PRODUCTION OF TANNASE BY A LOCAL STRAIN *ASPERGILLUS NIGER* FETL FT3 IN SUBMERGED CULTURE SYSTEM

by

SUMATHI A/P GANESHAN

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

FEBRUARY 2011

ACKNOWLEDGEMENT

My very special acknowledgement goes to my supervisor, Professor Dr. Darah Ibrahim for her continuous guidance and support throughout the research and writing of this manuscript. Her contributions and constructive criticism are the secrets to the success of this work. My special appreciation also goes to my co-supervisor, Professor Dr. Mohd. Jain Nordin Mohd. Kassim for his comments and suggestions throughout this study.

Besides, I would also like express my appreciation to the staffs of School of Biological Sciences, USM, especially Mr. Adrian and Ms. Jenny for assisting me with computer related problems. I would also like to thank Mr. Patchamuthu, Cik Jamilah and En. Johari for their technical assistance and help provided during my research in the Electron Microscope Unit and not forgetting also En. Rashid for the photographs he snapped for my work.

I would also like take this opportunity to thank my parents, brother and sister for their moral support and endless prayers throughout my studies. Thanks also to all my friends in the lab and hostel for always being there for me, especially at times when I really needed them. Lastly, I would also like extend my sincere thanks to Universiti Sains Malaysia for providing financial assistance under the Graduate Assistantship Scheme (Skim Biasiswa Khas, USM).

Sumathi, G.

2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

PENGHASILAN TANASE OLEH STRAIN TEMPATAN

***ASPERGILLUS NIGER* FETL FT3**

DALAM SISTEM KULTUR TENGGELAM

ABSTRAK

Kultur kulat penghasil tanase yang dicamkan sebagai *Aspergillus niger* FETL FT3 telah dipencilkan dari tempatan daripada ekstrak tanin terhidrolisis kulit kayu bakau *Rhizophora apiculata*. Keupayaan kultur kulat tersebut untuk meningkatkan penghasilan tanase ekstrasel telah ditambahbaikan dalam sistem fermentasi kultur tenggelam (SmF). Penambahbaikan pelbagai proses parameter untuk penghasilan maksimum tanase ekstrasel oleh kultur kulat tersebut dilakukan dengan sel bebas dan sel tersekat-gerak di dalam kelalang goncangan 250 ml. Seterusnya keadaan pengkulturan yang telah ditambahbaik di dalam kelalang goncangan dipindahkan ke bioreaktor angkut udara 2000 ml dan pembolehubah-pembolehubah tertentu ditambahbaikan semula. Dalam kelalang goncangan, penghasilan tanase ekstrasel yang maksimum iaitu 2.81 U/ml diperolehi selepas 4 hari pemfermentasian pada suhu 30^{o}C, menggunakan saiz inokulum 1% (i/i) 1×10^{6} spora/ml, pH awal medium 6.0 dan kelajuan goncangan 200 psm dalam 50 ml medium minimum Czapek Dox yang diubahsuai yang mengandungi 3 b%-C asid tanik, 0.8 b%-C glukosa dan 0.048 b%-N natrium nitrat dalam kes sel bebas. Sel tersekat-gerak di dalam pencuci periuk nilon telah menghasilkan tanase ekstrasel yang maksimum iaitu 3.98 U/ml, juga selepas 4 hari pemfermentasian menggunakan keadaan yang sama seperti sel bebas menggunakan 6 kepingan kiub pencuci periuk nilon. Walau bagaimanapun, aktiviti tanase yang rendah iaitu 2.14 U/ml dan 3.12 U/ml masing-masing diperolehi di

dalam bioreaktor angkut udara 2000 ml yang mengandungi 1800 ml medium pemfermentasian untuk kedua-dua sel bebas dan tersekat-gerak *Aspergillus niger* FETL FT3. Keadaan fermentasi yang telah ditambahbaik untuk penghasilan maksimum tanase ekstrasel iaitu 2.14 U/ml dan 3.12 U/ml masing-masing di dalam bioreaktor diperolehi selepas 4 hari pemfermentasian untuk kedua-dua sel bebas dan tersekat-gerak dengan saiz inokulum 1% (i/i) 8×10^5 spora/ml dan 2.0 vvm pengudaraan dalam kes sel bebas dan dengan saiz inokulum 1% (i/i) 4×10^5 spora/ml, 2.0 vvm pengudaraan dan 200 kepingan kiub pencuci periuk nilon dalam sel tersekat-gerak. Seterusnya, enzim yang dihasilkan ditulenkan sebanyak 12.75 kali ganda dengan 0.77% pemulihan melalui pemendakan amonium sulfate dan kromatografi turus penurasan gel Sephadex G-200. Berat molekul tannase yang ditulenkan dianggarkan sekitar 89 kDa melalui natrium dodesil sulfat-poliakrilamida gel elektroforesis (SDS-PAGE). Kajian tentang kesan suhu dan pH juga telah dijalankan ke atas aktiviti tanase yang telah ditulenkan. pH dan suhu optimum untuk aktiviti enzim ialah pada pH 5.0 dan 40^{o}C, masing-masing. Enzim tersebut juga menunjukkan kestabilan yang baik selama 1 jam sehingga suhu 50^{o}C dan kestabilan pH dalam julat yang terhad iaitu di antara pH 5.0 dan 6.0.

PRODUCTION OF TANNASE BY A LOCAL STRAIN

***ASPERGILLUS NIGER* FETL FT3 IN SUBMERGED CULTURE SYSTEM**

ABSTRACT

A tannase-producing fungal culture identified as *Aspergillus niger* FETL FT3 was isolated locally from hydrolysed tannin extract of *Rhizophora apiculata* barks. The potential of the fungal culture for enhanced extracellular production of tannase was improved in submerged fermentation (SmF) system. The improvement of various process parameters for maximum production of extracellular tannase from the fungal culture was carried out with free and immobilized cells in 250 ml shake flasks. Subsequently the improved cultivation conditions of shake flasks were transferred to a 2000 ml tubular air lift bioreactor and some selected variables were further improved. In shake flasks, maximum extracellular tannase production of 2.81 U/ml was obtained after 4 days of fermentation at 30^{o}C with 1% (v/v) of 1×10^{6} spores/ml inoculum size, initial medium pH 6.0 and agitation at 200 rpm in 50 ml of modified Czapek Dox minimal medium containing 3 wt%-C tannic acid, 0.8 wt%-C glucose and 0.048 wt%-N sodium nitrate in the case of free cells. The immobilized cells on nylon scouring mesh produced maximum extracellular tannase of 3.98 U/ml, also after 4 days of fermentation via conditions similar to that of free cells using 6 pieces of nylon scouring mesh cubes. However, lower tannase activities of 2.14 U/ml and 3.12 U/ml respectively were obtained in the 2000 ml tubular air lift bioreactor containing 1800 ml of the fermentation medium for both free and immobilized *Aspergillus niger* FETL FT3 cells. The improved fermentation conditions for the maximum production of extracellular tannase of 2.14 U/ml and 3.12 U/ml

respectively in the bioreactor were obtained after 4 days of fermentation for both free and immobilized cells with 1% (v/v) of 8×10^5 spores/ml inoculum size and 2.0 vvm aeration in the case of free cells and with 1% (v/v) of 4×10^5 spores/ml inoculum size, 2.0 vvm aeration and 200 pieces of nylon scouring mesh cubes in the immobilized cells. The enzyme produced was then purified 12.75 folds with a 0.77% yield through ammonium sulfate precipitation and Sephadex G-200 gel filtration column chromatography. The molecular weight of the purified tannase was estimated to be 89 kDa through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A study on the effects of temperature and pH was also carried out on the activity of the purified tannase. The pH and temperature optimum of the enzyme activity were found to be at pH 5.0 and 40^{o}C, respectively. The enzyme also showed good stability up to 50^{o}C for 1 hour and was stable over a narrow pH range of pH 5.0 to 6.0.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Tannin acyl hydrolase (E.C. 3.1.1.20) is commonly referred to as tannase and is an enzyme that hydrolyzes the ester bonds of tannic acid, to produce gallic acid and glucose (Haworth *et al*., 1958; Mondal *et al*., 2000). This unique enzyme was accidentally discovered by Tieghem (1867) in an experiment to demonstrate that the formation of gallic acid during fermentation is due to the action of fungus, and not to enzymes pre-existing in the galls, nor to oxidation by the air (Tieghem, 1867). He further identified the organisms as *Penicillium glaucum* and *Aspergillus niger* (Knudson, 1913; Lekha and Lonsane, 1997). Besides these microorganisms, various filamentous fungi (Hadi, *et al*., 1994; Bradoo *et al*., 1997; Seth and Chand, 2000; Banerjee *et al.*, 2001), bacteria (Deschamps *et al*., 1980; Skene and Brooker, 1995; Lekha and Lonsane, 1997; Kumar *et al*., 1999; Mondal and Pati, 2000) and yeast (Aoki *et al*., 1976a; Lekha and Lonsane, 1997) have also been reported to produce tannase.

Tannase is also an important enzyme and has various industrial applications including in the manufacture of instant tea (Coggon *et al*., 1975) and wine (Aguilar and Gutierrez-Sanchez, 2001; Vaquero *et al*., 2004). It is also used in the manufacture of gallic acid (Pourrat *et al*., 1985, 1987; Misro *et al*., 1997; Kar *et al*., 1999) and also as additive for detannification of food (Lekha and Lonsane, 1997). In addition to that, it is also used as a sensitive analytical probe for determining the

structure of naturally occurring gallic acid esters (Haslam and Tanner, 1970). The enzyme is also important for treating tannery effluents (Bradoo *et al*., 1997) and decomposition of plant organic matter (Garcia-Conesa *et al*., 2001). Recently, the enzyme has also been found to be useful in improving vegetable fermentation and also to treat vegetable waste such as olive mill waste waters (Ayed and Hamdi, 2002; Kachouri *et al*., 2005).

Based on preliminary screening of various local isolates, *Aspergillus niger* FETL FT3 was found to be the most potential tannase producer and thus was selected for this study. Studies on tannase production by *Aspergillus niger* FETL FT3 was carried out extensively in submerged fermentation system and the regulatory aspects of tannase production by this fungus in submerged fermentation showed that tannases are induced by tannic acid or by some of its derivatives (Knudson, 1913). However, the initial concentration of tannic acid used was found to be a crucial factor for growth and tannase induction, as high concentration of tannic acid may be toxic to the microorganism. Bajpai and Patil (1997) reported that the optimum concentration of gallotannin to induce tannase production by *Aspergillus niger* was 10% (w/v) concentration, while its tolerance limit to gallotannin was 20% (w/v) concentration, whereas Bhat *et al*. (1997) found *Aspergillus niger* to tolerate up to 15% (w/v) tannin in a test medium. The production of tannase by microorganisms was also found to be highly dependent on environmental conditions and therefore, the best combinations of various physical (cultural conditions) and physiological parameters (medium compositions) for maximum tannase production was determined by step by step improvement by changing one control variable at a time while holding the rest constant. Besides freely suspended cells, immobilized cells

system was also considered for tannase production because immobilized cell systems was reported to offer many advantages over freely suspended cells such as simple reuse of the biomass, easier liquid-solid separation and minimal clogging in continuous-flow systems (Arica *et al*., 1993; Tieng and Sun, 2000). Further, due to wide applications of tannases in many industries, quick purification procedure to keep the process inexpensive and characterization with respect to optimum temperature and pH conditions required for maximum tannase activity and stability was also carried out. Tannase has many potential applications in the food, pharmaceutical and chemical industries but due to the shortage, high cost and limited knowledge of the enzyme, the use of tannase in large-scale application is limited at present. Therefore, it is hoped that the economic benefits of tannase production and sufficient information of the enzyme can help improve the overall viability of the process.

1.2 RESEARCH OBJECTIVES

The present study was aimed to achieve the following objectives:

1. To isolate, screen and identify a new potential fungal tannase producer from hydrolysable tannin extract of *Rhizophora apiculata* barks.
2. To improve various physical parameters (cultural conditions) specifically temperature, initial medium pH, agitation speed and inoculum size and physiological parameters (carbon and nitrogen sources) of tannic acid concentration, various sugars as second carbon source and various inorganic

nitrogen sources for the maximum production of extracellular tannase by the selected isolate in submerged fermentation systems in shake flask.

3. To improve inoculum size and aeration rate for the maximum production of extracellular tannase by the selected isolate in a 2 liter tubular air lift laboratory bioreactor.
4. To purify and characterize the tannase produced by the selected isolate.

CHAPTER 2

LITERATURE REVIEW

2.1 TANNASE

Tannase (Tannin acyl hydrolase, E. C. 3.1.1.20) is an extracellular hydrolase enzyme that is induced in the presence of tannic acid with gallic acid as an end product (Kar *et al*., 2003). It catalyzes the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid, thereby releases gallic acid residues from glucose (Lekha and Lonsane, 1997; Ramirez-Coronel *et al*., 2003). Tannase can be obtained from various sources and is used commercially in many industries.

2.1.1 ISOZYMES OF TANNASE

Beverini and Metche (1990) reported that tannase is composed of a mixture of two separate isoenzymes, tannase I and tannase II. Tannase I has been reported to have specificities for ester linkage of galloyl esters of alcohol moiety, whereas tannase II has been reported to have strong affinity for galloyl esters of gallic acid (Beverini and Metche, 1990) in substrates such as tannic acid, methylgallate and m-digallic acid. Among these enzymes, gallic acid esterase is predominant (Lekha and Lonsane, 1997). The identities of these two enzymes were also confirmed by Haslam *et al*. (1961) and Haslam and Stangroom (1966).

2.1.2 SUBSTRATE SPECIFICITY OF TANNASE

Tannase hydrolyses only ester compounds of gallic acid. However, not all ester compounds of gallic acid are hydrolyzed by tannase. Some ester compounds of gallic acid which are insoluble in water such as stearyl, lauryl and cetyl gallate are not hydrolyzed by tannase (Iibuchi *et al*., 1972). Examples of substrates that are hydrolyzed by tannase are tannins such as tannic acid, methyl gallate, ethyl gallate, n-propylgallate and isoamyl gallate (Belmares *et al*., 2004; Aguilar *et al*., 2007). Tannase activity also appears to be related to the number of ester bonds in galloyl esters in which galloyl esters with more ester bonds were better substrates for tannase (Farias *et al*., 1994).

2.1.2.1 TANNINS AS SUBSTRATE FOR TANNASE

Tannins are defined as naturally occurring water-soluble polyphenolic compounds of varying molecular weight in the plant kingdom. These phenolic compounds differ from others by having the ability to precipitate proteins from solutions (Aguilar *et al*., 2007). In plant kingdom, these tannins are found in leaves, fruits, bark and wood (Albertse, 2002; Vaquero *et al*., 2004; Aguilar *et al*., 2007). Tannins also occur in many fruits and vegetables (Chung *et al*., 1998) and in nutritionally important forage trees, shrubs, legumes, cereals and grains (Sabu *et al*., 2006). Tannins are often considered to be nutritionally undesirable because they often form complexes with protein, starch and digestive enzymes and cause a reduction in nutritional values of food (Chung *et al*., 1998; Sabu *et al*., 2006). These tannins bind readily with proteins and other macromolecules to form indigestible or insoluble complexes,

thereby not only reducing nutritional value of the animal feed (McLeod, 1974; Rhoades and Cates, 1976) but also presenting undesirable taste and colouration in tea (Sanderson and Coggon, 1974) and beer (Lekha and Lonsane, 1997). Tannins are also considered to be the plant's secondary metabolic products because they play no direct role in the plant metabolism (Bhat *et al*., 1998; Albertse, 2002; Aguilar *et al*., 2007).

Tannins can be divided into four major groups: gallotannins, ellagitannins, condensed tannins and complex tannins (Aguilar *et al*., 2007) on the basis of their structure and properties (Figure 2.1).

2.1.2.1(a) GALLOTANNINS AND ELLAGITANNINS (HYDROLYSABLE TANNINS)

Hydrolysable tannins are polyphenolic compounds composed of esters of gallic acid (gallotannin) or ellagic acid (ellagitannin) with a sugar as a central core (Bhat *et al*., 1998). In the gallotannin (Figure 2.1), each molecule is usually composed of a core of D-glucose and 6 to 9 galloyl (single monomer of gallic acid) groups. These simple esters are extended by attachment of additional galloyl residues to the phenolic galloyl-OH groups to yield side-chains of variable length (Niehaus and Gross, 1997). Upon hydrolysis, gallotannin yields glucose and gallic acid (Lekha and Lonsane, 1997; Chen and Chung, 2000). The ellagitannin (Figure 2.1), in contrast, contain one or more hydroxy-diphenol residues, which are linked to glucose as a diester in addition to gallic acid (Chen and Chung, 2000). Therefore,

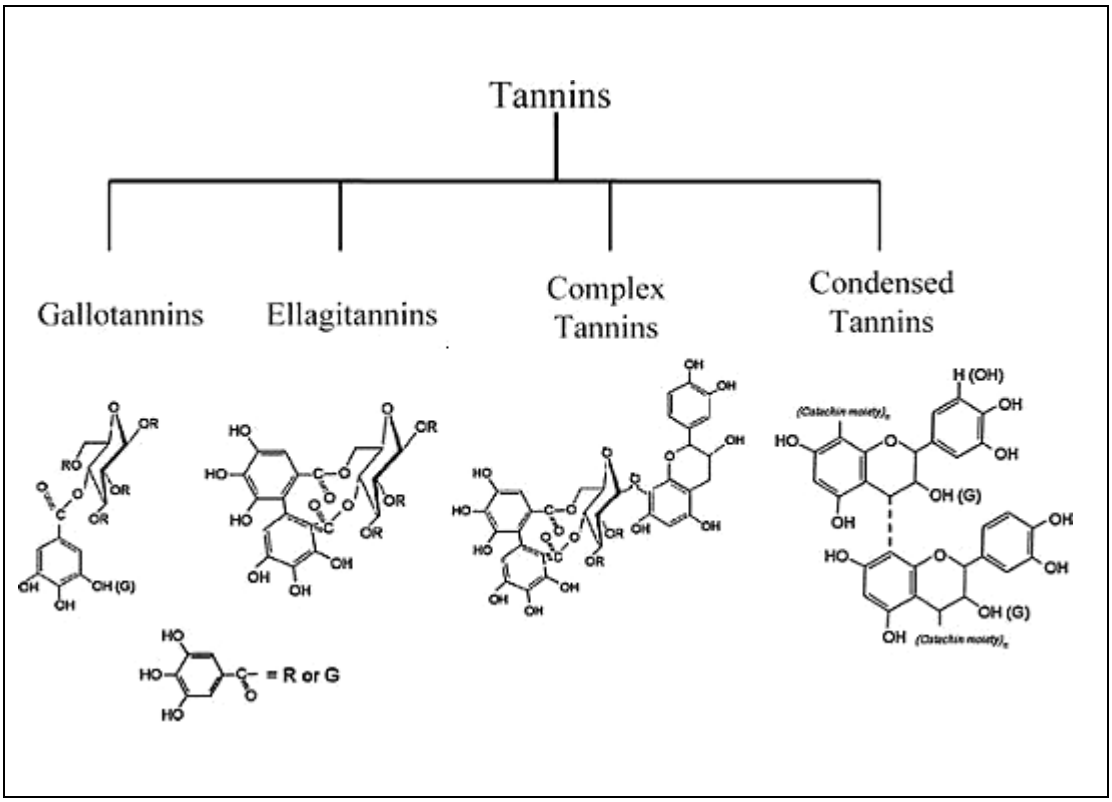

Figure 2.1: Main chemical structures of the tannin. (adapted from Aguilar *et al.*, 2007). R, gallate; G, digallate.

hydrolysis of ellagitannins yields glucose and ellagic acid together with gallic acid (Lekha and Lonsane, 1997).

2.1.2.1(b) CONDENSED TANNINS

Condensed tannins are also known as proanthocyanidins, and consist of phenols of the flavon type flavonoids (Lekha and Lonsane, 1997; Bhat *et al*., 1998). Condensed tannin may also contain 2 to 50 or greater flavonoid units and can have complex structures because the flavonoid unit can differ for some substituents and because of the variable sites for interflavan bonds. Typical condensed tannin can be represented by the dimer procyanidin, to which molecules of flavan (catechin moieties) can be added as indicated (Figure 2.1). A very interesting difference between condensed tannins and hydrolysable tannins is the fact that condensed tannins do not contain any sugar residues as a core (Goodwin and Mercer, 1983) and are not readily degraded by tannase (Skene and Brooker, 1995).

2.1.2.1(c) COMPLEX TANNINS

Complex tannins (Figure 2.1) are generated through reactions between gallic or ellagic acids with catechins and glucosides (Aguilar *et al*., 2007). Therefore, complex tannins are considered to be an intermediate group that combines both characteristics of hydrolysable tannins and condensed tannins. This family of tannins is also called catechin tannins (Bhat *et al*., 1998).

2.1.3 MECHANISM OF ACTION OF TANNASE

Tannase is known to catalyze the breakdown of ester bonds from hydrolysable tannins and gallic acid esters (Figure 2.2). When the reaction substrate is the methyl gallate, tannase gives gallic acid and methanol as final products (Iibuchi *et al*., 1972). When the reaction substrate is methyl-m-digallate, the enzyme produces methyl gallate and gallic acid, without any traces of m-digallic acid as intermediate compounds and finally gives gallic acid and methanol as final products. This suggests that the enzyme liberates gallic acid from m-digalloyl esters first (Iibuchi *et al*., 1972) prior to other reactions. This is clearly shown in the main pathway of hydrolysis of tannic acid by tannase (Figure 2.3) where R_1 and R_2 are gallate and m-digallate, respectively. When the reaction substrate is tannic acid, tannase gives glucose and gallic acid as final reaction products (Haworth *et al*., 1958). Tannase hydrolyses tannic acid completely to gallic acid and glucose through intermediate compounds of 2,3,4,6-tetragalloyl glucose and two kinds of monogalloyl glucose (Iibuchi *et al*., 1972). However, the position of gallic acid of the two kinds of monogalloyl glucose is not known (Iibuchi *et al*., 1972) and has not been determined (Lekha and Lonsane, 1997). The monogalloyl glucoses are then hydrolyzed completely by the enzyme to glucose and gallic acid as final products of hydrolysis (Iibuchi *et al*., 1972).

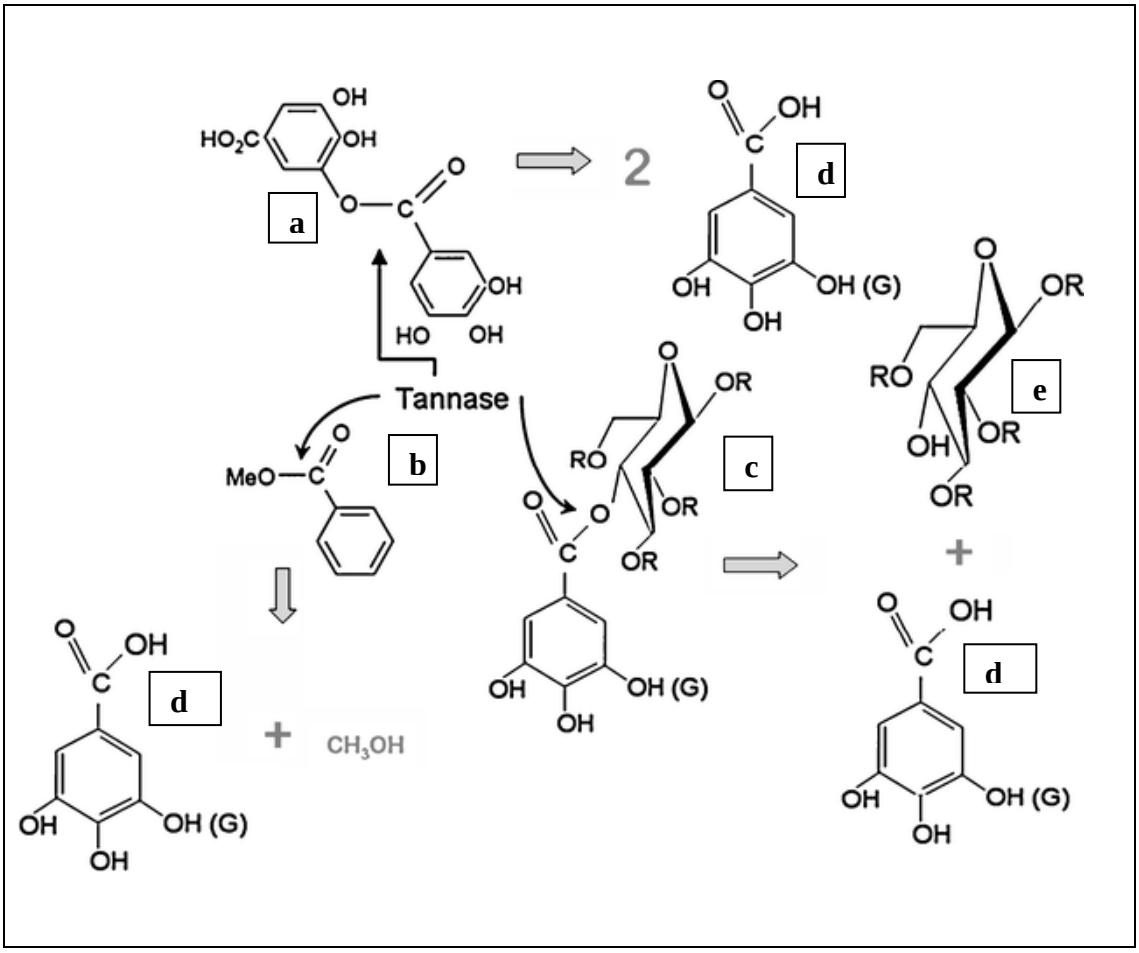

Figure 2.2: Mechanism of tannase action (adapted from Aguilar *et al*., 2007). a, m-digallic acid; b, methyl gallate; c, tannin; d, gallic acid; e, polygalloyl glucose.

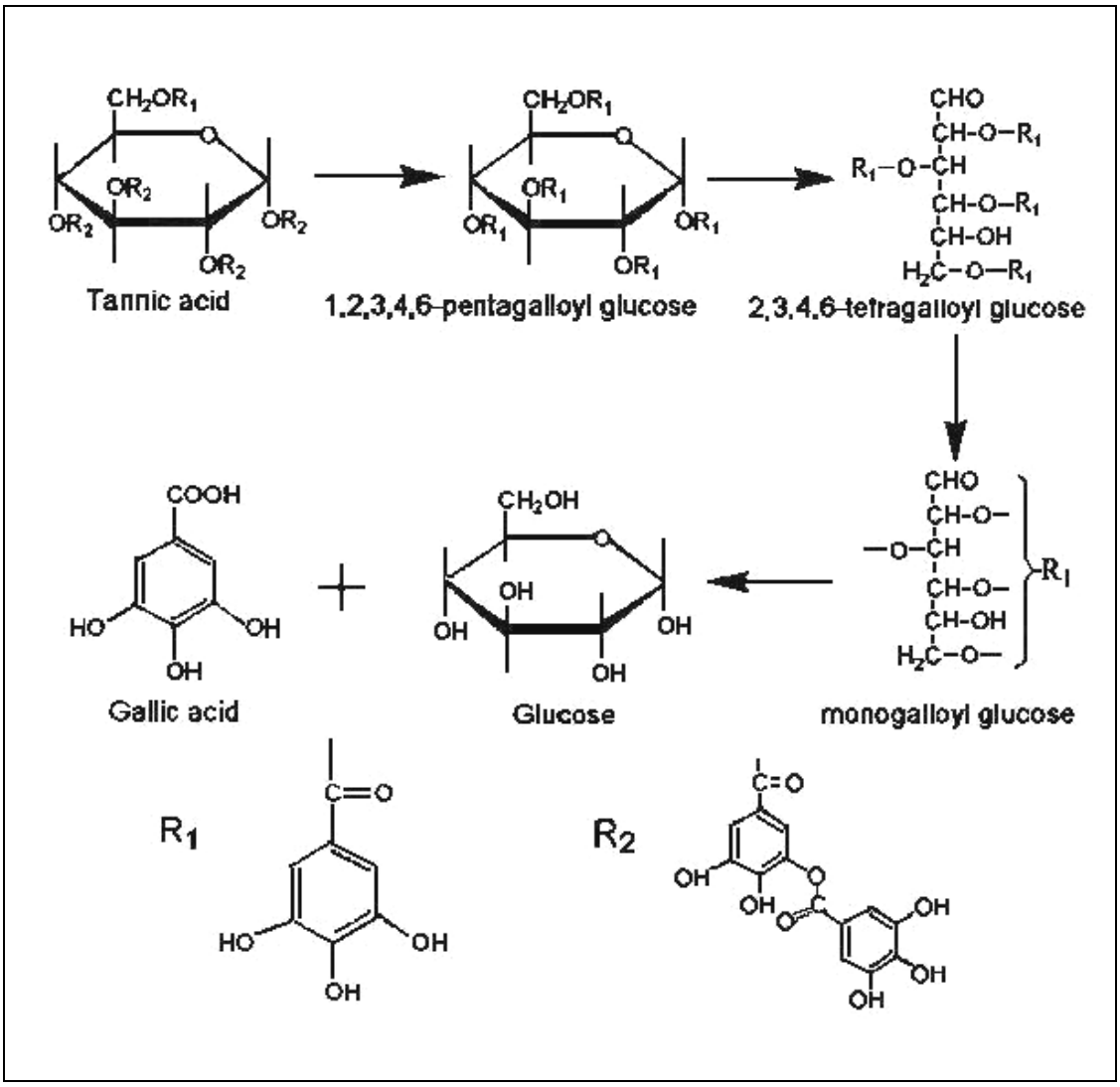

Figure 2.3: The main hydrolysis pathway of tannic acid by tannase (adapted from Iibuchi *et al*., 1972; Ong, 2005). R_1, gallate; R_2, digallate.

2.1.4 BIOSYNTHESIS OF TANNASE

Tannase is an inducible enzyme produced only in the presence of tannic acid or its end product, gallic acid (Knudson, 1913). Therefore, the minimum structural requirement for adaptive tannase formation is gallic acid. However, due to the fact that tannase is an esterase and that gallic acid contains no ester linkage, a test on induction of tannase by gallic acid as sole carbon source showed that it did not induce tannase activity (Aguilar *et al*., 2001a). Thus, tannase is produced only in the presence of tannic acid and this is evident from all the media reported for tannase production, where tannic acid was present as an inducer (Lekha and Lonsane, 1997). The minimum concentration of tannic acid that could stimulate the formation of tannase was found to be 0.1% (Knudson, 1913). Van de Lagemaat and Pyle (2005) reported that the mechanism of induction by tannic acid may have two phases. In the first phase, the presence of tannic acid or a combination of tannic acid and gallic acid in the growth medium induces the formation of intracellular and low levels of extracellular tannase, whereas in the second phase, the exhaustion of glucose in the medium increases the rate of extracellular tannase formation (Van de Lagemaat and Pyle, 2005).

2.2 SOURCES OF TANNASE

Tannase is known to be obtained from plant, animal and microbial sources (Vaquero *et al*., 2004). However, the most important source to obtain the enzyme is by microbial way, because the enzymes produced are more stable than similar ones obtained from other sources (Bhat *et al*., 1998; Purohit *et al*., 2006; Sabu *et al*.,

2006). Microorganism can also be cultured in large quantities in a short time by established methods of fermentation, thus they can produce an abundant and regular supply of the enzyme. Microbes can also be subjected to genetic manipulation more readily than plants and animals (Walsh and Headon, 1994; Aguilar and Gutierrez-Sanchez, 2001; Purohit *et al*., 2006; Sabu *et al*., 2006).

2.2.1 PLANTS

The role of tannase in green leaves has been reported to be much less obvious (Niehaus and Gross, 1997). Tannins in plants deter microorganisms, either by increasing resistance against pathogens or by protecting essential tissues such as wood against decay (Scalbert, 1991). It has also been recognized, that condensed tannins of *Acacia nigrescens* acted as an anti-defoliating agent against browsing by giraffe (Furstenburg and Van Hofen, 1994) and it was concluded from studies with *Epilobium*, *Cornus* or *Alnus* that the tannins in these plants were important in the defense against ruminants (Robbins *et al*., 1987). Thus, a tannin degrading enzyme in leaves would not make much sense as hydrosable tannins play a protective role in plant by direct astringency avoidance of herbivorous animals (Niehaus and Gross, 1997). However, the existence of this enzyme has been reported in the leaves of pedunculate oak (Niehaus and Gross, 1997). The existence of tannase in such green leaves thus is believed to play a role indirectly through their degradation products. Evidence has also been presented that the ellagitannin preferentially acted as a protoxin that releases insect growth inhibitor, particularly ellagic acid, upon hydrolytic cleavage (Klocke *et al*., 1986). In contrast to leaves, however, tannase has been discussed as major deastringency mechanism in fruit ripening (Ozawa *et al*.,

1987), whereby it contribute to these processes by loss of astringency through simple degradation of tannins (Niehaus and Gross, 1997). This is evident from a report on purification of tannase from divi-divi (*Caesalpinia coriaria*) fruit pods (Madhavakrishna and Bose, 1961).

2.2.2 ANIMALS

Low levels of tannase has been reported to be present in the rumen of cattle by Begovic and Duzic (1976), who also purified bovine tannase from the mucosal membrane of the rumen and small intestine of the cattle for the first time (Begovic and Duzic, 1977). The gall larvae that undergo development in plant galls also produce tannase to hydrolyze the tannic acid abundant in plant galls (Nierenstein, 1930). However, evidence suggests that animals depend mostly on symbiotic relationship with tannase producing gastrointestinal microbes to counter the anti-nutritional effects of dietary tannins (Bhat *et al*., 1998; Sasaki *et al*., 2005).

2.2.3 MICROORGANISMS

It has long been known that many microorganisms (Lewis and Starkey, 1969) especially several fungal species such as *Aspergillus* sp. (Lekha and Lonsane, 1997; Banerjee *et al*., 2001) and *Penicillum* sp. (Rajakumar and Nandy, 1983; Lekha and Lonsane, 1997) are capable of producing large amounts of tannase. Isolation of tannase from *Rhizopus oryzae* has also been reported (Hadi *et al*., 1994). Other tannase producers include bacteria (Deschamps *et al*., 1980; Skene and Brooker, 1995; Lekha and Lonsane, 1997; Kumar *et al*., 1999; Mondal and Pati, 2000) and

yeast (Aoki *et al*., 1976a; Lekha and Lonsane, 1997). Some of the tannase producing fungi, bacteria and yeast are shown in Table 2.1 (Aguilar *et al*., 2007). Microorganisms produce tannases, probably as a mode of invasion into the host plant by hydrolyzing tannins that are present in many herbaceous and woody plants (Lekha and Lonsane, 1997).

2.2.3.1 FILAMENTOUS FUNGI

Various filamentous fungi, mainly *Aspergillus niger* (Yamada *et al*., 1968; Pourrat *et al*., 1985; Barthomeuf *et al*., 1994; Lekha and Lonsane, 1994; Bhat *et al*., 1996; Bajpai and Patil, 1997; Pinto *et al*., 2001; Sharma *et al*., 2002; Ramirez-Coronel *et al*., 2003; Sharma and Gupta, 2003; Sabu *et al*., 2005a) and *Penicillium* (Rajakumar and Nandy, 1983) produce the enzyme tannase in the presence of tannic acid. Bajpai and Patil (1997) have recently reported the production of tannase by *Fusarium solani*, *Trichoderma viride* and *Aspergillus fischerii*. Furthermore, *Aspergillus japonicus* also produces extracellular tannase on simple and complex sugar substrates (Bradoo *et al*., 1997). Tannase have also been purified from culture broth of *Aspergillus oryzae* (Iibuchi *et al*., 1968; Lane *et al*., 1997; Garcia-Conesa *et al*., 2001), *Aspergillus flavus* (Adachi *et al*., 1968; Yamada *et al*., 1968), *Aspergillus foetidus* (Mukherjee and Banerjee, 2004; Purohit *et al*., 2006), *Aspergillus aculeatus* (Banerjee *et al*., 2001) and *Aspergillus awamori* (Seth and Chand, 2000; Mahapatra *et al*., 2005). It has also been reported that the fungus *Rhizopus oryzae* could also secrete the tannase enzyme (Hadi, *et al*., 1994; Misro *et al*., 1997; Mukherjee and Banerjee, 2004; Purohit *et al*., 2006).

Table 2.1: Microbial sources of tannase (adapted from Aguilar *et al*., 2007).

Microorganism	References
Bacteria	
Achromobacter sp.	Lewis and Starkey (1969)
Bacillus pumilus	Deschamps *et al*. (1983)
Bacillus polymyxa	Deschamps *et al*. (1983)
Corynebacterium sp.	Deschamps *et al*. (1983)
Bacillus cereus	Mondal *et al*. (2001)
Klebisella planticola	Deschamps *et al*. (1983)
Klebisella pneumoniae	Deschamps *et al*. (1983)
Pseudomonas solanaceanum	Deschamps *et al*. (1983)
Streptococcus bovis	Belmares *et al*. (2004)
Streptococcus gallolyticus	Sasaki *et al*. (2005)
Lactobacillus plantarum	Ayed and Hamdi (2002); Kostinek *et al*. (2007)
Lactobacillus paraplantarum	Nishitani and Osawa (2003); Nishitani *et al*. (2004)
Lactobacillus pentosus	Nishitani *et al*. (2004); Kostinek *et al*. (2007)
Lactobacillus acidophilus	Nishitani *et al*. (2004); Sabu *et al*. (2006)
Lactobacillus animalis	Nishitani *et al*. (2004)
Lactobacillus murinus	Nishitani *et al*. (2004)
Enterococcus faecalis	Goel *et al*. (2005)
Weissella paramesenteroides	Kostinek *et al*. (2007)
Leuconostoc fallax	Kostinek *et al*. (2007)
Leuconostoc mesenteroides	Kostinek *et al*. (2007)
Pediococcus acidilactici	Nishitani *et al*. (2004)
Pediococcus pentosaceus	Nishitani *et al*. (2004)
Citrobacter freundii	Belmares *et al*. (2004)
Selenomonas ruminantium	Belmares *et al*. (2004)
Yeasts	
Candida sp.	Aoki *et al*. (1976b)
Saccharomyces cerevisiae	Zhong *et al*. (2004)
Mycotorula japonica	Belmares *et al*. (2004)

Table 2.1: …Continued

Microorganism	References
Pichia sp.	Deschamps *et al*. (1983)
Debaryomyces hansenii	Deschamps *et al*. (1983)
Fungi	
Aspergillus niger	Bradoo *et al.* (1996); Rana and Bhat (2005); Cruz-Hernandez *et al*. (2006); Trevino-Cueto *et al*. (2007); Murugan *et al*. (2007)
Aspergillus japonicus	Bradoo *et al*. (1997)
Aspergillus gallonyces	Belmares *et al*. (2004)
Aspergillus awamori	Bradoo *et al*. (1996); Mahapatra *et al*. (2005)
Aspergillus fumigatus	Batra and Saxena (2005)
Aspergillus versicolor	Batra and Saxena (2005)
Aspergillus flavus	Yamada *et al*. (1968); Batra and Saxena (2005)
Aspergillus caespitosum	Batra and Saxena (2005)
Aspergillus oryzae	Bradoo *et al*. (1996)
Aspergillus aculeatus	Banerjee *et al*. (2001)
Aspergillus aureus	Bajpai and Patil (1997)
Aspergillus fischeri	Bajpai and Patil (1997)
Aspergillus rugulosus	Bradoo *et al*. (1996)
Aspergillus terreus	Bajpai and Patil (1997)
Aspergillus foetidus	Banerjee *et al*. (2005)
Penicillium notatum	Ganga *et al*. (1977)
Penicillium islandicum	Ganga *et al*. (1977)
Penicillium chrysogenum	Bradoo *et al*. (1996)
Penicillium digitatum	Bradoo *et al*. (1996)
Penicillium acrellanum	Bradoo *et al*. (1996)
Penicillium carylophilum	Bradoo *et al*. (1996)
Penicillium citrinum	Bradoo *et al*. (1996)
Penicillium charlessi	Bradoo *et al*. (1996); Batra and Saxena (2005)
Penicillium variable	Batra and Saxena (2005)
Penicillium glaucum	Lekha and Lonsane (1997)
Penicillium crustosum	Batra and Saxena (2005)
Penicillium restrictum	Batra and Saxena (2005)
Penicillium glabrum	Van de Lagemaat and Pyle (2005)
Trichoderma viride	Bradoo *et al*. (1996)
Trichoderma hamatum	Bradoo *et al*. (1996)

Table 2.1: …Continued

Microorganism	References
Trichoderma harzianum	Bradoo *et al*. (1996)
Fusarium solani	Bradoo *et al*. (1996)
Fusarium oxysporium	Bradoo *et al*. (1996)
Mucor sp.	Belmares *et al*. (2004)
Paecilomyces variotii	Mahendran *et al.* (2006); Battestin and Macedo (2007)
Rhizopus oryzae	Hadi *et al.* (1994); Purohit *et al*. (2006)
Cryphonectria parasitica	Farias *et al*. (1994)
Heliocostylum sp.	Bradoo *et al*. (1996)
Cunnighamella sp.	Bradoo *et al*. (1996)
Syncephalastrum racemosum	Bradoo *et al*. (1996)
Neurospora crassa	Bradoo *et al*. (1996)

2.2.3.2 BACTERIA

Bacteria have generally been considered to be highly sensitive to tannins, but some isolates have been observed to survive and even degrade tannins (Basaraba, 1966). Over the past decade, many bacterial species have been reported to produce tannase. The production of tannase have been observed in the cultivated strains of *Bacillus pumilus, Bacillus polymyxa, Corynebacterium* sp. and *Klebsiella pneumoniae*, with chestnut bark extract as the sole carbon source (Deschamps *et al*., 1983). The production of tannase have also been reported in *Bacillus licheniformis* (Mondal and Pati, 2000; Mondal *et al*., 2000). Several studies also reported that bacterial species such as *Streptococcus gallolyticus* (Osawa *et al*., 1995a) and *Lonepinealla koalarum* isolated from alimentary tracts of koalas, goat or sheep (Osawa *et al*., 1995b; McSweeney *et al*., 1999) also showed tannase activity. An anaerobic diplococoid bacteria that is able to degrade hydrolysable tannins has also been isolated from the ruminal fluid of a goat (Nelson *et al*., 1995), and an enterobacteria that can degrade hydrolysable tannin-protein complexes has been isolated from the koala alimentary tract (Osawa, 1992). These isolated ruminal bacteria is thought to play a role in the anaerobic detoxification of tannin containing feeds and it is possible that these bacteria also derive energy from them through syntrophic associations in the rumen (Nelson *et al*., 1995). More recently, tannase activity was found in *Lactobacillus* strains isolated from human feces and fermented food (Osawa *et al*., 2000) and also from *Lactobacillus plantarum* (Ayed and Hamdi, 2002; Vaquero *et al*., 2004). *Lactobacillus* with tannase activity have also been isolated from the faeces of the Japanese large wood mouse (Sasaki *et al*., 2005) and from sheep wastes (Sabu *et al.,* 2006).