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Fungal Tannase: A Journey from Strain Isolation to Enzyme Applications

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ABSTRACT

Tannase is an important enzyme and plays an important role in plant polyphenol degradation as well as conversion to very important pharmaceutical products. Even though this enzyme is widely applied to different food and beverage industries, its industrial production, proper induction and catalysis mechanism still remain limited. This review presents an illustrated revision on fungal tannase. Emphasis had been laid on fungal strains which can produce tannase, different fermentation processes of tannase production, tannase purification, structural and functional characteristics of this enzyme, different enzyme assay techniques and applications of this enzyme. A probable mechanism of tannin degradation by fungus is briefly described.

Keywords: Application, fungal tannase, production, properties, tannase assay

CONTENTS

INTRODUCTION

Tannins are plant polyphenolic compounds having high antimicrobial activity and easily precipitate any protein due to their complexation with proteins and enzymes. However, few organisms are known to have the ability to survive in a tannin-rich environment by production of tannase (Vermeire and Vandamme 1988). Tannin acyl hydrolase (E. C. 3.1.1.20) or tannase was accidentally discovered in 1867 by Van Tieghem (Aguilar *et al.* 2007). This adaptive, inducible hydrolase catalyzes the hydrolysis of depside and ester bonds in varied substrates like, gallo tannins; gallic acid esters; epicatechin gallate; epigallocatechin-3-gallate, releasing glucose and galic acid (Bajpai and Patil 1996; Bradoo *et al.* 1997; Lekha and Lonsane 1997; Banerjee *et al.* 2001; Banerjee and Pati 2007; Kasieczka-Burnecka *et al.* 2007; Robledo *et al*. 2008; Mahapatra and Banerjee 2009; Lokeswari *et al*. 2010; Renovato *et al*. 2011; Costa *et al*. 2012). Tannase expand its commercial importance for not only its pharmaceutical important end product gallic acid, but also, there are several applications of treated substrate of tannase. From the last few decades tannase is widely applied in the production of instant tea, clarification of beer and fruit juices, manufacture of coffee flavored soft drinks, improvement in the flavor of grape wine and as an analytical probe for determining the structures of naturally occurring gallic acid esters (Bajpai and Patil 1996; Lekha and Lonsane 1997). Although, tannase can obtained from plant, animal and microbes but the most common and important source to obtain this enzyme is from microbes due to better production, low costing and high stability of microbial tannase (Lekha and Lonsane 1997). Among the microorganisms fungi are the most predominant source of tannse producer. At present most of the commercialized tannase is produced by fungi like tannase of Biocon (India), Kikkoman (Japan) ASA Special enzyme GmbH (Germany) and JFC GmbH (Germany).

There are few reviews published on microbial tannase. In this communication attention has been paid to fungal tannase; from production to application.

SUBSTRATE FOR TANNASE PRODUCTION

Tannins are naturally occurring water soluble polyphenolic plant secondary metabolites with varying molecular weights ranging from 300 D to 3000 D (Haslam 1989). It is the

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Invited Review

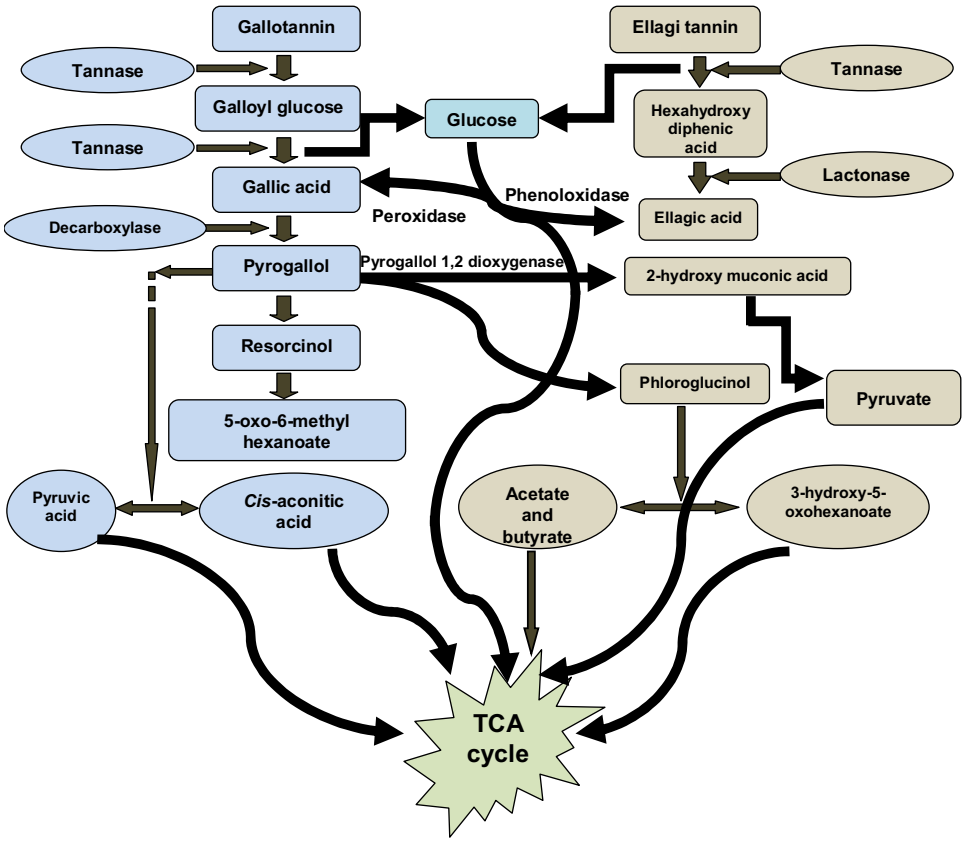

Fig. 1 Schematic diagram representing tannin degradation and metabolism by fungi.

second most abundant plant phenolics (Aguilar *et al.* 2007). Tannins are divided mainly into four groups; hydrolysable tannins or gallotannins, ellagitannins, complex tannins and condensed tannins. At present some special types of tannins are reported that have both hydrolysable and condensed part and are called catechin tannins (Graham 1992). Based on their structural characteristics hydrolysable tannins like, gallotannins are easily degraded by yeast and molds. But, others are more resistant towards microbial degradation because of their highly intricate structures. Most of the hydrolysable tannins, a few of the ellagitannins and some seed, leaf tannins are commonly used by fungus as their soul carbon source and utilization depends on the enzyme; tannase produced by the fungus.

MECHANISM OF TANNIN METABOLISM

In 1913, Knudson reported that filamentous fungi could degrade tannin. Fungal degradation of gallotannins and ellagitannins are well described by several researchers (Nishira 1961; Iibuchi *et al.* 1972; Lekha and Lonsane 1997; Bhat *et al.* 1998; Gonzalez *et al.* 2012). Tannase hydrolyzed tannins yielding gallic acid and glucose. This gallic acid then decraboxylated by gallic acid decarboxylase to form pyrogallol, which ultimately converted to pyruvic acid or cis-aconituc acid or 5-oxo-6-methyl hexanoate or 3-hydroxy-5-oxohexanoate or 2-hydroxy muconic acid by different pathways and ultimately enters to the TCA cycle (Watanabe 1965; Mahadevan and Sivaswamy 1985; William *et al.* 1986; Bhat *et al.* 1998; Gonzalez *et al.* 2012). A schematic diagram of tannin metabolism is presented in **Fig. 1**.

FUNGAL TANNASE PRODUCERS

Although fungi is the most dominant tannase producer among all microbes but tannase producers do not belongs to wide variety of fungal genera. Most of the research out puts clearly indicated that *Aspergillus* sp. and *penicillium* sp. are the most common tannase producers. Some other molds like *Fusarium* sp., *Mucor* sp., *Pacilomyces* sp. *Hyalopus* sp. also reported as tannase producers but the list is not very long. In consideration of yeasts, only a few tannase producing species were reported (Banerjee and Pati 2007). A detailed list of fungal tannase producers were presented here for better observations (**Table 1**).

PRODUCTION OF TANNASE

Tannase is an inducible enzyme and tannic acid is the most common inducer reported for tannase synthesis from fungi. Many research findings indicated that some other chemical compounds like gallic acid, pyrogallol, methyl gallate, glucose, etc. induces tannase expression (Bajpai and Patil 1997). However, the actual mechanism by which tannic acid or other inducers accelerate tannase synthesis has not been established and sometimes these inducers played a negative role in tannase synthesis (Deschamps *et al.* 1983; Aguilar *et al.* 2001). Fungal tannase production is also varied with supporting chemical components and physical conditions used during tannase production. Generally, optimization of tannase production were done by doing variation in tannic acid percentage, percentage of moisture, rate of oxygenation, addition of supplementary nitrogen sources, addition of supplementary phosphate, addition of supplementary salts, concentration of supplementary nitrogen, salts and phosphate, pH of the medium, incubation time, incubation temperature, etc. Different fungal strain needed different combinations of these variables for maximum tannase biosynthesis. But from overall observation some range can be predicted for fungal tannase synthesis. Most of the tannase producing fungi favors acidic pH range (pH 3.0 to 6.5), high oxygen level, 28 to 35°C incubation temperature for enzyme secretion. The incubation period varied from 24 to 120 h.

MODE OF FERMENTATION

Different fermentation procedures were developed for fungal tannase production. Liquid submerged fermentation,

Table 1 List of fungal strains reported as tannase producers.

Fungi	Reference
Filamentous fungi	
Aspergillus acolumaris	Batra and Saxena 2005
Aspergillus aculeatus	Van Diepeningen *et al*. 2004
Aspergillus aculeatus DBF 9	Banerjee *et al.* 2001; Banerjee *et al.* 2007b
Aspergillus alliaceus	Batra and Saxena 2005
Aspergillus amstellodemi	Batra and Saxena 2005
Aspergillus aureus	Bajpai and Patil 1997
Aspergillus awamori	Seth and Chand 2000
Aspergillus awamori nakazawa	Mahapatra *et al.* 2005
Aspergillus awamori BTMFW032	Beena *et al.* 2010; Beena *et al.* 2011
Aspergillus awamori MTCC9299	Beniwal and Chhokar 2010; Chhokar *et al.* 2010
Aspergillus brasiliensis	Van Diepeningen *et al*. 2004
Aspergillus caespitosum	Batra and Saxena 2005
Aspergillus carbonarius	Van Diepeningen *et al*. 2004
Aspergillus carneus	Batra and Saxena 2005
Aspergillus candidus	Murugan and Al-Sohaibani 2010
Aspergillus ficuum	Lu and Chen 2009
Aspergillus fischeri	Bajpai and Patil 1997
Aspergillus fisheri	Batra and Saxena 2005
Aspergillus flavus	Paranthaman *et al.* 2009
Aspergillus foetidus	Banerjee *et al*. 2005; Mukherjee and Banerjee 2006; Naidu *et al.* 2008
Aspergillus fumigatus	Batra and Saxena 2005
Aspergillus gallonyces	Belmares *et al.* 2004
Aspergillus giganteus	Batra and Saxena 2005
Aspergillus heteromorphus	Van Diepeningen *et al*. 2004
Aspergillus janus	Batra and Saxena 2005
Aspergillus japonicus	Bradoo *et al.* 1997; Van Diepeningen *et al.* 2004
Aspergillus javipes	Batra and Saxena 2005
Aspergillus nidulans	Batra and Saxena 2005
Aspergillus niger	Bradoo *et al.* 1996; Ramírez-Coronel *et al.* 2003; Yu and Li 2005; Lokeswari and Raju 2007; Sharma *et al.* 2007; Paranthaman *et al.* 2009; Rodríguez-Duran *et al.* 2011
Aspergillus niger Aa-20	Trevino *et al.* 2007
Aspergillus niger ATCC 16620	Sabu *et al.* 2005
Aspergillus niger FETL FT3	Darah *et al.* 2011
Aspergillus niger GH1	Mata-Gomez *et al.* 2009; Ramos *et al.* 2011
Aspergillus niger MTCC 2425	Bhardwaj *et al.* 2003; Rana and Bhat 2005
Aspergillus niger PSH	Robledo *et al.* 2008
Aspergillus niger van Teighem	Sharma and Gupta 2003
Aspergillus nivens	Batra and Saxena 2005
Aspergillus ornatus	Cruz-Hernández *et al.* 2005
Aspergillus oryzae	Zhong *et al.* 2004; Paranthaman *et al.* 2008
Aspergillus parasiticus	Bajpai and Patil 1996; Batra and Saxena 2005
Aspergillus penicilliniformis	Batra and Saxena 2005
Aspergillus recurriero	Batra and Saxena 2005
Aspergillus ruber	Kumar *et al.* 2007
Aspergillus rugulosus	Bradoo *et al.* 1996
Aspergillus sojae	Yamada *et al.* 1968
Aspergillus stellatus	Batra and Saxena 2005
Aspergillus striatus	Batra and Saxena 2005
Aspergillus tamari	Costa *et al*. 2008
Aspergillus terreus	Bajpai and Patil 1997; Batra and Saxena 2005
Aspergillus terricola	Cruz-Hernández *et al.* 2005
Aspergillus tubingensis	Van Diepeningen *et al.* 2004
Aspergillus variecol	Batra and Saxena 2005
Aspergillus versicolor	Batra and Saxena 2005
Aspergillus usamii	Yamada *et al.* 1968
Aspergillus ustus	Yamada *et al.* 1968
Aspergillus SHL 6	Huang *et al.* 2005
Chaetomium sp.	Bhat *et al.* 1998
Cryphonectria parasitica	Farias *et al.* 1994
Cunnighamella sp.	Bradoo *et al.* 1996
Cylindrocladiella peruviana	Peterson *et al.* 2009
Cylindrocarpon sp.	Bhat *et al.* 1998
Doratomyces stemonitis	Peterson *et al.* 2009
Fusarium flocciferum	Mendonça *et al.*
Fusarium oxysporium	Bradoo *et al.* 1996
Fusarium solani	Bradoo *et al.* 1996; Bajpai and Patil 1997
Fusarium subglutinans	Hamdy 2008
Fusarium spp.	Murugan *et al.* 2007
Heliocostylum sp.	Bradoo *et al.* 1996
Hyalopus sp.	Mahapatra and Banerjee 2009
Hymenoscyphus ericae	Bending and Read 1996

Table 1 (Cont.)

Fungi	Reference
Filamentous fungi (Cont.)	
Mariannaea camptospora	Peterson *et al.* 2009
Mucor sp.	Belmares *et al.* 2004
Neosartorya fischeri	Aguilar *et al.* 2001
Neurospora crassa	Bradoo *et al.* 1996
Paecilomyces variotii	Battestin and Macedo 2007
Penicillium acrellanum	Bradoo *et al.* 1996
Penicillium aculeatum	Batra and Saxena 2005
Penicillium atramentosum	Selwal *et al.* 2011
Penicillium awrentiogiseum	Batra and Saxena 2005
Penicillium canescens	Sariozlu and Kivanc 2009
Penicillium capsulatum	Batra and Saxena 2005
Penicillium carylophilum	Bradoo *et al.* 1996
Penicillium carymbiferum	Batra and Saxena 2005
Penicillium charlessi	Bradoo *et al.* 1996; Batra and Saxena 2005
Penicillium chrysogenum	Bradoo *et al.* 1996; Nuero and Reyes 2002
Penicillium citrinum	Bradoo *et al.* 1996
Penicillium commune	Cruz-Hernández *et al.* 2005
Penicillium concentricum	Peterson *et al.* 2009
Penicillium corylophilum	Batra and Saxena 2005
Penicillium crustosum	Batra and Saxena 2005
Penicillium digitatum	Bradoo *et al.* 1996; Batra and Saxena 2005
Penicillium expansum	Yamada *et al.* 1968; Batra and Saxena 2005
Penicillium frequentans	Sariozlu and Kivanc 2009
Penicillium glabrum	Van de Lagemaat and Pyle 2005
Penicillium glaucum	Lekha and Lonsane 1997
Penicillium granulatum	Batra and Saxena 2005
Penicillium islandicum	Ganga *et al.* 1977
Penicillium javanicum	Yamada *et al.* 1968
Penicillium notatum	Ganga *et al.* 1977
Penicillium oxalicum	Yamada *et al.* 1968
Penicillium purpurogenum	Sariozlu and Kivanc 2009
Penicillium purpurogenum PAF6	Jana *et al.* 2012
Penicillium restrictum	Batra and Saxena 2005
Penicillium rugulosum	Batra and Saxena 2005
Penicillium spiculisporum	Batra and Saxena 2005
Penicillium spinulosum	Sariozlu and Kivanc 2009
Penicillium spp.	Murugan *et al.* 2007
Penicillium variable	Saxena and Saxena 2004; Batra and Saxena 2005; Sharma *et al.* 2007
Penicillium zacinthae	Sariozlu and Kivanc 2009
Raffaelea quercivora	Imai *et al.* 2009
Rhizoctonia sp.	Bhat *et al.* 1998
Rhizopus oryzae	Hadi *et al.* 1994; Purohit *et al.* 2005; Mukherjee and Banerjee 2006
Syncephalastrum racemosum	Bradoo *et al.* 1996
Trichoderma atroviride	Peterson *et al.* 2009
Trichoderma hamatum	Bradoo *et al.* 1996
Trichoderma harzianum	Bradoo *et al.* 1996
Trichoderma spp.	Murugan *et al.* 2007
Trichoderma viride	Bradoo *et al.* 1996; Bajpai and Patil 1997
Trichoderma viride MTCC 167	Lokeswari *et al.* 2010
Verticillium sp. P9	Kasieczka-Burnecka *et al.* 2007
Yeasts	
Arxula adeninivorans	Boer *et al.* 2009; Boer *et al.* 2011
Aureobasidium pullulans DBS66	Banerjee and Pati 2007
Candida nitrativorans	Kumar *et al.* 1999
Candida sp.	Aoki *et al.* 1976
Candida utilis	Shi *et al.* 2005
Debaryomyces hansenii	Deschamps *et al.* 1983
Mycotorula japonica	Belmares *et al.* 2004
Pichia adzetti	Kumar *et al.* 1999
Pichia spp.	Deschamps *et al.* 1983
Saccharomyces cerevisiae	Zhong *et al.* 2004

liquid surface fermentation and solid state fermentation were well studied for tannase production (Yamada 1967; Doi *et al.* 1973; Barthomeuf *et al.* 1994; Hadi *et al.* 1994; Lekha and Lonsane 1994; Chatterjee *et al.* 1996; Bradoo *et al.* 1997; Banerjee *et al.* 2001; Mahapatra and Banerjee 2009). Among these, liquid submerged and solid state fermentation were mostly applied process for tannase production.

Submerged fermentation

Tannase production in liquid submerged fermentation (SmF) is a very common techniques used from very beginnings and it has many positive sides like easy control of variables, isolation of enzyme, utilization of whole substrate, short incubation time, etc. Beena *et al.* (2010) worked on *Aspergillus awamori* BTMFW032 which produced acido-

philic tannase in SmF with high specific activity (2761.89 IU). Paranthaman *et al.* (2009) reported that under submerged fermentation *Aspergillus flavus* produced maximum tannase activity of 30.12 (U/g/min) in 96 h at 35°C with 2% tannic acid. Costa *et al.* (2012) reported that *Aspergillus tamari* produced highest tannase when 2% tannic acid or 2% gallic acid was used as carbon source; maximal tannase production (14.8 U/ml and 20.4 U/ml) was obtained after 48 h fermentation. Aguilar *et al.* (2001) studied on extracellular tannase yield from *Aspergillus niger* Aa-20 (12 U/ml) using tannic acid as the main carbon source under submerged fermentation. Tannase production by *Aspergillus oryzae* is increased and optimum activity 32.62U/ml was recorded at 40°C, pH of 5.0 after the optimum incubation period of 24 h when 1.5% (w/v) of pure tannic acid was used (Lokeswari 2010). Bradoo *et al.* (1997) and Rajakumar and Nandy (1983) reported that maximum extracellular tannase was produced from *Aspergillus japonicas* and *Penicillium chrysogenum* when 2% tannic acid was used in fermentation medium as soul carbon source. Bajpai and Patil (1997) reported that *Aspergillus niger*, *Aspergillus fischeri*, *Trichoderma viridae* and *Fusarium solani* produced maximum tannase in submerged fermentation when they were cultured with initial gallotannin concentration of 10, 3, 2 and 3%, respectively. *Aspergillus aculaetus* DBF 9 produced maximum tannase when cultured with 3% natural tannin of *Cassia siamea* (Banerjee *et al.* 2007a). Maximum Tannase production from *Aspergillus niger* occurred in the culture broth containing 1-2% (w/v) tannic acid and 0.05-0.1% (w/v) glucose (Lokeswari and Raju 2007). Lokeswari (2012) investigated tannase production under SmF in the medium containing extract of cashew testa tannins (*A. occidentale*) using *Aspergillus oryzae*. Tannase production by the organism was found to be maximal in medium containing 0.5% (w/v) of crude tannin of *A. occidentale* with medium pH 5.0 and of 48 h incubation time, at 40°C. Beniwal and Chhokar (2010) reported that maximum tannase production (1.45 U/ml) was obtained from *Aspergillus awamori* MTCC 9299 in pH and incubation temperature of 5 and 35°C, respectively, in 48 h incubation when the level of agitation speed was at 125 rpm. Optimum enzyme production by *Trichoderma viride* MTCC 167 was noticed at 48 h incubation (Lokeswari *et al.* 2010). The production of intracellular and extracellular tannase by *Aspergillus niger* van Tieghem MTCC 2425 was reported by Rana and Bhat (2005) where the enzyme production reached a peak activity by 48 h and thereafter showed a significant decline. Lekha *et al.* (1994) and Sabu *et al.* (2005) reported that after 96 h fermentation *Aspergillus niger* produced maximum extra-cellular tannase. It was also reported that *Rhizopus oryzae* produced maximum tannase at 120 h (Chaterjee *et al.* 1996). Banerjee *et al.* (2001) found maximum extracellular tannase production by *A. aculaetus* DBF9 after 36 h. Various physico-chemical parameters were optimized to obtain maximum enzyme production by *Asperillus* SHL 6. Both the tannase activity and yield of ellagic acid have a maximum when sucrose used as the additional carbon source. Organic nitrogen source especially peptone and about 5 g/l tannin concentration is favourable for the high tannase activity and ellagic acid production from valonia tannin. Maximum tannase activity occurred between 48 and 72 h of growth of the microorganism at 28°C, pH 4-5 (Huang *et al.* 2005). Saxena and Saxena (2004) have optimized the production of extracellular tannase by *Penicillium variable* on natural tannin isolated from the fruit of *Terminalia chebula*. The maximum tannase activity was found at pH 5.0 and 5.8 g/50 ml of the substrate concentration used in the medium after 72 h incubation. *Aspergillus niger*, *Aspergillus awamori* MTCC 9299, have been reported to have their optimal media pH 5.0 for tannase synthesis (Srivastava and Kar 2009; Beniwal and Chhokar 2010). *Aspergillus aculaetus* DBF9 showed highest tannase production at pH 5.5 in submerged fermentation (Banerjee *et al.* 2001) *Aspergillus awamori* MTCC 9299, *Fusarium subglutinans* produced maximum tannase when they were cultured at 35°C (Hamdy 2008; Beniwal and Chhokar 2010). Lokeswari and Raju (2007) reported that the pH, incubation period and temperature optima of tannase production in submerged culture by *Aspergillus niger* was found at 5.5, 36 h and 35°C, respectively. In an another report (Sharma *et al.* 2007) optimization for tannase production by *Aspergillus niger* showed that 5% tannic acid, 0.8% sodium nitrate, pH 5.0, 5×10^7 spores/50 ml inoculum density, 150 rpm agitation speed and 48 h incubation period were optimum for tannase production. The optimum process conditions for tannase synthesis by *Aspergillus awamori* was 60 h incubation with an initial tannic acid concentration of 35.0 gm/l, yielding 771 IU of intracellular tannase per gram dry cell weight (Seth and Chand 2000). *Aspergillus japonicus* produces maximum extracellular tannase activity (33.06 U/ml) after 24 h incubation at 30°C and pH 6.6 with 0.2% glucose and 2% tannic acid in Czapek-Dox's minimal medium (Bradoo *et al.* 1997). Tannase production by *Aureobasidium pullulans* DBS66 was studied by Banerjee and Pati (2007). The organism produced maximum tannase in presence of 1% tannic acid after 36 h incubation at 0.1% (w/v) glucose concentration with $(NH_4)_2HPO_4$ as nitrogen source and shaking speed of 120 rpm.

Boer *et al.* (2011) optimized tannase production using transgenic *Arxula adeninivorans* strains. They have reported that transgenic Arxula strain containing ANAN1 expression module produce 51,900 U/l of tannase activity after 142 h fermentation. The recombinant tannase production was induced by tannic acid and gallic acid (Boer *et al.* 2009). The enzyme is indistinguishable with its wild type. They have mentioned that recombinant strain produced tannase as much as four times higher than its wild type in same culture conditions. In 2004, Zhong *et al.* isolate a tannase gene from *A. Oryzae* and cloned it in *Pichia pastoris* where tannase were expressed. The large amount of recombinant tannase (7000 IU/l) was produced by this organism in fed batch culture.

Solid state fermentation

Over the last few decades tannase production through solid-state culture system (Lekha and Lonsane 1994; Chaterjee *et al.* 1996) was studied efficiently by several researchers because tannase expressed in solid state fermentation (SSF) had higher titer with more stable in spite of temperature and pH changes in comparison with those obtained by submerged culture (Aguilar *et al.* 1999). Rana and Bhat (2005) studied on production of tannase by *Aspergillus niger* van Tieghem MTCC 2425 under submerged, surface and solid state fermentation. They reported that in SSF tannase was synthesized within a short period of time (96 h) compared to other two fermentation techniques. Aguilar *et al.* (1999), Mata-Gomez *et al.* (2009), Mahapatra and Banerjee (2009) also reported such type of better tannase production in SSF.

Jana *et al.* (2012) reported about tannase production by *Penicillium purpurogenum* PAF6. Different plant materials were used as substrate and enzyme productivity was found in the following order: tamarind seed > haritaki > pomegranate > tea leaf waste > arjun fruit. *Penicillium purpurogenum* PAF6 produced the maximum tannase in SSF after 48 h of cultivation at 30°C, substrate: moisture ratio of 1:3, 1% (w/v) urea, 0.1% (w/v) diammonium hydrogen concentration with additional 4% (w/v) tannic acid supplementation. Rodríguez *et al.* (2011) studied SSF for tannase production by *Aspergillus niger* in packed-bed bioreactors using polyurethane foam as an inert support. The organism produced maximum tannase in the medium containing 50 g/l tannic acid at 30°C and pH 4.0. Lekha and Lonsane (1994) used sugar cane pith bagasse as an inert support for tannase production by *Aspergillus niger* PKL 104 where 6% tannic acid was additionally supplemented as soul carbon source. Trevino *et al.* (2007) reported that *Aspergillus niger* Aa-20 produced maximum tannase (2479.59 U/l) in SSF within 12 h of fermentation using discontinuous polyurethane matrix as a inert support. Ramirez-Coronel *et al.*

(2003) also worked on tannase production from *Aspergillus niger* in SSF with polyurethane foam as an inert support. Rodrigues *et al.* (2007) reported *Aspergillus oryzae* produce tannase by SSF with cashew apple bagasse as substrate. The supplementation with 60 ml water/100 g of substrate, 2.5% tannic acid and ammonium sulphate considerably improved the tannase production using this organism. Banerjee *et al.* (2007b) studied tannase production through SSF by *Aspergillus aculeatus* DBF9 where wheat bran was used as a solid substrate. The organism produced maximum tannase after 72 h incubation at 30ºC with 80% initial moisture content. Additional 5% tannic acid was supplemented to the solid medium as soul carbon source for *Aspergillus aculeatus* DBF9. *Aspergillus heteromorphus* MTCC 5466 was reported as a tannase producer in SSF where fermentation was carried out using wattle, quebracho, myrobalan and bahera fruit powder as substrate. Bahera fruit (*Terminalia belerica*) was found as best substrate for *Aspergillus heteromorphus* MTCC 5466 and maximum tannase production was found in medium with 60% v/w moisture, 4% v/w inoculum; 1.5% w/w corn steep liquor as nitrogen source, 3:7 w/w Behera fruit and wheat bran, 72 h incubation at 32°C (Prasad *et al.* 2011). Reddy and Rathod (2012) investigated tannase production by *Penicillium purpurogenum* BVG7 under SSF with different natural substrate namely, acacia pods, red gram husk, sorghum husk and spent tea powder having tannic acid content 0.48, 0.36, 0.20 and 0.12 mg/g of substrate, respectively. pH 5.5 was found most suitable for tannase synthesis using all the substrates. Tannase production level in different materials were recorded as follows: red-gram husk (34.0 U/ml) > acacia pod (33.5 U/ml) > sorghum husk (33.1 U/ml) > spent tea powder (30.0 U/ml). In their study tannase and gallic acid production by *P. purpurogenum* BVG7 were observed maximum at 30°C. Battestin and Macedo (2007) reported on use of coffee husk and rice bran as substrate for tannase synthesis under SSF by *Paecilomyces variotii* and maximum tannase synthesis was recorded when this organism grown for five days in SSF with 15% (w/w) tannic acid concentration, ammonium nitrate as additional nitrogen and residual substrate of 50:50 of coffee husk: rice bran. Kumar *et al.* (2007) isolated an *Aspergillus rubber* which produced highest tannase (69 U/g dry substrate) in SSF with jamun leaves as substrate at 30°C after 96 h incubation with tap water as moistening agent and medium pH 5.5. Trevino-Cueto *et al.* (2006) reported that when *Aspergillus niger* Aa-20 cultured in SSF with 70% initial moisture content, pH of 5.5 for 43 h and *Larrea tridentata* cov. was used as the sole carbon source, inducer and solid support then maximum tannase was synthesized. Sabu *et al.* (2005) used Palm kernel cake and tamarind seed powder as substrate and NH_4NO_3, $MgSO_4$, NaCl supplementation for tanase production under SSF by *A. niger* ATCC 16620. Pinto *et al.* (2003) reported that *A. niger* 3T5B8 produced maximal tannase under solid state fermentation after 24 h incubation with medium containing 15% tannic acid concentration, 37.5% initial moisture, 1.7% ammonium sulphate, 2.0% sodium phosphate.

Recently some new processes were applied for better tannase production from fungi. Kar *et al.* (1999) described a modified solid state fermentation (MSSF) process for simultaneous production of gallic acid and tannase by *Rhyzopus oryzae* and they have got 1.7 times higher tannase production with this process (Kar and Banerjee 2000). Van de Lagemaat and Pyle (2001) stated a continuous solid state fermentation system for the fungal tannase production. Banerjee *et al.* (2005) reported a co-culture system for tannase production. Yu and Li (2005) reported microencapsulation of fungal mycelia for tannase synthesis. Darah *et al.* (2011) studied the production of tannase in submerged fermentation using immobilized cells of *Aspergillus niger* FETL FT3 and the tannase production of 3.98 IU/ml was reported. They also mentioned that the production of tannase by immobilized fungal cell is 41.64% higher than traditional SmF culture of the same *A. niger*. Recently, Beena *et al.* (2011) tried with slurry state fermentation for maximum tannase production by *Aspergillus awamori* BTMFW032. In this study they have got maximum tannase within 18 h incubation in a medium containing 26.6% (w/v) *Garcinia cambogia* leaf, supplemented with 0.1% tannic acid for tannase induction at 40°C incubation temperature, medium pH 5.0 and sea water. Authors suggested that sea water played an inducer role in tannase expression of *A. awamori* BTMFW032.

PURIFICATION OF TANNASE

Tannase has been purified from variety of fungi. Tannase produced both as intracellular and extracellular form and thus purification started either from mycelia extract or from culture filtrate (Yamada *et al.* 1968; Banerjee *et al.* 2001). At first tannase was partially purified and concentrated by ammonium sulphate precipitation method (Rajkumar and Nandy 1983) or acetone precipitation (Iibuchi *et al.* 1968; Bevenini and Metche 1990; Gupta *et al.* 1997; Mahapatra and Banerjee 2009). Different percentages of ammonium sulphate were used for tannase precipitation. The precipitated tannase was dialyzed for salting out and thus partially purified tannase obtained. Partial purification of tannase using polyethylene glycol, polyvinyl alcohol, acetone and dextran has also been reported (Beverini and Metche 1990). Aoki *et al.* (1976) reported that ammonium sulphate could not precipitate the tannase from *Candida* sp. As tannase is acidic in nature (Adachi *et al.* 1968) so the second step employed in most cases was anion-exchange chromatography (Rajkumar and Nandy 1983). DEAE-cellulose was commonly used for purification (Yamada *et al.* 1968; Fumihiko and Kiyoshi 1975; Rajkumar and Nandy 1983). The last step employed in tannase purification was gel-filtration chromatography (Rajkumar and Nandy 1983; Iibuchi *et al.* 1968). Different kinds of sephadex like G-200, G-150, G-100 were used by most workers (Iibuchi *et al.* 1968; Yamada *et al.* 1968; Aoki *et al.* 1976; Rajkumar and Nandy 1983; Sharma *et al.* 1999; Bhardwaj *et al.* 2003). A common purification method was schematically represented in **Fig. 2**.

Recently, Gaikaiwari *et al.* (2012) reported a new method for tannase purification. They used reverse micellar extraction (RME) using ionic surfactants which provide an attractive option for concentration and purification of *Aspergillus allahabadi* intracellular tannase. They demonstrated that CTAB–isooctane system was most appropriate as a surfactant for purification of *Aspergillus allahabadi* tannase. Under the specific circumstances, 12.7-fold purification, 81.2% recovery and 3-fold concentration of tannase with a process time of 45 min was found by them.

TANNASE ASSAY METHODS

From the very beginning petri plate screening technique was extensively used, where tannase activity was detected by formation of clear zone surrounding the fungal colony (Bhat *et al.* 1996; Bradoo *et al*. 1996). Several researchers tried to develop assay method of tannin acyl hydrolase. Among them Dhar and Bose (1964) is pioneer in this field. They have developed a method using methyl gallate as a substrate. Haslam and Tanner (1970) used another method where *p*-nitrophenyl gallic acid was used as a substrate. Iibuchi *et al.* (1967) and Deschamps *et al.* (1983) used another method where absorbace were measured at 310 nm and 260 nm after protein precipitation of residual gallotannin. Katwa *et al.* (1981) described an assay method for immobilized tannase. Tannase was assayed with polyacrylamide gel, collagen and Duolite-S-762 as matrices and end product gallic acid was spectrophotometrically determined. The kinetic parameters of the enzymatic reaction have been studied and an assay procedure has been formulated. Jean *et al.* (1981) developed a gas chromatographic method for the satisfactorily determination of gallic acid after enzymatic hydrolysis of methyl gallate by fungal tannase. In this method a specific, quantitative analysis of the enzyme is possible. A colorimetric method for determination of rel-

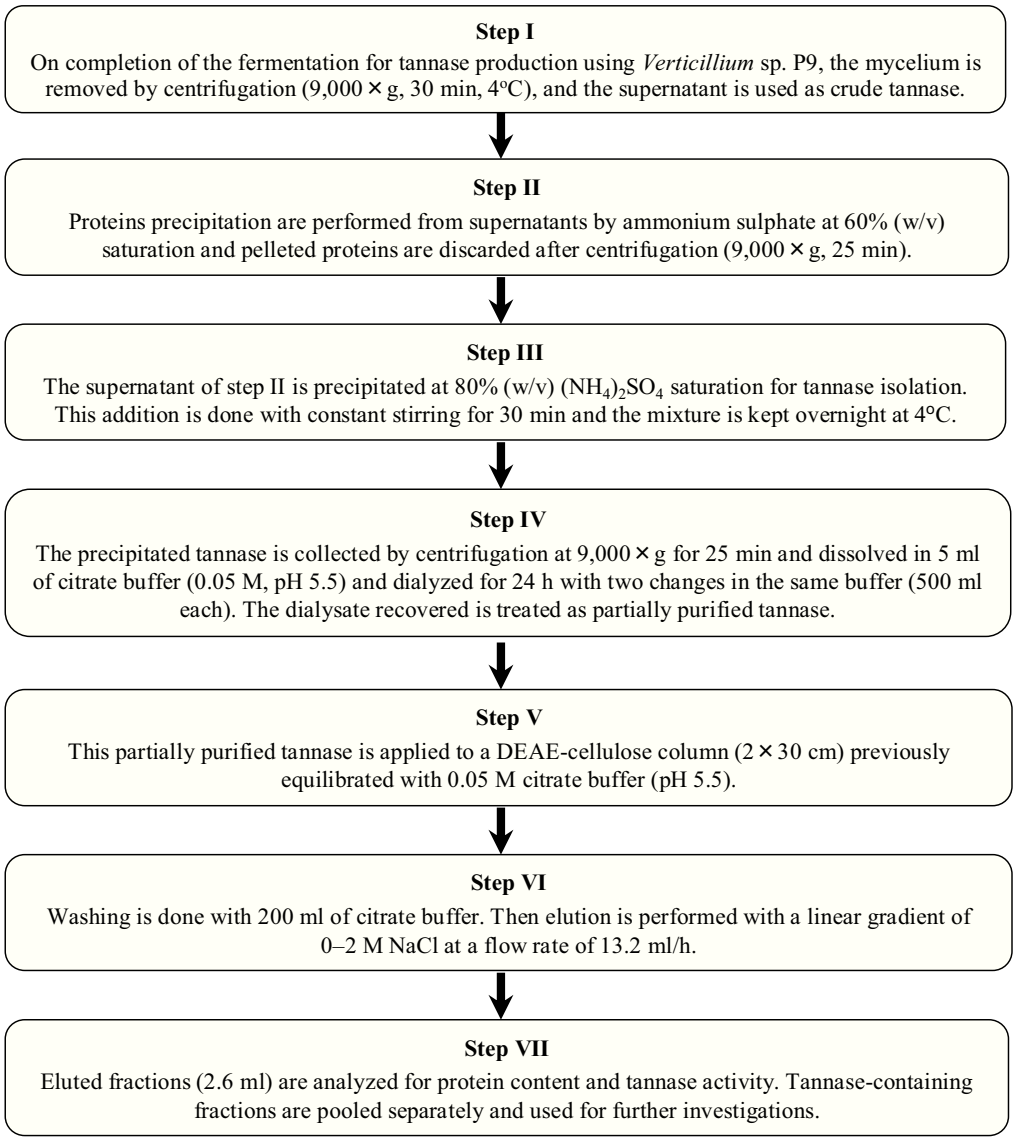

Fig. 2 Methods of *Verticillium* sp. P9 tannase purification (based on Kasieczka-Burnecka *et al*. 2007).

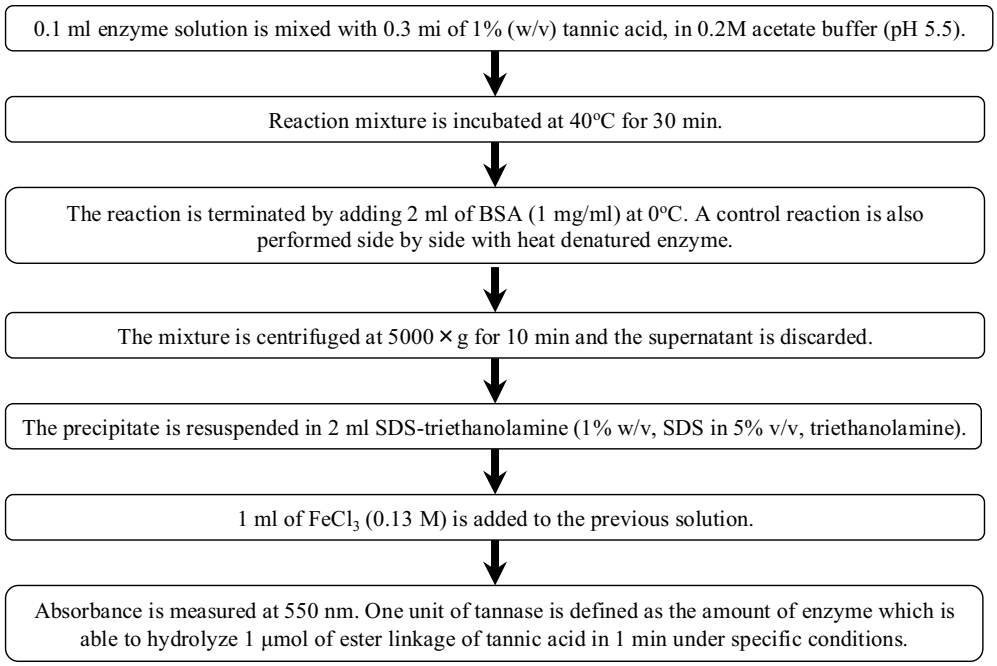

Fig. 3 Tannase assay protocol by the method of Mondal *et al.* 2001 (based on Mahapatra and Banerjee 2009).

Table 2 Molecular characteristics of some fungal tannase.

Fungus name	Molecular weight (Da)	Carbohydrate content (%)	Reference
Emericella nidulans (=*Aspergillus nidulans*)	302 000	50%	Gonçalves *et al.* 2011
Aspergillus flavus	192 000	25.4%	Yamada *et al.* 1968; Adachi *et al.* 1971
Aspergillus niger	186 000	43%	Barthomeuf *et al.* 1994; Parthasarathy and Bose 1976
Aspergillus oryzae	300 000	22.7%	Hatamoto *et al.* 1996
Aspergillus niger van Tieghem	185 000	-	Rana and Bhat 2005
Candida sp. K-1	250 000	61.9%	Aoki *et al.* 1976
Aspergillus awamori BTMFW032	230 000	8.02%	Beena *et al.* 2010
Aspergillus niger GH1	225 000	7.1%	Mata-gomez *et al.* 2009
Chryphonectria parasitica	240 000	64%	Aoki *et al.* 1976
Arxula adeninivorans	320 000	-	Boer *et al.* 2009
Verticillium sp. P9 TAH 1	155 000	11%	Kasieczka-Burnecka *et al.* 2007
Verticillium sp. P9 TAH 2	155 000	26%	Kasieczka-Burnecka *et al.* 2007
Pichia pastoris	90 000	-	Zhong *et al.* 2004
Aspergillus niger MTCC 2425	185 000	-	Bhardwaj *et al.* 2003
Aspergillus oryzae	63 000	-	Hatamoto *et al.* 1996
Paecilomyces variotii	87 300 and 71 500	-	Battestin and Macedo 2007
Paecilomyces variotii	149 800	-	Mahendran *et al.* 2006
Cryphonectria parasitica	240 000	-	Farias *et al.* 1994
Aspergillus niger ATTC 16620	149 000	-	Sabu *et al.* 2005
Aspergillus niger N 888	165 000	-	Sabu *et al.* 2005
Penicillium variable	310 000	-	Sharma *et al.* 2008
Aspergillus awamori MTCC 9299	101 000	-	Chhokar *et al.* 2010

eased gallic acid was described by Skene and Brooker (1995). Sharma *et al.* (2000) described a new method for assay of microbial tannase (tannin acyl hydrolase) based on the formation of chromogen between gallic acid and rhodanine. Another colorimetric method was described by Mondal *et al.* (2001). Here they have detected the changes in optical density of tannic acid at 530 nm after enzymatic reaction. The residual tannic acid was measured by BSA precipitation method. Chang *et al.* (2006) developed a stopped-flow manifold to assay and characterize immobilized tannase. In this method immobilized enzyme reactor was inserted within the tube-type electrode pair (cell constant = 103.2 cm^{-1}) for a real-time conductometric measurement by which activities and kinetic parameters (K_m values) for propyl gallate, methyl gallate and tannic acid were investigated. Ramirez *et al.* (2008) describes a new technique for tannase assay. In this report they demonstrated a new microplate high-throughput assay technique which allowed the detection of tannase activity as low as 1.7 mU/ml. Iacazio *et al.* (2000) described a tannase assay method where they used protocatechuic acid *p*-nitrophenyl ester, 5 as substrate and upon tannase action p-nitrophenol was released and measured spectrophotometrically either at 350 nm (pH <6.0) or at 400 nm (pH 6.0-7.0). A commonly used tannase assay method is schematically represented in **Fig. 3**.

PROPERTIES OF TANNASE

The most well studied field in tannase research is physicochemical characterization of the enzyme. So many research articles were published where different properties of tannase discussed. It was well established that fungal tannase both from molds and yeast is a glyco protein (Aoki *et al.* 1976; Rajakumar and Nandy 1983; Kasieczka-Burnecka *et al.* 2007; Costa *et al.* 2012) and depending on the fungal strain and culture conditions percentage of sugar varies from 5.4 to 64% (**Table 2**) (Aoki *et al.* 1976b; Beverini and Metche 1990; Albertse 2002; Zhong *et al.* 2004; Kasieczka-Burnecka *et al.* 2007; Costa *et al.* 2012).

It has been reported that fungal tannase formed with two or more subunits and sometimes this subunits were linked by disulfide linkages (Hatamoto *et al.* 1996; Kasieczka-Burnecka *et al.* 2007; Beena *et al.* 2010). Ramírez-Coronel (2003) purified and characterized an *A. niger* tannase which is active in monomeric and dimeric iso-forms of 90 and 180 kDa, respectively; Boer and co-workers (2009) found that tannase from the dimorphic yeast *Arxula adeninivorans* is composed of homo-tetramer with subunits of 80 kDa; Beena *et al.* (2010) reported a tannase of *A. awamori* formed by six identical subunits of 37.8 kDa. It has been reported that native tannase of *A. oryzae* consists of four pairs of two types of subunits; 30 and 34 kDa, respectively linked together by disulfide bonds, forming a hetero-octamer of 310 kDa (Hatamoto *et al.* 1996). Two types of tannase produced from *Verticillium* sp. P9 are multimeric; each consist of 40 and 46 kDa subunits (Kasieczka-Burnecka *et al.* 2007). Tannase from *Emericella nidulans* (=*Aspergillus nidulans*) is a glycoprotein contains two protein of 45.8 and 52 kDa, suggesting constituted by three copies of each subunit (Goncalves *et al.* 2011). The tannase of *Cryphonectria parasitica* was a tetramer composed of four subunits with a molecular weight of 58 kDa (Farias *et al.* 1994).

Tannase synthesized by fungi is a mixture of esterase and depsidase (Toth 1944; Haslam *et al.* 1961; Beverini and Metche 1990) with molecular weight between 50 and 320 kDa (**Table 2**) (Kasieczka Burnecka *et al.* 2007; Mata-Gomez *et al.* 2009; Beena *et al.* 2010; Costa *et al.* 2012).

The iso-electric points of tannase from several fungi were varied from strain to strain. *A. oryzae* tannase showed a pI value of near to pH 4.0 (Iibuchi *et al.* 1968). Tannase from *A. niger*, *A. awamori* BTMFW032 and *A. niger* GH1 has an isoelectric point 3.8, 4.4 and 3.5, respectively (Ramirez-Coronel *et al.* 2003; Mata-Gomez *et al.* 2009; Beena *et al.* 2010). Tannase by *A. niger* LCF 8 has a pI of 4.3 (Barthomeuf *et al.* 1994). The pI of *Cryphonectria parasitica* tannase was 4·6-5·1 (Farias *et al.* 1994).

Studies on tannase activity inhibition and protein sequencing analysis indicated that the active site of this enzyme contains threonine, serine and methionine (Barthomeuf *et al.* 1994; Gonzalez *et al.* 2012). Adachi et al. (1971) studied with radioactive isotopes and it was noticed by them that tannase activity was inhibited by isopropylfluorophosphate. From their study it was suggested that the amino acid sequences of the active site of tannase contained threonine, serine and methionine. In 2008, Sharma et al. reported that tannase from *Penicillium variable* IARI 2031 was inhibited about 72% and 81% residual activity by phenyl methyl sulphonyl fluoride (PMSF) and *N*-ethylmaleimide. From this experiment it was concluded that the isolated tannase was a class of serine hydrolases.

In general, properties of tannase like pH stability, pH optimum, temperature stability, temperature optimum, isoelectric point, effect of metal ions, EDTA, organic solvents depends strongly upon the culture conditions and the fungal strain (Yamada *et al.* 1968; Adachi *et al.* 1971; Iibuchi *et al.* 1972; Aoki *et al.* 1976; Chae and Yu 1983; Rajakumar and

Table 3 Different pH and temperature for optimum tannase activity and stability.

Fungus name	Optimum pH	pH stability	Optimum temperature (°C)	Temperature Stability (°C)	Reference
Aspergillus niger LCF 8	6.0	3.5 - 8.0	35	>50°C	Barthomeuf *et al.* 1994
Penicillium variable IARI 2031	5	-	50	-	Sharma *et al.* 2008
Aspergillus awamori nakazawa	5.0	-	35	-	Mahapatra *et al.* 2005
Emericella nidulans (=*Aspergillus nidulans*)	5.0	4.0-5.0	45	22–50	Goncalves *et al.* 2011
Aspergillus flavus	5.0-5.5	-	50-60	-	Adachi *et al.* 1968
Aspergillus niger van Tieghem	5.0-6.0	-	30	-	Rana and Bhat 2005
Aspergillus niger van Tieghem	6.0 and 4.5	6.0	60	>60	Sharma *et al.* 1999
Candida sp.	6.0	-	50	-	Aoki *et al.* 1976
Cryphonectria parasitica	5.5	-	30	-	Farias *et al.* 1994
Paecilomyces variotii	5.5	-	55	-	Battestin and Macedo 2007
Penicillium chrysogenum	5.0-6.0	4.5-6.0	30-40	-	Rajkumar and Nandy 1983
Verticillium sp. P9 TAH 1	5.5	-	25	-	Kasieczka-Burnecka *et al.* 2007
Verticillium sp. P9 TAH 2	5.5	-	20	-	Kasieczka-Burnecka *et al.* 2007
Aspergillus aculeatus DBF9	5.0	4.0-6.0	-	-	Banerjee *et al.* 2001
Aspergillus oryzae (Kikokoman tannase)	5.0-5.5	3.5-5.5	40	40	www.kikkoman.co.jp
Hyalopus sp.	6.5	5.0-8.0	60	>50	Mahapatra and Banerjee 2009
Aspergillus niger Aa20	6.0	-	60-70	-	Ramirez-Coronel *et al.* 2003
Paecilomyces variotii	6.0	4.0-8.0	40	30-50	Mahendran *et al.* 2006
Rhizopus oryzae and *Aspergillus foetidus*	5.0	-	40	-	Mukherjee and Banerjee 2006
Aspergillus awamori MTCC 9299	5.5	-	30	-	Chhokar *et al.* 2010

Nandy 1983; Barthomeuf *et al.* 1994; Farías *et al.* 1994; Albertse 2002; Ramírez-Coronel *et al.* 2003; Kar *et al.* 2003; Kasieczka-Burnecka *et al.* 2007; Battestin and Macedo 2007; Mata-Gomez *et al.* 2009). Tannase from fungi has a optimum pH range of 4.0 to 7.0 and temperature range of 20 to 70°C for maximum hydrolyzing activity and a pH range of 3.0 to 8.0 and temperature range 30 to 40°C was found most suitable for enzyme stability (**Table 3**) (Farías *et al.* 1994; Albertse 2002; Ramírez-Coronel *et al.* 2003; Kasieczka-Burnecka *et al.* 2007; Battestin and Macedo 2007; Ramos *et al.* 2011; Renovato *et al.* 2011; Reddy and Rathod 2012). Beena *et al.* (2010) reported that tannase from marine *Aspergillus awamori* BTMFW032 had two pH optima, pH 2.0, pH 8.0 and enzyme was stable only at pH 2.0 for 24 h. Costa *et al.* (2012) reported that tannase produced by *Aspergillus tamari* showed pH stability in a broad range, pH 3.0 to 9.0. The effect of different metal ions on tannase activity was described by several researchers. From their research papers it was noticed that metal ion effect on tannase activity drastically changes from fungi to fungi and from metal ion to metal ion. Research findings showed that some metal ions induce tannase activity whereas some others sharply decreased the enzyme activity (Mukherjee and Banerjee 2006; Kar *et al.* 2003; Chhokar *et al.* 2010). Effect of EDTA on tannase activity was also varied with tannase source organism. Aoki *et al.* (1976) reported that EDTA had no significant inhibitory activity on yeast tannase where as Iibuchi *et al.* (1968) reported that tannase from *Aspergillus oryzae* completely inactivated in presence of EDTA. Effect of different organic solvents on tannase activity were demonstrated by Chhokar *et al.* (2010) and except butanol and benzene, which induced tannase activity, all other solvents completely inhibit the enzyme activity.

APPLICATIONS OF TANNASE

Tannase is widely utilized in industrial sector, food and in pharmacy. The main applications are discussed as follows:

- Tannase is used for production of gallic acid. This gallic acid is a pharmaceutically important compound, needed for production of trimethoprim and propyl gallate (Lekha and Lonsane 1997; Aguilar *et al*. 2007; Banerjee *et al*. 2007a; Chavez-Gonzalez *et al.* 2012). Gallic acid is also important in preparation of different cosmetics, hair products, lubricants, adhesives, dyes, photographic film development (Aguilar *et al*. 2007; Banerjee *et al*. 2007a).
- Tannase treatment reduces the bitter taste, dark colour and formation of sediment during preparation and preservation of different fruit juices (Rout and Banerjee 2006; Belur and Mugeraya 2011; Chavez-Gonzalez *et al.* 2012).
- Tannase is used as clarifying agent in wine preparation. In wines tannins become oxidized to quinines and turbidity formed. This problem can be reduced by application of tannase.
- In beer preparation tannase is used for reduction of turbidity, which can form in absence of tannase by the formation of complexes between protein content of beer and tannin, added in the form of hops (Belmares *et al.* 2004; Belur and Mugeraya 2011).
- Tannase is used for the manufacture of instant tea. It helps to remove insoluble precipitants without hampering the high aromatic contents and colour of soluble instant tea (Bajpai and Patil 1996; Lekha and Lonsane 1997; Banerjee and Pati 2007; Banerjee *et al*. 2007b; Natarajan 2009).
- Tannase is used for animal feed production. Different animal feeds like sorghum contains high tannin, which is toxic for animal cells. Tannase application can reduce this toxicity and anti nutritional factors and thus useful for animal feed production (Lekha and Lonsane 1997; Madeira *et al*. 2012).
- Tannase is used in detoxification of tannery effluents, rich in polyphenols (Suseela and Nandy 1985; Murugan and Al-Sohaibani 2010).
- Tannse is extensively used in leather industry for leather tanning (Sivashanmugam and Jayaraman 2011).

However, tannase applications are limited till date due to its high production cost (Van de Lagemaat and Pyle 2006; Belur and Mugeraya 2011).

CONCLUDING REMARKS

Hundred forty five years ago tannase was first discovered form fungus and to date hundreds of research papers were published in this field. So many patents were taken by several scientists. But demands in this field of research do not touch the real peak. Till date costing in production, purification of tannase is much higher. So, from last few decade researchers from several countries tried different natural and agricultural byproducts for tannase production using tradition and new fermentation methods. Recently, the molecular biology and genetics of tannase were also studied by different researchers. For the development of this enzyme research more research is needed. From their research publication it was seems that interest has been grown

rapidly and results were positive. More research is needed and it hopes that, tannase will be available chiefly in the market.

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