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. 1988). 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 gallic 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). Tannins 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 tannase 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...
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 ellagittannins 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 decarboxylated by gallic acid decarboxylase to form pyrogallol, which ultimately converted to pyruvic acid or cis-aconitic 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., *Paecilomyces* 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>
<thead>
<tr>
<th>Table 1</th>
<th>List of fungal strains reported as tannase producers.</th>
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<tbody>
<tr>
<td><strong>Fungi</strong></td>
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<td><em>Aspergillus acolumaris</em></td>
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<td>Banerjee et al. 2001; Banerjee et al. 2007b</td>
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<td><em>Aspergillus amstellodemi</em></td>
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<td><em>Aspergillus aureus</em></td>
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<td><em>Aspergillus awamori BTMFW032</em></td>
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<td><em>Aspergillus ficuum</em></td>
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<td><em>Cunninghamella sp.</em></td>
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<td><em>Cylindrocladiella peruviana</em></td>
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<td><em>Helicostylum sp.</em></td>
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<td><em>Hymenoscyphus ericae</em></td>
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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.

**Table 1** (Cont.)

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<td>Trichoderma viride MTCC 167</td>
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<tr>
<td>Verticillium sp. P9</td>
<td>Kasiiezka-Burnecka et al. 2007</td>
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</tbody>
</table>

**Yeasts**

| Arxula adeninivorans | Boer et al. 2009; Boer et al. 2011 |
| Aureobasidium pullulans DBS66 | Banerjee and Pati 2007 |
| Candida adhaerens | Kumar et al. 1995 |
| 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 azetelli | Kumar et al. 1999 |
| Pichia spp. | Deschamps et al. 1983 |
| Saccharomyces cerevisiae | Zhong et al. 2004 |

**Submerged fermentation**

Tannase production in liquid submerged fermentation (SmF) is a very common technique 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 sub-
merged fermentation \textit{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 \textit{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 and 96 h, respectively. Braddock et al. (1997) reported that intracellular tannase yield from \textit{Aspergillus niger} Aa-20 (12 U/ml) using tannic acid as the main carbon source under sub-
merged fermentation. Tannase production by \textit{Aspergillus oryzae} is increased and optimum activity 32.62 U/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). Braddock et al. (1997) and Raja-
kumar and Nandy (1983) reported that maximum extracel-
ular tannase was produced from \textit{Aspergillus japonicas} and \textit{Penicillium chrysogenum} when 2% tannic acid was used in fermentation medium as sole carbon source. Bajpai and Patil (1997) reported that \textit{Aspergillus niger}, \textit{Aspergillus fis-
cheri}, \textit{Trichoderma viridae} and \textit{Fusarium solani} produced maximum tannase in submerged fermentation when they were cultured with initial gallo-tannin concentration of 10, 3, 2 and 1% (w/v), respectively. \textit{Aspergillus} gen-
duced maximum tannase when cultured with 3% natural tannin of \textit{Cassia siamea} (Banerjee et al. 2007a). Maximum Tannase production from \textit{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 (\textit{A. oc-
cidente}) using \textit{Aspergillus niger} tannase. Production by the organism was found to be maximal in medium con-
taining 0.5% (w/v) of crude tannin of \textit{A. occidentale} with medium pH 5.0 and of 48 h incubation time, at 40°C. Beni-
wal and Chhokar (2010) reported that maximum tannase production (1.45 U/ml) was obtained from \textit{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 produ-
cion by \textit{Trichoderma viride} MTCC 167 was noticed at 48 h incubation (Lokeswari et al. 2010). The production of intracellular and extracellular tannase by \textit{Aspergillus niger} van Tieghem MTCC 2425 was reported by Rana and Bhat (2005) where the enzyme production reached a peak activ-
ity by 48 h and thereafter showed a significant decline. Lekha et al. (1994) and Sabu et al. (2005) reported that after 96 h fermentation \textit{Aspergillus niger} produced maxi-
mal tannase. Maximum tannase yield by \textit{Penicillium purpuro-
genus oryzae} produced maximum tannase at 120 h (Chaterje et al. 1996). Banerjee et al. (2001) found maximum extra-
cellular tannase production by \textit{A. aculeata} DBF9 after 36 h. Various physico-chemical parameters were optimized to obtain maximum enzyme production by \textit{Aspergillus SHL} 6. Both the tannase activity and yield of ellagic acid have a maximum when sucrose used as the additional carbon source. In the following reports the sucrose source was about 5 g/l tannin concentration is favourable for the high tannase activity and ellagic acid production from \textit{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 opti-
mized the production of extracellular tannase by \textit{Penicil-
lium variabile} on natural tannin isolated from the fruit of \textit{Terminalia chebula}. The maximum tannase activity was found at pH 5.0 and 5.8 g/50 ml of the substrate concentra-
tion used in the medium after 72 h incubation. \textit{Aspergillus niger}, \textit{Aspergillus awamori} MTCC 9299, have been re-
ported to have their optimal media pH 5.0 for tannase synthesis (Srivastava and Kar 2009; Beniwal and Chhokar 2010). \textit{Aspergillus aculeata} DBF9 showed highest tannase pro-
duction at pH 5.5 in submerged fermentation (Banerjee et al. 2001) \textit{Aspergillus awamori} MTCC 9299, \textit{Fusarium sub-
glutinans} produced maximum tannase when they were cult-
tured 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 \textit{Aspergillus niger} was found at 5.5, 36 h and 35°C, respectively. In another report (Sharma et al. 2007) optimization for tannase production by \textit{Aspergillus niger} showed that 5% tannic acid, 0.8% sodium nitrate, pH 5.0, 5×10³ spores/50 ml inoculum density, 150 rpm agita-
tion speed and 48 h incubation period were optimum for tannase production. The optimum process conditions for tannase synthesis by \textit{Aspergillus awamori} was 60 h incuba-
tion 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). \textit{Aspergillus japonicus} pro-
duces 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 (Brado et al. 1997). Tannase production by \textit{Aureobasidium pul-
lulans} 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 concentra-
tion with (NH4)2HPO4 as nitrogen source and shaking speed of 120 rpm.

Boer et al. (2011) optimized tannase production using transgenic \textit{Arxula adenia}virans strains. They have re-
ported that transgenic Arxula strain containing ANAN1 expres-
sion module produce 51,900 U/I 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 tann-
ase as much as four times higher than its wild type in same culture conditions. In 2004, Zhong et al. isolate a tannase gene from \textit{A. Orzoe} and cloned it in \textit{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-
ostate culture system (Lekha and Lonsane 1994; Chaterje 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 sub-
merged culture (Agular et al. 1999). Rana and Bhat (2005) studied on production of tannase by \textit{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. Agular 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 \textit{Penicillium purpurogenum} PAF6. Different plant materials were used as substrate and enzyme productivity was found was increased by 20 fold. The following order: tamarrind seed > haritaki > pome-
granate > tea leaf waste > arjun fruit. \textit{Penicillium purpuro-
genum} 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 concentra-
tion with additional 4% (w/v) tannic acid supplementa-
tion. Rodriguez et al. (2011) studied SSF for tannase pro-
duction by \textit{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 \textit{Aspergillus niger} PKL 104 where 6% tannic acid was additionally supplemented as soul carbon source. Treviso et al. (2007) reported that \textit{Aspergillus niger} Aa-20 produced maximum tannase (2479.59 U/l) in SSF within 12 h of fermentation using discontinuous poly-
urethane matrix as an inert support. Ramirez-Coronel et al.
(2003) also worked on tannase production fromAspergillus 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 byAspergillus oryzae DBF9 where gem blan was used as a solid substrate. The organism produced maximum tannase after 72 h incubation at 30°C with 80% initial moisture content. Additional 5% tannic acid was supplemented to the solid medium as sole 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% w/v moisture, 4% w/v 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 byPenicillium purpureogenum BVG7 under different natural substrates such as red gram 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 byP. purpureogenum 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 byPaecilomyces 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 whenAspergillus 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 NH4NO3, MgSO4, NaCl supplementation for tannase production under SSF by A. niger ATCC 16620. Pinto et al. (2003) reported thatA. 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. The procedure 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 Rhizopus 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. (2006) new process for a well 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 SSF 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 ofA. 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 ammonium sulphate was reported (Beverini and Metche 1996). Aoki et al. (1976) reported that ammonium sulphate could not precipitate the tannase fromCandida 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; Funahiko and Kiyoshi 1975; Rajkumar and Nandy 1983). The last step employed 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, Gaikawari 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–isoctane 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 hydrolyase. Among them Dhari 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 wherep-nitrophenyl gallic acid was used as a substrate. Iibuchi et al. (1967) and Deschamps et al. (1983) used another method where absorbance was measured at 310 nm and 260 nm after protein precipitation of residual gallo-tannin. Katwa et al. (1981) described an assay method for two-stage tannase. Tannase was assayed with polyacryl-amide 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-
On completion of the fermentation for tannase production using *Verticillium* sp. P9, the mycelium is removed by centrifugation (9,000 × g, 30 min, 4°C), and the supernatant is used as crude tannase.

Proteins precipitation are performed from supernatants by ammonium sulphate at 60% (w/v) saturation and pelleted proteins are discarded after centrifugation (9,000 × g, 25 min).

The supernatant of step II is precipitated at 80% (w/v) (NH₄)₂SO₄ saturation for tannase isolation. This addition is done with constant stirring for 30 min and the mixture is kept overnight at 4°C.

The precipitated tannase is collected by centrifugation at 9,000 × g for 25 min and dissolved in 5 ml of citrate buffer (0.05 M, pH 5.5) and dialyzed for 24 h with two changes in the same buffer (500 ml each). The dialysate recovered is treated as partially purified tannase.

This partially purified tannase is applied to a DEAE-cellulose column (2 × 30 cm) previously equilibrated with 0.05 M citrate buffer (pH 5.5).

Washing is done with 200 ml of citrate buffer. Then elution is performed with a linear gradient of 0–2 M NaCl at a flow rate of 13.2 ml/h.

Eluted fractions (2.6 ml) are analyzed for protein content and tannase activity. Tannase-containing fractions are pooled separately and used for further investigations.

0.1 ml enzyme solution is mixed with 0.3 ml of 1% (w/v) tannic acid, in 0.2M acetate buffer (pH 5.5).

Reaction mixture is incubated at 40°C for 30 min.

The reaction is terminated by adding 2 ml of BSA (1 mg/ml) at 0°C. A control reaction is also performed side by side with heat denatured enzyme.

The mixture is centrifuged at 5000 × g for 10 min and the supernatant is discarded.

The precipitate is resuspended in 2 ml SDS-triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine).

1 ml of FeCl₃ (0.13 M) is added to the previous solution.

Absorbance is measured at 550 nm. One unit of tannase is defined as the amount of enzyme which is able to hydrolyze 1 μmol of ester linkage of tannic acid in 1 min under specific conditions.
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 (1995). Sharma et al. (2000) described a new technique for tannase assay. In this report they demonstrated a new assay method is schematically represented in Fig. 3.

PROPERTIES OF TANNASE

The most well studied field in tannase research is physico-chemical 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 glycoprotein (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; Alberse 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). Ramirez-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 adeni-nivorans 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-octa-mer 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 pl 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 pl of 4.3 (Barthomeuf et al. 1994). The pl of Cryphonectria parasitica tannase was 4-6-5.1 (Farias et al. 1994).

Studies on tannase activity inhibition and protein se-queencing 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 variabile 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, iso-electric 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 2 Molecular characteristics of some fungal tannases.

<table>
<thead>
<tr>
<th>Fungus name</th>
<th>Molecular weight (Da)</th>
<th>Carbohydrate content (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emericella nidulans (=Aspergillus nidulans)</td>
<td>302 000</td>
<td>50%</td>
<td>Gonçalves et al. 2011</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>192 000</td>
<td>25.4%</td>
<td>Yamada et al. 1968; Adachi et al. 1971</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>186 000</td>
<td>43%</td>
<td>Barthomeuf et al. 1994; Parthasarathy and Bose 1976</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>300 000</td>
<td>22.7%</td>
<td>Hatamoto et al. 1996</td>
</tr>
<tr>
<td>Aspergillus niger van Tieghem</td>
<td>185 000</td>
<td></td>
<td>Rana and Bhat 2005</td>
</tr>
<tr>
<td>Candida sp. K-1</td>
<td>250 000</td>
<td>61.9%</td>
<td>Aoki et al. 1976</td>
</tr>
<tr>
<td>Aspergillus awamori BTMFW032</td>
<td>230 000</td>
<td>8.02%</td>
<td>Beena et al. 2010</td>
</tr>
<tr>
<td>Aspergillus niger GH1</td>
<td>225 000</td>
<td>7.1%</td>
<td>Mata-gomez et al. 2009</td>
</tr>
<tr>
<td>Chryphonectria parasitica</td>
<td>240 000</td>
<td>64%</td>
<td>Aoki et al. 1976</td>
</tr>
<tr>
<td>Arxula adelinorvaros</td>
<td>320 000</td>
<td></td>
<td>Boer et al. 2009</td>
</tr>
<tr>
<td>Verticillum sp. P9 TAH 1</td>
<td>155 000</td>
<td>11%</td>
<td>Kasieczka-Burnecka et al. 2007</td>
</tr>
<tr>
<td>Verticillum sp. P9 TAH 2</td>
<td>155 000</td>
<td>26%</td>
<td>Kasieczka-Burnecka et al. 2007</td>
</tr>
<tr>
<td>Pichia pastoris</td>
<td>90 000</td>
<td></td>
<td>Zhong et al. 2004</td>
</tr>
<tr>
<td>Aspergillus awamori MTCC 2425</td>
<td>185 000</td>
<td></td>
<td>Bhardwaj et al. 2003</td>
</tr>
<tr>
<td>Aspergillus oryzae</td>
<td>63 000</td>
<td></td>
<td>Hatamoto et al. 1996</td>
</tr>
<tr>
<td>Paecilomyces variotii</td>
<td>87 300 and 71 500</td>
<td></td>
<td>Battestin and Macedo 2007</td>
</tr>
<tr>
<td>Paecilomyces variotii</td>
<td>149 800</td>
<td></td>
<td>Mahendran et al. 2006</td>
</tr>
<tr>
<td>Cryphonectria parasitica</td>
<td>240 000</td>
<td></td>
<td>Farias et al. 1994</td>
</tr>
<tr>
<td>Aspergillus niger ATTC 16620</td>
<td>149 000</td>
<td></td>
<td>Sabu et al. 2005</td>
</tr>
<tr>
<td>Aspergillus niger N 888</td>
<td>165 000</td>
<td></td>
<td>Sabu et al. 2005</td>
</tr>
<tr>
<td>Penicillium variabile</td>
<td>310 000</td>
<td></td>
<td>Sharma et al. 2008</td>
</tr>
<tr>
<td>Aspergillus awamori MTCC 9299</td>
<td>101 000</td>
<td></td>
<td>Chhokar et al. 2010</td>
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</tbody>
</table>
Nandy 1983; Barthomeuf et al. 1994; Farias 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) (Farias 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 (Banerjee 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 libuchi 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.

APPLICATONS 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; Aguilera et al. 2007a; 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 (Aguilera 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).
- Tannase 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

<table>
<thead>
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<th>Table 3 Different pH and temperature for optimum tannase activity and stability.</th>
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<tr>
<td><strong>Fungus name</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>Aspergillus niger LCF 8</td>
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<tr>
<td>Penicillium variabile IARI 2031</td>
</tr>
<tr>
<td>Aspergillus awamori nakazawa</td>
</tr>
<tr>
<td>Emericella nidulans (=Aspergillus nidulans)</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
</tr>
<tr>
<td>Aspergillus niger van Tieghem</td>
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<tr>
<td>Aspergillus niger van Tieghem</td>
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<tr>
<td>Candida sp.</td>
</tr>
<tr>
<td>Cryphonectria parasitica</td>
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<tr>
<td>Paecilomyces variotii</td>
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<tr>
<td>Penicillium chrysogenum</td>
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<tr>
<td>Verticillium sp. P9 TA1</td>
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<tr>
<td>Verticillium sp. P9 TA2</td>
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<tr>
<td>Aspergillus aculeatus DBF9</td>
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<tr>
<td>Aspergillus oryzae (Kikokoman tannase)</td>
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<tr>
<td>Hyalospora sp.</td>
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<tr>
<td>Aspergillus niger Aa20</td>
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<tr>
<td>Paecilomyces variotii</td>
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<tr>
<td>Rhizopus oryzae and Aspergillus foetidus</td>
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<tr>
<td>Aspergillus awamori MTCC 9299</td>
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