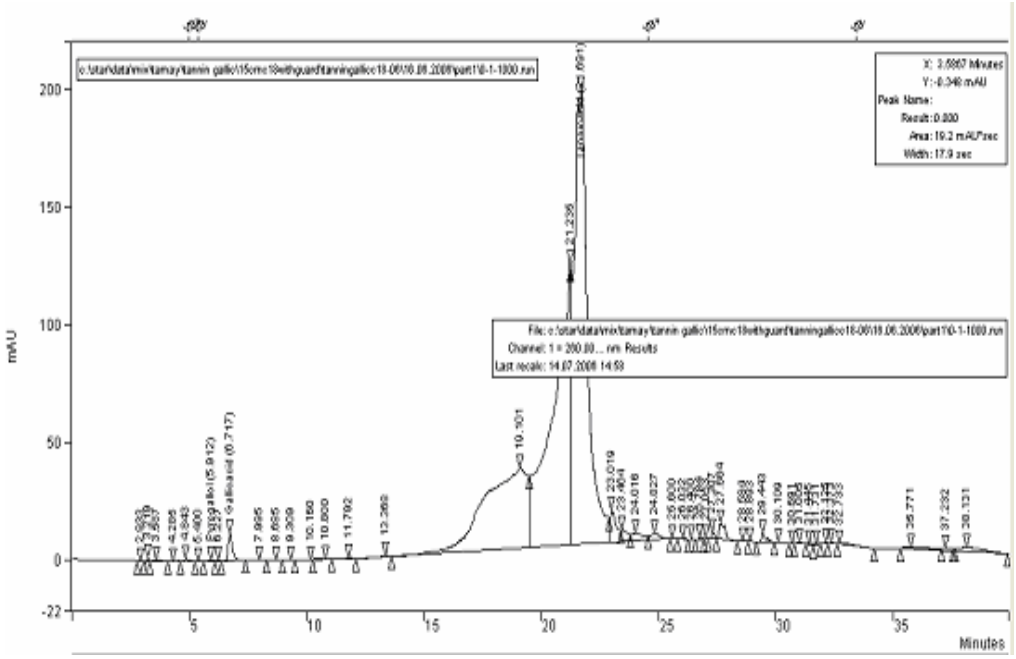

Figure F.2 HPLC results of sample withdrawn from the 0th day of cultivation

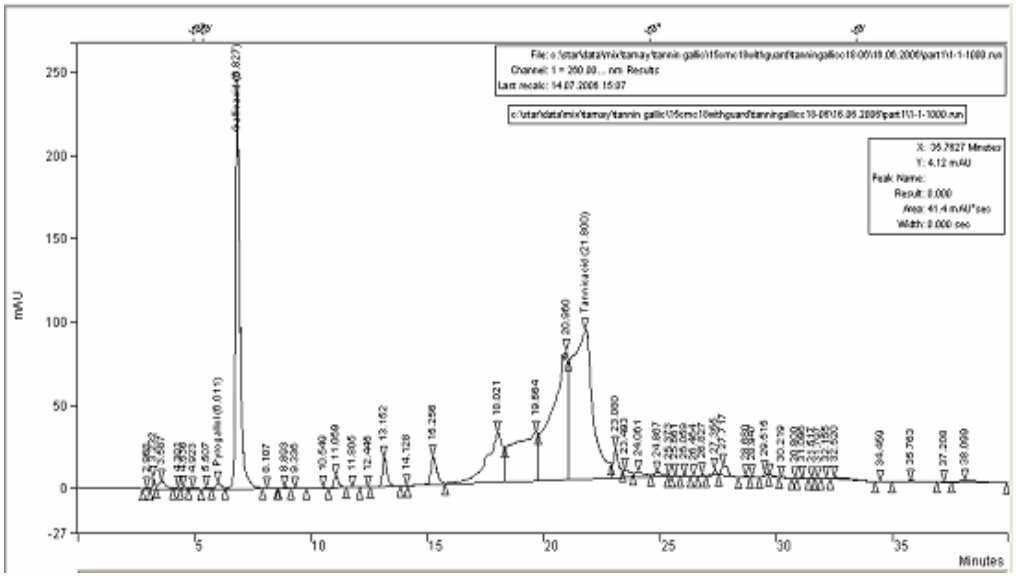

Figure F.3 HPLC results of sample withdrawn from the 1st day of cultivation

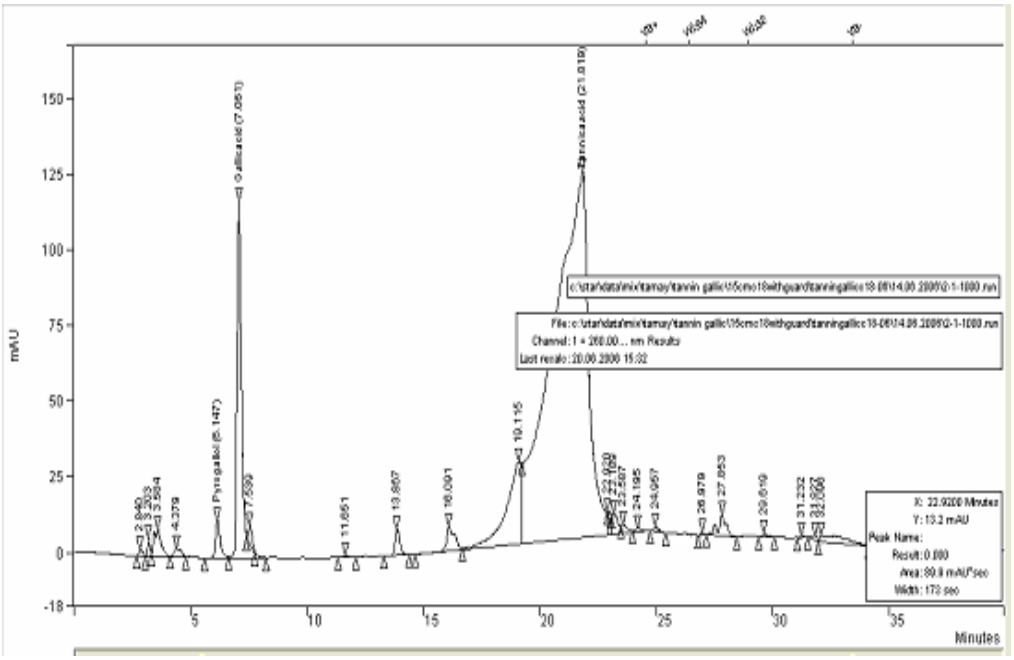

Figure F.4 HPLC results of sample withdrawn from the 2nd day of cultivation

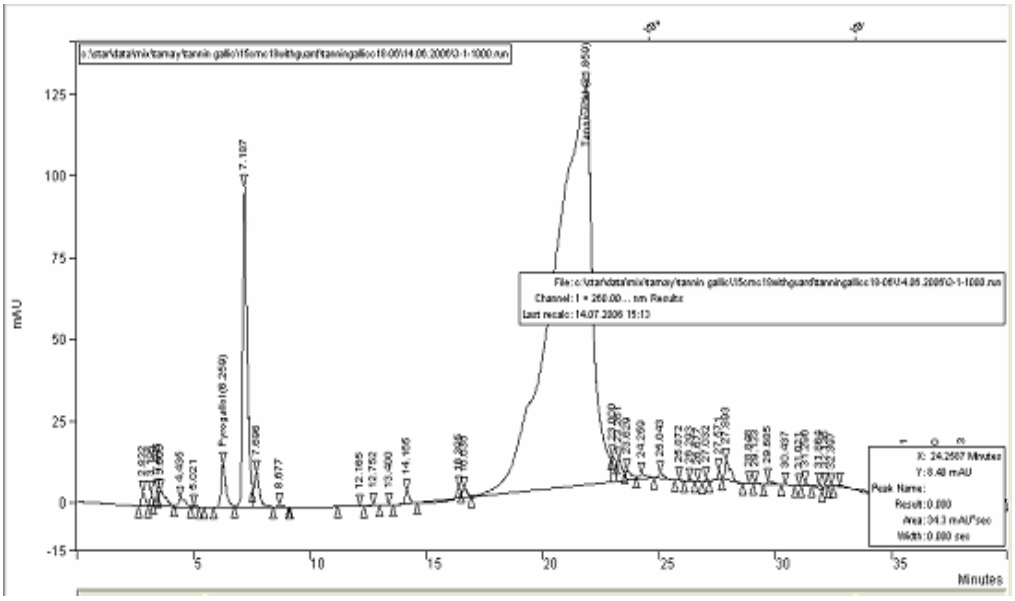

Figure F.5 HPLC results of sample withdrawn from the 3rd day of cultivation

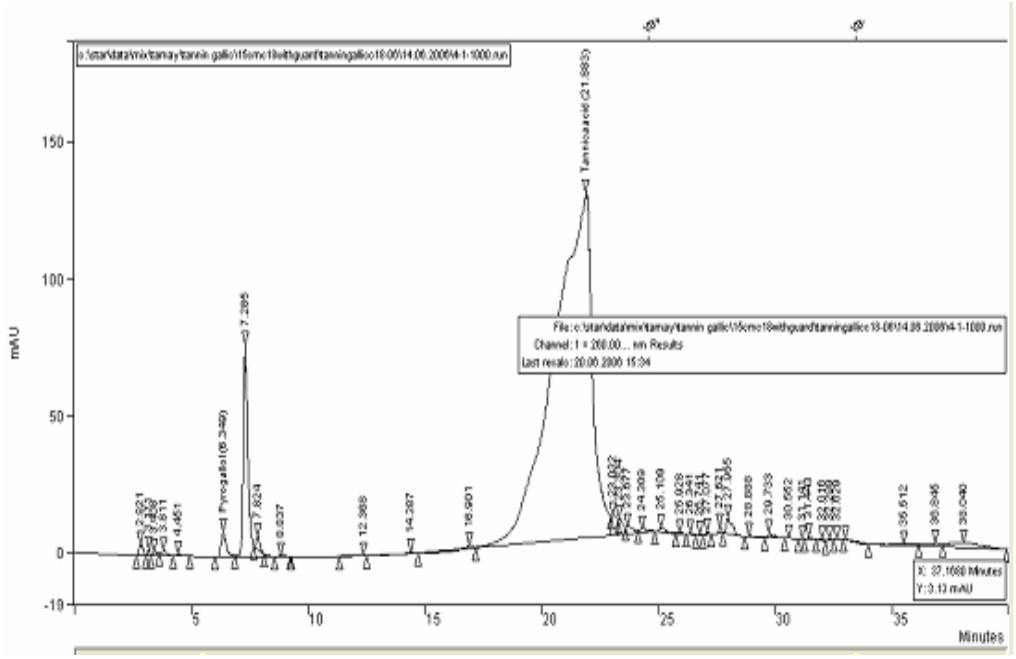

Figure F.6 HPLC results of sample withdrawn from the 4th day of cultivation

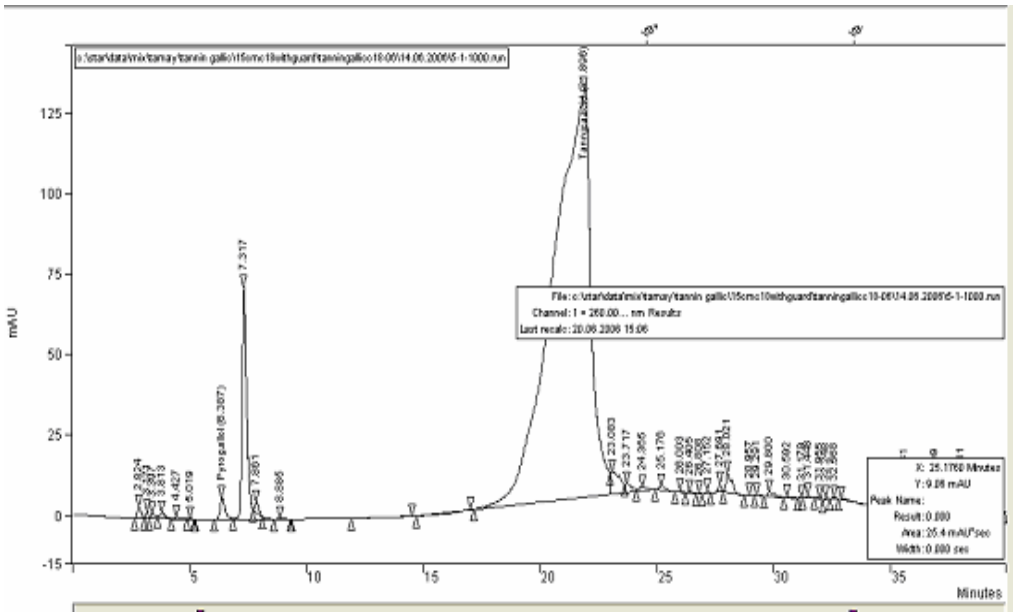

Figure F.7 HPLC results of sample withdrawn from the 5th day of cultivation

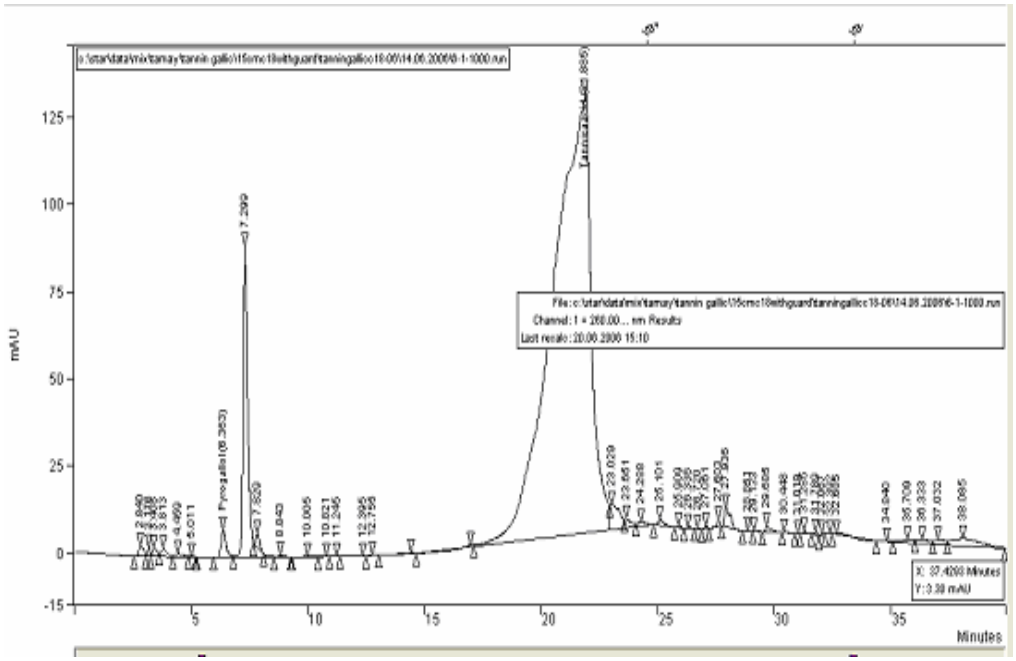

Figure F.8 HPLC results of sample withdrawn from the 6th day of cultivation

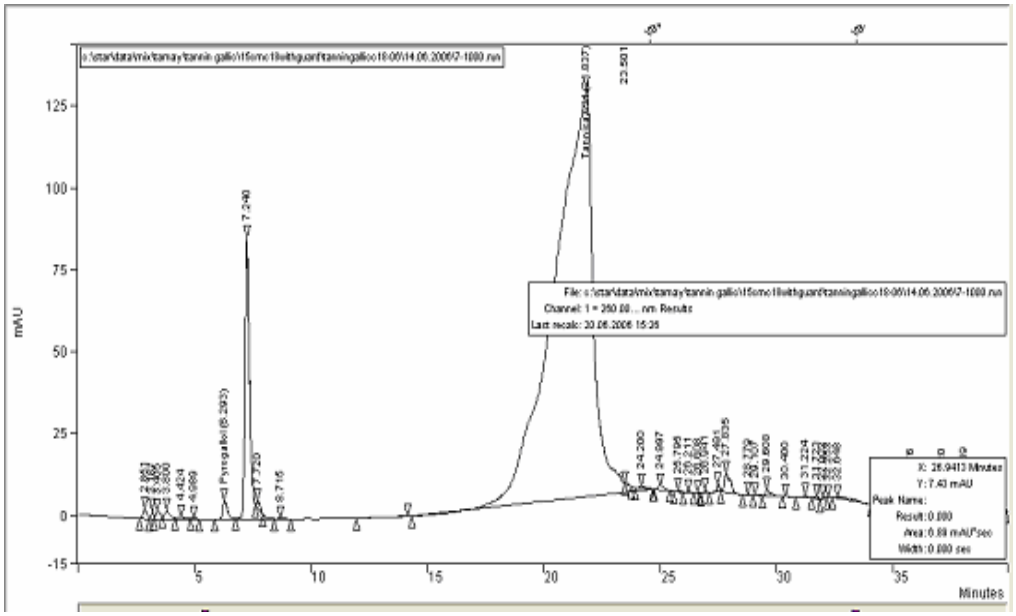

Figure F.9 HPLC results of sample withdrawn from the 7th day of cultivation

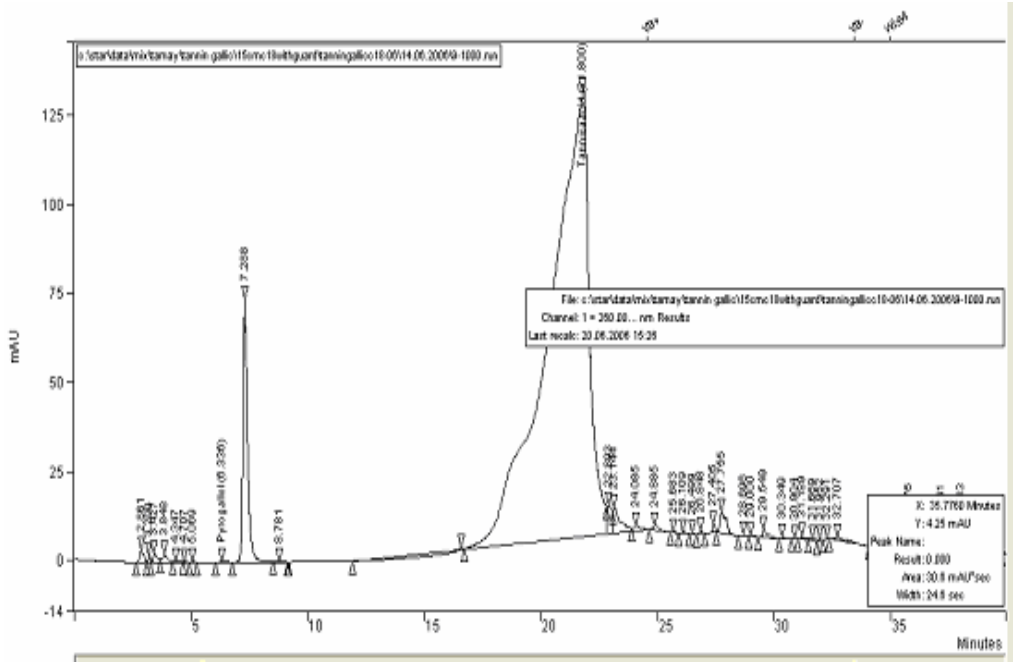

Figure F.10 HPLC results of sample withdrawn from the 9th day of cultivation