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MINI-REVIEW

Microbial tannases: advances and perspectives

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Abstract In the last years, tannase has been the subject of a lot of studies due to its commercial importance and complexity as catalytic molecule. Tannases are capable of hydrolyzing complex tannins, which represent the main chemical group of natural anti-microbials occurring in the plants. The general outline of this work includes information of the substrates, the enzyme, and the applications. This review considers in its introduction the concepts and history of tannase and explores scientific and technological aspects. The "advances" trace the route from the general, molecular, catalytic, and functional information obtained under close to optimal conditions for microbial production through purification, description of the enzyme properties, and the commercial applications to the "perspectives" including expression studies, regulation, and potential uses; aspects related to the progress in our understanding of tannin biodegradation are also included.

Keywords Tannase · Tannins biodegradation · Microbial sources · Advances · Perspectives

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Introduction

Tannin acyl hydrolase (E.C. 3.1.1.20) is commonly referred as tannase, an enzyme accidentally discovered by Tieghem (1867) in an experiment of formation of gallic acid into an aqueous solution of tannins, where grew two fungal species later identified as *Penicillium glaucum* and *Aspergillus niger* (Lekha and Lonsane 1997). Tannase catalyses the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. The enzyme is used in food and beverage processing; however, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large-scale application.

The major commercial applications of the tannases reside in the elaboration of instantaneous tea or of acorn liquor and in the production of gallic acid (Coggon et al. 1975; Chae and Yu 1983; Pourrat et al. 1985; Lekha and Lonsane 1997; García-Nájera et al. 2002; Belmares et al. 2004), the latter being an important intermediary compound in the synthesis of the antibacterial drug, trimethroprim, used in the pharmaceutical industry (Sittig 1988) and also in the food industry; gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant. Moreover, tannase is used as a clarifying agent in some wines, beers, fruit juices, and in refreshing drinks with coffee flavour (Lekha et al. 1993; Lekha and Lonsane 1994; Belmares et al. 2004). Its commercial production is

through microorganisms using submerged culture (SmC), where it is intracellularly expressed, implying additional costs during its production (Lekha and Lonsane 1994; Belmares et al. 2004). Tannase is commercialized by Biocon (India), Kikkoman (Japan), ASA special enzyme GmbH (Germany), and JFC GmbH (Germany) with different catalytic units depending on the product presentation. Tannases from Kikkoman and Biocon are produced by solid state culture (SSC).

An excellent review about the production and applications of tannase was reported by Lekha and Lonsane (1997), summarizing a lot of useful information generated in almost 130 years of research. During the last 10 years, novel and interesting information has been published. This review pretends to present and discuss these contributions to know the advances and perspectives in the knowledge and application of the microbial tannases.

Substrates

Tannins are naturally occurring polyphenolic compounds with varying molecular weights that occur naturally in the plant kingdom. These phenolic compounds differ from others by having the ability to precipitate proteins from solutions. In the plant kingdom, these tannins are found in leaves, bark, and wood. Tannins are considered to be plants' secondary metabolic products because they play no direct role in plant metabolism. After lignin, tannins are the second most abundant group of plant phenolics. One of the major characteristics of tannins is their ability to form strong complexes with protein and other macromolecules such as starch, cellulose, and minerals (Lekha and Lonsane 1997; Aguilar and Gutiérrez-Sánchez 2001). It is widely accepted that tannins are divided into four major groups: gallotannins, ellagitannins, condensed tannins, and complex tannins (Fig. 1). Gallotannins are characterized by the presence of several molecules of organic acids, such as gallic, digallic, and chebulic acids, esterified to a molecule of glucose. On the other hand, ellagitannins have building blocks of ellagic acid units linked to glucosides. Molecules with a core of quinic acid instead of glucose have also been considered as ellagitannins. To maintain its binding capacity, gallotannins, and ellagitannins must have more than two acidic unit constituents esterified to the glucose core. Gallotannins can easily be hydrolysed under mild acid or alkaline conditions, either in hot water or enzymatically (López-Ríos 1984). Ellagitannins are more stable than gallotannins. Condensed tannins or proanthocyanidins are complex compounds made of flavonoid building blocks (from 2 over 50) that are not considered to be easily hydrolyzable (Ramirez-Coronel et al. 2004). Among the major constituents are catechin derivatives such as cyanidin and delphinidin, which are responsible for the astringent taste of fruit and wines (Sanchez 2001). The negative effect of tannins relates not only to taste but also directly to animal nutrition with the known capacity of tannins to bind macromolecules, rendering them indigestible (Mendez 1984; Goel et al. 2005). This results in the formation of stable complexes with enzymes and minerals otherwise required by ruminal microorganisms (Goel et al. 2007). The bitter taste also is the reason why the feed intake is reduced. However, low tannin concentrations in feed have been shown to result in an increase in nitrogen assimilation in ruminants, rendering higher growth rates and milk production (Nip and Burns 1969). Complex tannins can be generated through reactions between gallic or ellagic acids with catechins and glucosides.

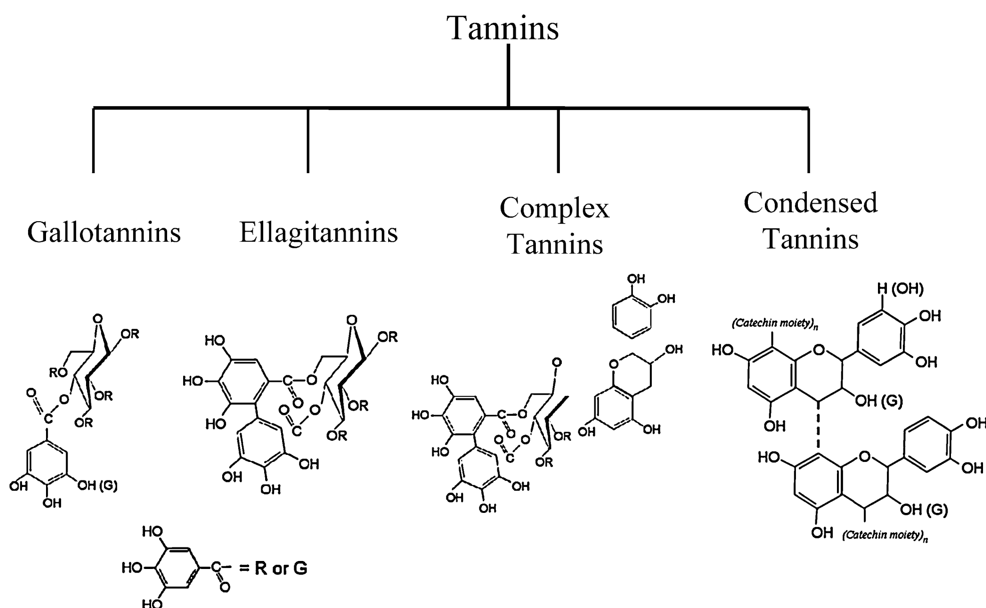

Fig. 1 Main chemical structures of the tannins

Tannins have a range of effects on various organisms—from toxic effects on animals to growth inhibition of microorganisms. Some microbes are, however, resistant to tannins and have developed various mechanisms and pathways for tannin degradation in their natural habitat. Microbial degradation of condensed tannins is, however, less documented than that of gallotannins in both aerobic and anaerobic environments (Bhat et al. 1998). Condensed tannins are not hydrolysed by "classical tannases" (Contreras-Dominguez et al, 2006), with initial degradation steps carried out by mono- or di-oxygenases. However, further studies are needed to further characterize the degradation of condensed tannins. Tannases generally act on gallotannins. However, in the particular case of ellagitannins, the information is scarce and confuse (Scalbert 1991; Vivas et al. 2004), mainly due to their chemical complexity and diversity of kinds of ellagitannins. Saavedra et al. (2005) reported that the production of ellagic acid has not been explored due to its high production cost and the great amount of subproducts generated as a result of ellagitannin biodegradation. This results in serious problems regarding the recovery and purification of ellagic acid. To date, there is no published data on ellagitannin degradation using biological methods (microbial or enzymatic). However, it is known that the selective hydrolysis of galloyl groups of the ellagitannin phyllianmblinin is catalyzed by tannase (Zhang et al. 2001). Yoshida et al. (1999) reported the production of nobotannin K from complex ellagitannins through catalysis of tannases. However, the biochemical mechanism was not clearly explained.

Vattem and Shetty (2002, 2003) reported on ellagic acid production from cranberry pomace fermented by a SSC using *Lentinus edodes*, attributing the catalysis to the enzyme β-glucosidase. Huang et al. (2005) described a new valonea tannin hydrolase as responsible for the biodegradation of valonea tannins. This enzyme is itself a tannin acyl hydrolase.

Mingshu et al. (2006) reported in their review information generated by Vaquero et al. (2004) on tannase production by species of *Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus*, using as carbon sources gallotannins, ellagitannins, and condensed tannins present in muscadine grapes. However, Vaquero et al. (2004) only considered the tannase production by lactobacilli without evaluation of ellagic acid released from ellagitannins.

Ramirez-Coronel et al. (2004) purified a thermostable bi-functional tannases from *Aspergillus*. The enzyme also had *b*-glucosidase activity. Unpublished results from the same group indicated that, in the presence of both tannic acid and cellobiose as substrates and of the purified enzyme, only the glucosidase activity was present. While studying the degradation of ellagitannins, Vattem and Shetty (2003) also observed glucosidase activity. In addition, Lee and Talcott (2005) incubated ellagitannins from muscadine grape seeds with commercial tannase and α-glucosidase, and they found that tannase activity was not expressed, while α-glucosidase showed high reactivity to ellagitannins. However, further studies on the subject are required to understand the microbial degradation process of such molecules.

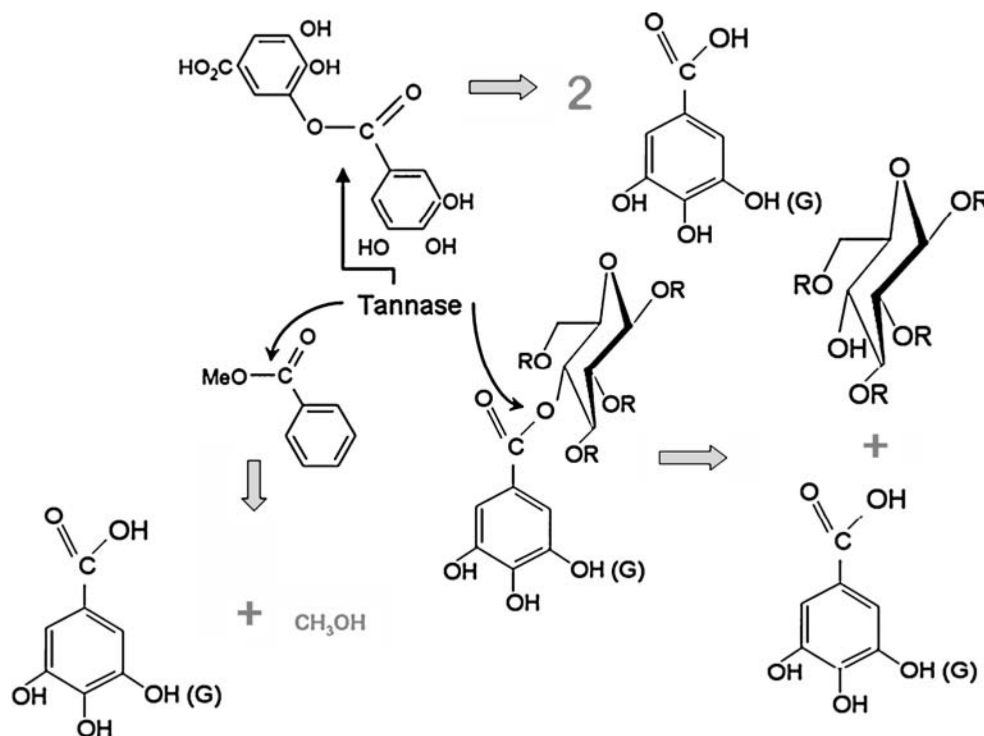

Fig. 2 Mechanism of action of tannase

Enzyme

Tannase catalyses the breakdown of hydrolyzable tannins such as tannic acid, methyl gallate, ethyl gallate, *n*-propylgallate, and isoamyl gallate (Fig. 2). It is well known that tannase hydrolyses the ester bonds of tannic acid, although tannic acid is known to denature proteins. Tannase hydrolyses tannic acid completely to gallic acid and glucose through 2,3,4,6,-tetragalloyl glucose and two kinds of monogalloyl glucose. This is supported by the facts that the same products can be detected in the hydrolysate of 1,2,3,4,6,-pentagalloyl glucose and that gallic acid of methyl.*m*.digallate is liberated first.

Albertse (2002) reported that for a true enzyme-substrate complex to form, the criteria to consider are the following: (a) There should be no restriction on the structure of an alcohol composing a substrate ester, although the acid should be gallic acid; (b) any phenolic hydroxyl might react with the binding site of the enzyme and prevent the enzyme from forming a true enzyme-substrate (ES) complex; and (c) an ester bond or carboxyl does not link to the enzyme by itself because an ester or carboxylic compound is not hydrolyzed by or inhibits the enzyme unless it has phenolic hydroxyls.

Although tannase is present in plants, animals, and microorganisms, it is mainly produced by the latter. Tannase is produced by bacteria, yeasts, and fungi (Table 1).

The production and applications of tannase have been extensively studied; researches related to strain isolation and improvement, process development, and application of tannases have resulted in a great number of scientific publications and patents. Table 2 presents some of the published patents regarding tannase production and application.

Filamentous fungi of the *Aspergillus* genus and bacteria of the *Bacillus* genus have been widely used for tannase production (Mondal et al. 2001a, b; Pinto et al. 2001). Although tannase production by *Aspergillus* can occur in the absence of tannic acid, this fungus (mainly *A. niger*) tolerates tannic acid concentrations as high as 20% without having a deleterious effect on both growth and enzyme production (Van Diepeningen et al. 2004; Cruz-Hernández et al. 2006). Studies on tannase production by *Aspergillus* have been carried out in submerged and solid-state cultures. Depending on the strain and the culture conditions, the enzyme is induced and expressed with different levels of activity, showing different production patterns. Phenolic compounds such as gallic acid, pyrogallol, methyl gallate, and tannic acid induces tannase synthesis (Bajpai and Patil 1997). However, the induction mechanism has not been clearly demonstrated, and there is some controversy about the role of some of the hydrolyzable tannin constituents as related to the synthesis of tannase (Deschamps et al.1983; Aguilar et al. 2001a). For instance, gallic acid, one of the

Table 1 Microbial sources of tannase

Microorganism	Reference
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. (2001b)
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. (1976)
Saccharomyces cerevisiae	Zhong et al. (2004)
Mycotorula japonica	Belmares et al. (2004)
Pichia spp.	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); Treviño-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)

Table 1 (continued)

Microorganism	Reference
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)
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. (2005); Battestin and Alves-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)

structural constituents of some hydrolyzable tannins, such as tannic acid, has been reported as an inducer of tannase synthesis under submerged fermentation, while it represses tannase synthesis under solid-state fermentation. Nevertheless, independent of the involved mechanism, it has been well accepted that, due to the complex composition of the hydrolyzable tannins, some of their hydrolysis products induce tannase synthesis (Aguilar et al. 2002).

The addition of carbon sources such as glucose, fructose, sucrose, maltose, and arabinose to the culture medium at initial concentrations from 10 to 30 g/l improves tannase production by *A. niger* (Bradoo et al. 1997). Nitrogen requirements can be supplied by different organic and inorganic sources. Inorganic nitrogen can be supplemented as ammonium salts (sulphate, carbonate, chloride, nitrate, monohydrated phosphate) or nitrate salts (sodium, potassium, or ammonium). Other nutritional requirements such as potassium, magnesium, zinc, phosphorous, and sulfur are supplied as salts. Although *A. niger* does not require cofactor supplementation, folic and pantothenic acid are eventually added to the cultured medium (Belmares et al. 2004).

Table 2 Published patents regarding tannase production and application

Year	Title	Patent no.
1974	Conversion of green tea and natural tea leaves using tannase	USP3812266
1975	Production of tannase by *Aspergillus*	JP7225786
1975	Tea soluble in cold water	UKP1280135
1976	Extraction of tea in coldwater	GP2610533
1976	Enzymatic solubilization of tea cream	USP3959497
1985	Gallic acid ester(s) preparation	EP-137601
1985	Preparation of gallic acid esters, e.g., propylgallate	EP-137601
1985	Enzymatic treatment of black tea leak	EP135222
1987	Preparation of tannase	JP62272973
1987	Manufacturing of tannase with *Aspergillus*	JP62272973
1988	Production of tannase by *Aspergillus oryzae*	JP63304981
1988	Elaboration of tannase by fermentation	JP63304981
1989	Preparation of spray-concrete coating in mining shaft	SUP1514947
1989	Antioxidant catechin and gallic acid preparation	JP01268683
1989	Tannase production by culture of *Aspergillus tamarii*	EP-339011
1989	New *Aspergillus niger* B1 strain	EP307071
1989	Tannase production process by *Aspergillus* and its application to obtain gallic acid	EP339011
1992	Tannase preparation method	JP4360684
1995	Enzymatic clarification of tea extracts	USP5445836
1997	DNA fragment containing a tannases gene, a recombinant plasmid, a process for producing tannases, and a promoter	USP5665584
2000	Tea concentrate prepared by enzymatic extraction and containing xanthan gum that is stable at ambient temperature	USP6024991
2000	Producing theaflavin	USP6113965
2004	Compositions based on vanilloid–catechin synergies for	USP6759064
2006	Diagnostic agent and test method for colon cancer using tannases as index	USP7090997
2006	Isolation of a dimmer di-gallate a potent endothelium-dependent vasorelaxing compound	USP7132446

Tannase production in submerged culture by *Aspergillus* sp. is improved at high aeration rates. It is favored at 30–33°C and initial pH values from 3.5 to 6.5. The maximal enzymatic activity is attained after 1 to 3 days of cultivation. Tannase production has been mostly studied in submerged fermentation. Strain improvement for tannase production from co-culture of *A. foetidus* and *Rhizopues oryzae* was reported by Purohit et al. (2006) using ultraviolet light, heat, and 3-nitro,5-methylguanidine mutagenesis.

However, several studies have reported interesting advantages between the tannase produced by SSC in relation with that produced by SmC. On this topic, several reports have been published (Barthomeuf et al. 1994; García-Peña 1996; Chaterjee et al. 1996; Lekha and Lonsane 1997; García-Peña et al. 1999; Ramírez-Coronel et al. 1999; Aguilar et al. 1999, 2001a and b, 2002; Kar et al. 2002; Viniegra-González et al. 2003; Van de Lagemaat and Pyle 2001, 2005; Rana and Bhat 2005; Pinto et al. 2006). In those, attractive advantages were indicated: the high-production titles (up to 5.5 times more than in SmC), the extracellular nature of the enzymes, and the stability towards wide pH and temperature ranges (Lekha and Lonsane 1994). Aguilar et al. (1999) reported productivities of 6.667 and 1.275 UE/Lh for SSC and SmC, respectively. Maximum tannase activity expressed intracellularly is also 18 times more in SSC than in SmC, while the extracellular activity is 2.5 times higher in SSC that in SmC.

It is important to note that such higher tannase activity levels in SSC than SmC have been clearly associated with the concomitant production of proteolytic activities in the latter culture system (Aguilar et al. 2002; Viniegra-González et al. 2003). Also, in SSC, the tannase produced exhibits a higher tolerance to wide range of pH and temperature (Lekha and Lonsane 1994; Rana and Bhat 2005).

Table 3 shows the materials used as supports of SSC for tannase production. Also, it presents the tannin-rich materials employed in both culture systems (SmC and SSC) for the production of the microbial enzyme.

For optimization of tannase production, Pinto et al. (2003) evaluated the tannic acid/wheat bran ratio, different moisture levels, addition of supplementary nitrogen sources, addition of supplementary phosphate, and concentration of supplementary nitrogen and phosphate added to the medium. Their results showed that the best medium was with 15% of tannic acid, 37.5% of initial moisture, 1.7 ammonium sulphate, and 2.0% of sodium phosphate. The presence of phosphate was of great importance for optimization because it promoted the increase in the synthesis level and a very expressive decrease in the maximum production time, from 72 to 24 h of fermentation. The optimized process promoted an increase of 861% in yield and 2783% in productivity.

Hatamoto et al. (1996, 1997) cloned and sequenced the gene-encoding tannase, and a structural study of the enzyme subunit from *A. oryzae* gave the possibility to manipulate the producer systems to increase and to improve the levels of tannase activity. In that study, authors reported that gene sequence did not have any introns. They found that the gene code for a 588-amino acid sequence with an 18-amino acid signal sequence and a molecular weight of approximately 64,000 Da. They hypothesized that their tannase consisted out of two subunits with molecular weights of 30,000 and 33,000 Da linked by a disulfide bond. The tannase gene was transcribed as a single polypeptide chain, after which, the 18 amino acid signal sequence was cleaved off, and the polypeptide chain was cleaved into two subunits. It was reported that the single polypeptide chain was cleaved by a KEX-II-like protease in two separate polypeptide chains. They concluded that native tannase consisted of four pairs of the two subunits, forming a hetero-octamer with a molecular weight of about 300,000 Da (Fig. 3).

Table 3 Materials used as supports of SSC for tannase production and tannin-rich materials used as enzyme inducer in SSC and SmC

Traditional supports	Reference
Sugarcane bagasse	Lekha and Lonsane (1994); García-Peña et al. (1999)
Wheat bran	Chaterjee et al. (1996); Sabu et al. (2005)
Polyurethane foam	Ramirez-Coronel et al. (1999); Aguilar et al. (2001b); Van de Lagemaat and Pyle (2001, 2005)
Tamarind seed powder	Sabu et al. (2005)
Palm kernel cake	Sabu et al. (2006)
Tannin rich materials	
Chestnut bark	Deschamps et al. (1983)
Tara (*Caesalpinia spinosa*) tannins	Pourrat et al. (1985)
Gall nuts (*Quercus infectoria*)	Barthomeuf et al. (1994)
Rhus coriaria leaves	Barthomeuf et al. (1994)
Fruits of *Terminalia chebula*	Banerjee et al. (2005)
Pod cover of *Caesalpinia digyna*	Banerjee et al. (2005)
Quercus aegylops tannins	Shi et al. (2005)
Jawar leaves (*Sorghum vulgaris*)	Kumar et al. (2006)
Amla leaves (*Phyllanthus emblica*)	Kumar et al. (2006)
Jamun leaves (*Syzygium cumini*)	Kumar et al. (2006)
Ber leaves (*Zyzyphus mauritiana*)	Kumar et al. (2006)
Creosote bush leaves	Treviño-Cueto et al. (2007)

Albertse (2002) sequenced the genes of positive isolates of *Aspergillus* and expressed a tannase gene in *Saccharomyces cerevisiae*, evaluating the characterization and comparison of the recombinant tannase with published properties of other tannases. However, the recombinant tannase was expressed in very low amounts, probably due to the phenomenon exhibited in yeasts to hyper-glycosylate glycoproteins, therefore, expressing proteins that are often catalytically inactive or different than the native enzymes.

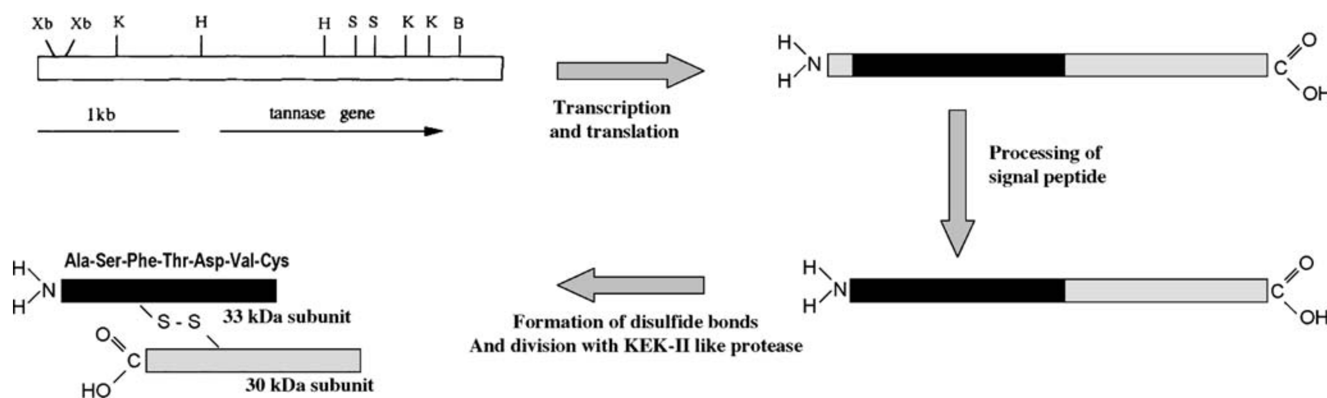

Fig. 3 Descriptive scheme of the proposed post-translational modification of tannase precursor (Hatamoto et al. 1996)

Recently, Cerda-Gomez et al. (2006) reported the use of conserved tannase gene sequences from five different *Aspergillus* species (Fig. 4) to design a set of primers (Tan1, Tan2), which later were used to amplify by polymerase chain reaction (PCR) a DNA segment of 435 bp from four different *Aspergillus* strains (Fig. 5). Zhong et al. (2004) reported the heterologous expression of *A. oryzae* tannase, suggesting that the *Pichia pastoris* system represents an attractive means of generating large quantities of tannases (7,000 IU/l) for both research and industrial purpose.

On the other hand, tannase extraction strongly depends on the fermentation system used. As tannase is mostly extracellular when produced by SSC, it can be easily extracted with water or a buffer. Two to three volumes of the agent extraction is well mixed with the fermented mass and pressed to obtain the enzymatic extract. Tannase location during its production by SmC depends on the cultivation time (Rajkumar and Nandy 1983). It is mainly intracellular at the beginning of the culture, and it is further secreted to the culture medium. However, up to 80% of tannase remained bound to the mycelium when the maximum overall tannase titter is attained. Bound tannase can be extracted after cell-wall hydrolysis with digestive enzymes such as chitinase.

The cells can also be mechanically disrupted to recover the bound tannase. An extracellular tannase produced by solid-state cultures of *A. niger* was purified to homogeneity from the cell-free culture broth by preparative isoelectric focusing and by fast protein liquid chromatography (FPLC) using anion-exchange and gel-filtration chromatography (Ramirez-Coronel et al. 2003). SDS-PAGE analysis, as well as gel localization studies of purified tannase, indicated the presence of two enzyme forms.

Tannase produced by *A. awamori nakazawa* was purified and characterized by Mahapatra et al (2005). Tannase from this new isolate exhibited optimum activity at 35°C and at a pH of 5.0. Urea concentrations higher than 3 M were inhibitory. Increasing concentrations of sodium lauryl sulfate (SLS) also led to decrease in activity. Two percent SLS was inhibitory. Increasing concentrations of ethylenediaminetetraacetic acid (EDTA) had an inhibitory effect on tannase. Tannase was found to be a glycoprotein. Circular dichroism analysis of purified fractions of tannase indicates that the β-sheet structure in tannase was predominant, indicating its globular nature.

Because a fraction of the produced tannase remains bound to the cell under submerged fermentation conditions, the produced biomass may be recycled as a biocatalyst. Several strategies can be used for tannase concentration or purification and immobilization after extraction from the biomass (submerged fermentation) or from the culture medium (submerged solid state fermentation). To increase the specific activity of the enzymatic preparation, tannase should be concentrated. For that, classical methods such as salt or solvent precipitation, ultrafiltration followed by ion

Fig. 4 Tannase gene sequence from five different *Aspergillus* species used for primer design (Cerda-Gomez et al. 2006)

ATTACGAGTGCGGGTCCTTTGGGCCCACCTCCCATCCGTGTCTATTATACCCTGTT
GCTTCGGCGGGCCCGCCG**GRTAGAACTGGTACCA**CTTGTCGGCCGCCGGGGGG
GCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACTACGAG
TGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGG
CGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCGT
GCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGAT
TGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA
GAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAG
TCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTTTC**ACCT**
CTCCTGGCAGTYGCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
GCATATCCAAATAAGCGGAAA

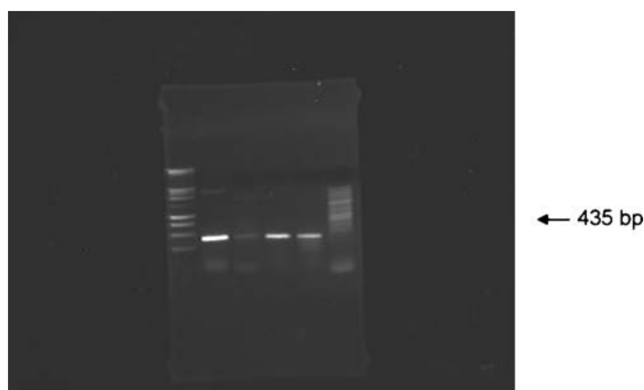

Fig. 5 Amplification of a tannase gene segment (435 bp) from *Aspergillus* strains. *Lanes 1* and *6* Molecular marker, *lane 2* GH1 strain, *lane 3* ESH strain, *lane 4* PSH strain, and *lane 5* GS strain (Cerda-Gomez et al. 2006)

exchange or size exclusion chromatography, as well as solvent extraction can be used (Lekha and Lonsane 1994).

Tannases of *Aspergillus* strains have a molecular weight around 150–350 kDa. Their activity and stability pH are 5–6.0 and 3.5–8.0, respectively, while optima temperatures from 35 to 40°C have been reported (Farias et al. 1994). Tannase is stable for several months at 30°C. Tannase produced by *Penicillium* strains present similar characteristics in terms of pH and temperature activity and stability. When tannic acid is used as substrate, Km values of 11.25 and 0.048 mM were obtained with *Aspergillus* and *Penicillium*, respectively (Yamada et al. 1968; Adachi et al. 1971; Ibuchi et al. 1972; Aoki et al. 1976; Chae and Yu 1983).

Ramirez-Coronel et al. (2003) produced by SSC, a single tannase present in monomeric and dimeric forms with molecular masses of 90 and 180 kDa. The tannase had an isoelectric point of 3.8, a temperature optimum of 60–70°C, and a pH optimum of 6.0. The substrate specificity of the tannase was determined by high performance liquid chromatography (HPLC) analysis of tannin substrates and products. The enzyme was able to remove gallic acid from both condensed and hydrolyzable tannins. Internal sequences were obtained from each of the gel-purified and trypsin-digested tannase forms. The peptide sequences obtained from both forms were identical to sequences within a β-glucosidase from *A. kawachii* (Fig. 6). The purified tannase was tested for β-glucosidase activity and was shown to hydrolyze cellobiose efficiently. However, no β-glucosidase activity was detected when the enzyme was assayed in the presence of tannic acid.

Tannase requires the presence of metal ions to express its full catalytic activity; in this sense, it is important to know the kind of ions and their concentrations in achieving maximal reaction efficiency. The effect of metal ions on tannase activity was studied by Kar et al. (2003). One mMMg^{+2} or Hg^{+} activated tannase activity. Ba^{+2},Ca^{+2}, Zn^{+2}, Hg^{+2}, and Ag^{+} inhibited tannase activity at 1.0 mM concentration, and Fe^{+3} and Fe^{+2} completely inhibited tannase activity. Ag^{+}, Ba^{+2}, and Hg^{+2} competitively inhibited tannase activity (Mukherjee and Banerjee 2005). Among the anions studied, 1 mM bromide or S_2O_3 enhanced tannase activity. Other chemical substances reported to play a role in the tannase activity are the surfactants and chelators. Kar et al. (2003) reported that palmitic acid and oleic acid enhanced tannase activity, whereas stearic acid inhibited tannase activity; also, SLS and Triton X-100 inhibited tannase activity. Urea stimulated tannase activity at a concentration of 1.5 M. Among the chelators, 1 mM EDTA or 1,10-*o*-phenanthrolein inhibited tannase activity. Dimethyl sulfoxide and *b*-mercapto ethanol inhibited tannase activity at 1 mM concentration, whereas soybean extract inhibited tannase activity at concentrations varying from 0.05 to 1.0% (*w*/*v*). Among the nitrogen sources selected, ammonium ferrous sulfate, ammonium sulfate, ammonium nitrate, and ammonium chloride enhanced tannase activity at 0.1% (*w*/*v*) concentration.

Applications

With respect to the uses of tannase, one of the major applications of tannase is in the manufacturing of instantaneous tea. Tannase applications in food and beverage industrial products contribute to remove the undesirable effects of tannins (Boadi and Neufeld 2001).

Enzymatic treatment of fruit juices to reduce the bitterness has got advantages such as the higher quality of juice due to the lower haze and non-deterioration of juice quality. New fruit juices (pomegranate, cranberry, raspberry, cold tea, etc.) have recently been acclaimed for their health benefits, in particular, for its disease-fighting antioxidant potential. The presence of high tannin content in those fruits is responsible for haze and sediment formation, as well as for color, bitterness, and astringency of the juice upon storage. Due to the inability of conventional fruit juice debittering processes to remove the bitterness effectively, enzymatic debittering should be preferred. Preliminary results reported by Rout and Banerjee (2006) for pomegranate juice demonstrated that tannase treatment resulted in 25% degradation of tannin, while a combination of tannase and gelatin (1:1) resulted in 49% of tannin degradation. However, further studies in this topic are needed.

Other important application of tannase is the production of gallic acid and propylgallate, fine-chemicals employed as antioxidants in foods, cosmetics, hair products, adhesives, and lubricant industry (Kar et al. 2002; Yu et al. 2004; Yu and Li 2006). Both antioxidants (gallic acid and prolygallate) have been biosynthesized in fungal SmC (Gaathon et

Fig. 6 Alignment of the amino acid sequences obtained from the 90- and 180-kDa forms of the *A. niger* tannase with the deduced amino acid sequence of *A. kawachii* β-D-glucosidase (*Glc*; accession no. BAA19913, gi|2077896|). Thirteen sequences were obtained from the 180-kDa form (*D180*) and two sequences from the 90-kDa form (*M90*). Identical amino acids are *shaded* (Ramirez-Coronel et al. 2003)

Glc	MRFTLIEAVA	LTAVSLASAD	ELAYSPPYYP	SPWANGQGDW	AQAYQRAVDI	VSQMTLAEKV	60
D180					RAVDI	VSQMTLAEKV	
M90							
Glc	NLTTGTGWEL	ELCVGQTGGV	PRLGVPGMCL	QDSPLGVRDS	DYNSAFPSGM	NVAATWDKNL	120
D180						NL	
M90							
Glc	AYLRGKAMGQ	EFSDKGADIQ	LGPAAGPLGR	SPDGGRNWEG	FSPDLAPSGV	LFAETIKGIQ	180
D180	AYLRGKAMGQ	EFSDK		NWEG	FSPDLAPSGV	LFAETIKGIQ	
M90	GKAMGQ	EFSDK					
Glc	DAGVVATKH	YIAYEQEHFR	QAPEAQCYGF	NISESGSANL	DDKTMHELYL	WPFADAIRAG	240
D180	DAGVVATK				TMHELYL	WPFADAIR	
M90							
Glc	GDVDYDSGTS	YWGTNLTVSV	LNGTVPQWRV	DDMAVRIMAA	YYKVGRDRLN	TPPNFSSWTR	360
D180			V	DDMAVRIMAA	YYK LN	TPPNFSSWTR	
M90							
Glc	DEYGYKYYYV	SEGPYEKVNH	YVNVQRNHSE	LIRRIGADST	VLLKNDGALP	LTGKERLVAL	420
D180	DEYGYK			RIGADST	VLLKNDGALP	LTGK	
M90							
Glc	IGEDAGSNPY	GANGCSDRGC	DNGTLAMGWG	SGTANFPYLV	TPEQAISNEV	LKNKNGVFTA	480
D180						NGVFTA	
M90							
Glc	TDNWAIDQIE	ALAKTASVSL	VFVNADSGEG	YINVDGNLGD	RKNLTLWRNG	DNVIKAAASN	540
D180	TDNWAIDQIE	ALAKTASVSL	VFVNADSGEG	YINVDGNLGD	R		
M90							
Glc	CNNTIVIIHS	VGPVLVNEWY	DNPNVTAILW	GGLPGQESGN	SLADVLYGRV	NPGAKSPFTW	600
D180						SPFTW	
M90							
Glc	GKTREAYQDY	LVTEPNNGNG	APQEDFVEGV	FIDYRGFDKR	NETPIYEFGY	GLSYTTFNYS	660
D180	GK						
M90							
Glc	GDASYGQDSS	DYLPEGATDG	SAQPILPAGG	GPGGNPRLYD	ELIRVSVTIK	NTGKVAGDEV	780
D180						VAGDEV	
M90							
Glc	PQLYVSLGGP	NEPKIVLRQF	REITLQPSEE	TKWSTTLTRR	DLANWNVEKQ	DWEITSYPKM	840
D180	PQLYVSLGGP	NEPK	ITLQPSEE	TKWSTTLTR			
M90							
Glc	VFVGSSSRKP	PLRASLPTVH					
D180	VFVGSSSR	ASLPTVH					
M90							

al. 1989; Kar and Banerjee 2000; García-Nájera et al. 2002; Sharma and Gupta 2003), in SSC (Kar and Banerjee 2000), and in modified SSC (Kar et al. 2002) using microencapsulated tannase in chitosan and as free enzyme (Yu et al. 2004), and by mycelium-bound tannase in organic solvent like 1-propanol (Yu and Li 2006). This last is an important contribution to discover the new reactions of tannase in non-conventional media because the industrial biocatalysis has grown considerably in recent years for its potential applications in fine-chemical, agrochemical, perfumery, flavor, pharmaceutical, and drug industries. Advantages of the tannase action of non-aqueous system lie in dramatically higher substrate solubility, the ability to use tannase synthetically rather than hydrolytically, and the capability to modify native selectivity by simply tailoring the reaction medium rather than the tannase itself.

Tannase has been also immobilized to reuse its catalytic action in agarose, chitosan, alginate, ceolite, and different derivatives of silicious materials can be used for tannase immobilization (Sharma et al. 2002; Sharma and Gupta 2003; Mahendran et al. 2005). Abdel-Naby et al. (1999) immobilized tannase from *A. oryzae* on various carriers. However, the enzyme immobilized on chitosan glutaraldeheyde showed the highest activity. The bound enzyme retained 20.3% of the original specific activity. On the other hand, Sharma et al. (2002) immobilized tannase from *A. niger* on concavalin A-sepharose via bioaffinity interaction. The immobilized preparation was quite stable to reuse; there was no loss of enzyme activity after three cycles, and it retained 81% activity even after the sixth cycle. Ester hydrolysis using the immobilized enzyme led to a 40% conversion into gallic acid as compared with 30% obtained with the free enzyme.

A stopped-flow manifold was developed to assay and characterize immobilized tannase. The immobilized enzyme reactor was inserted within the tube-type electrode pair (cell constant=103.2 cm^{-1}) for a real-time conductometric measurement. Tris buffer (2 mM, pH=7.0) was used as

the carrier for sensitivity improvement. The activities and kinetic parameters (Km values) for propyl gallate, methyl gallate, and tannic acid were reported by Chang et al. (2006). Raab et al. (2007) immobilized fungal tannase on Eupergit C to produce complex tannins based on catechins esterified with gallic acid.

The gallic acid is used in the pharmaceutical industry for the synthesis of antibacterial drugs and in the food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallates (García-Nájera et al. 2002).

Tannase may play an important role in plant cell-wall degradation by cleaving some of the cross-links existing between cell-wall polymers (García-Conesa et al. 2001). Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries, which represent serious environmental problems (Van de Lagemaat and Pyle 2001; Gammoun et al. 2006). Also, it can be used in the preparation of animal feeding using as culture support the mycelial wastes from penicillin manufacture (Nuero and Reyes 2002). Bioactive polyphenolic compounds can be produced by tannases applied to lentil flours (Dueñas et al. 2007).

Concluding remarks

Tannins are complex and diverse molecules in structure. It is, therefore, clear that “tannase” is a generic term that groups a number of enzymes from different organisms that are all able to hydrolyze tannins. The use of tannase from different microbial sources may have benefits for different areas such as food, beverage, cosmetic, and pharmaceutical industries, as well as environmental depollution. A tannase may be efficient on one substrate and not on another. For that, more effort is needed to develop specific tannases to answer specific needs. In this sense, solid-state fermentation presents more advantages than the submerged type of culture. However, new bioreactors open the possibility to improve the tannase productivity in both culture systems. Study of biodegradation of ellagitannins is a field that remains unclear. In addition, improvements on tannase immobilization are needed for the development of cheaper processes. For that, enzymatic preparations with improved catalytic characteristics must be developed, and new conditions of reaction should be tested. Expression studies have been conduced but more efforts are necessary to improve and optimize the production of tannase under new culture conditions. To fully elucidate the kinetics of the recombinant espressed tannase, future expression will be required in an established microbial expression host. Finally, studies on the structure and protein engineering should be conduced to enhance the levels of activity of tannase.

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