Appl Biochem Biotechnol (2011) 165:382–395
DOI 10.1007/s12010-011-9258-3

Differential Properties of *Aspergillus niger* Tannase Produced Under Solid-State and Submerged Fermentations

Jaqueline Renovato · Gerardo Gutiérrez-Sánchez · Luis V. Rodríguez-Durán · Carl Bergman · Raúl Rodríguez · Cristóbal Noe Aguilar

Received: 16 December 2010 / Accepted: 4 April 2011 /
Published online: 19 April 2011

Abstract Significant differences on structure, stability, and catalytic properties of tannase were found when this enzyme was produced under solid-state and submerged fermentations (SSF and SmF) by *Aspergillus niger*. The specific activity was 5.5 times higher on SSF than in SmF. Significant differences in isoelectric points of tannases were found. The pH optima for both types of enzyme was found at 6 and the pH stability of SSF and SmF tannase were at 6 and 5–8, respectively. The optimal temperature range was from 50 to 60 °C for SmF tannase and 60 °C for SSF tannase, and both enzyme types showed tolerance to high temperatures (60–70 °C). The SSF tannase showed a major specificity for methyl gallate substrate while SmF tannase for tannic acid. All metal ions tested, had an activity inhibition from 30–46% on SSF tannase. SDS-PAGE analysis as well as gel localization studies of both SSF and SmF purified tannases showed a single band with a molecular weight of 102 and 105 kDa, respectively. Different levels of glycosylation were found among SSF and SmF purified tannases. This is the first report about structural differences among tannase produced under SSF and SmF and this study provides basis for explanation of the stability and catalytic differences observed previously for this two tannase types.

Keywords Tannase · Catalysis · Stability · Glycosylation · *Aspergillus niger*

Introduction

Tannin acyl hydrolase (E.C.3.1.1.20) or tannase is an inducible enzyme that catalyzes the hydrolysis of ester and depside linkages of gallotannins to give gallic acid and glucose, and

J. Renovato · L. V. Rodríguez-Durán · R. Rodríguez · C. N. Aguilar (✉)
Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila,
Blvd. V. Carranza and González Lobo, ZIP 25280 Saltillo, Coahuila, Mexico
e-mail: cristobal.aguilar@uadec.edu.mx

G. Gutiérrez-Sánchez · C. Bergman
Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens, GA 30602-4712, USA

other tannins including gallocatechins and alyl-gallates to give respective products [28]. These catalytic properties give tannase several important applications in food, feed, chemical, and pharmaceutical industries. It is mainly used in the processing of iced tea, acorn liquor, and production of gallic acid from plant sources with high tannin contents. Gallic acid is used in the production of antioxidants and it is an intermediate complex in the production of the antibiotic trimethoprim [4, 6].

Study of tannase represents a scientific relevance topic because hydrolytic as well as synthetic tannase capability in suitable solvent systems [44, 50]. The complex catalytic molecules like tannase have reached a relevant commercial import. Nevertheless, important applications of this enzyme are currently limited due to inadequate information on the basic characteristics such as physicochemical and catalytic properties, regulation mechanisms, and production cost [6].

Tannase is mostly produced by fungi [19, 20, 23, 32, 33, 37], but some yeasts [16] and bacteria [9, 21, 22, 35, 36, 41, 47] have been reported as producers of this enzyme. At industrial levels tannase is mainly produced by *Aspergillus* species under submerged fermentation (SmF). The drawback of tannase produced under SmF, is that it is expressed mainly intracellularly by fungi and additional costs for purification need to be considered [30, 40].

Tannase production has been studied under submerged and solid-state fermentation (SSF); literature reports that enzymatic production under SSF has many advantages in comparison to that under SmF. Among them are the higher titers of enzyme production, extracellular nature of enzyme, and the low protease production [48]. But at industrial level, the use of SmF is advantageous because of ease of sterilization, and process control is easier to engineer in this system [31].

Several authors have compared tannase productivity values in different fermentation systems trying to explain how the fermentation system affects fungi physiology. In such comparisons have been included important aspects such as diffusion of nutrients, growth patterns, titers of enzymatic productivity, and induction and repression patterns [6]. However, there is no information about structural differences among tannase produced under both systems.

This study reports important differences in structure, stability, and catalysis of tannase produced under SmF and SSF by a wild *Aspergillus niger* to evidence the effect of the fermentation system on enzyme functionality and specificity.

Materials and methods

Chemicals

Tannic acid, gallic acid, and rodhanine were purchased from Sigma Aldrich (St. Louis, MO, USA). Methyl gallate was purchased from Fluka (St. Louis, MO, USA). All components of culture media were purchased in SumiLab (Saltillo, Coahuila, Mexico).

Microorganism and Culture Medium

A. niger (UAdeC-DIA collection) was used in the present studies. Spores were maintained in cryo-protector solution (milk 8.5% and glycerol 10% *w*/*v* dissolved in water) stored at −50 °C. Fungus was grown on potato-dextrose agar at 30 °C for 7 days. The spores were then scraped with 0.01% Tween 20 and counted in a Neubauer chamber.

The composition of culture medium was (g/L): KH_2PO_4 [1], $NaNO_3$, [3], $MgSO_4 \cdot 7H_2O$ (0.5), KCl (0.5), $FeSO_4$ (0.01). Liquid medium was autoclaved at 121 °C

for 20 min. Tannic acid solution was filter sterilized and added to a final concentration of 2% (*w*/*v*) to the culture broth. The pH was adjusted to 5.5 with 0.01 N NaOH.

Submerged Fermentation

The sterilized culture broth (tannic acid 2%) was transferred into 2-L capacity Erlenmeyer flasks and was inoculated with 1×10^6 spores/mL. The inoculated medium was incubated at 30 °C in an orbital shaker at 250 rpm for 42 h. Flasks were removed and biomass was separated by filtration through a Whatman No. 41 filter paper. The cell-free culture broth was assayed for extracellular tannase activity and protein concentration.

Solid-State Fermentation

Polyurethane foam (PUF) was used as support of this fermentation system. PUF was cut in cubes (0.5×0.5 cm×0.5 cm) and washed tree times with hot water (50 °C) and three times with cold deionized water. PUF cubes were then dried at 60 °C for 24 h. Dried PUF (15 g) was transferred into 2-L capacity Erlenmeyer flask and autoclaved at 121 °C for 20 min. Fermentation medium was inoculated with 1×10^6 spores/mL, added to Erlenmeyer flask containing PUF cubes and mixed thoroughly. The initial moisture content was 60%. Flasks were incubated at 30 °C for 42 h. The extracellular crude enzyme was obtained by washing the PUF with 250 mL of 100 mM sodium acetate buffer pH 5 with 100 mM NaCl. The extract was then filtrated through a Wathman No. 41 paper. The crude enzyme extract was assayed for extracellular tannase activity and protein content.

Concentration of Tannase

The crude extracts containing extracellular tannase from SSF and SmF were concentrated using an Amicon concentration unit with a 30 MWCO membrane (Millipore, Billerica, MA, USA). The concentrated enzymatic extract was dialyzed against water prior to isoelectric focusing separation (IEF).

Tannase Activity Assay

The tannase activity was evaluated by the spectrophotometric method reported by Sharma et al. [43] with some modifications. The reaction time was prolonged to 15 min and the reagents volumes were reduced to save volume of reagents and samples. The substrate (0.01 M methyl gallate in 50 mM sodium acetate buffer, pH 5.0), enzyme extract and buffer (0.05 M sodium acetate buffer, pH 5.0) were pre-incubated at 30 °C for 5 min. before the enzyme reaction was started. An aliquot of 100 μL of substrate were mixed with 100 μL of the buffer (control) or 100 μL of the enzyme preparation (test). Tubes were incubated at 30 °C for 15 min. Then 120 μL of methanolic rhodanine (0.667%; *w*/*v*) were added to tubes and were then kept at 30 °C for 5 min. After this, 80 μL of 0.5 M KOH were added to each tube and were incubated at 30 °C for 2.5 min. Finally, each tube was diluted with 1.6 mL distilled water, incubated at 30 °C for 10 min. Absorbance was read at 520 nm. The enzyme activity was calculated from the change in absorbance: $\Delta A_{520}=A_{\text{test}}-A_{\text{control}}$. One unit of enzyme was defined as 1 μmol of gallic acid formed per minute. Specific activity was expressed in units per milligram of protein.

Protein Determination

The protein concentration was determined using the Bradford Bio-Rad assay (Biorad. Hercules, CA, USA). BSA was used as standard.

Purification

Isoelectric Focusing Using Rotofor

The dialyzed crude extract (10.5 mL) was mixed with 3 mL of 20% pH 3–10 ampholytes (Biorad, Hercules, CA, USA), then brought to a volume of 60 mL with distilled water. Fractionation of the crude extract by IEF was performed in a preparative Rotofor cell (Biorad, Hercules, CA, USA) at constant power (12 W) for 4 h at 4 °C. Twenty fractions were harvest and their pH values and tannase activity were determined. The fractions with tannase activity were pooled and dialyzed against water prior to anion-exchange chromatography.

Anion-Exchange Chromatography

The fractions with tannase activity obtained from the IEF fractionation were dialyzed against 50 mM sodium acetate pH 5.0. The sample was loaded into 5 mL high Q column (Biorad, Hercules, CA, USA), previously equilibrated with 50 mM sodium acetate buffer pH 5 (buffer A). The enzyme was eluted using a linear gradient from 0% to 50% with 50 mM sodium acetate buffer pH 5 containing 1 M NaCl (buffer B) for 60 min. Eighty fractions were collected and analyzed for tannase activity. Fractions containing tannase activity were pooled and dialyzed against buffer A, prior to the second anion-exchange chromatography separation.

Second Anion-Exchange Chromatography

The sample from the first anion-exchange chromatography was loaded into a 1 mL High Trap Q column (Amersham Pharmacia, Piscataway, NJ, USA) previously equilibrated with buffer A. The sample was eluted from the column using a linear gradient from 0% to 50% buffer B at 0.5 mL/min for 80 min. Fractions containing tannase activity were pooled and dialyzed against 50 mM sodium acetate pH 5, prior to gel filtration chromatography.

Cation Exchange Chromatography

Cation exchange chromatography was used only for SmF sample obtained from the second anion-exchange chromatography. The sample was dialyzed against 50 mM sodium acetate buffer pH 3 and loaded into a 5-mL high S column (Biorad. Hercules, CA, USA) equilibrated previously with 50 mM sodium acetate buffer pH 3. The enzyme was eluted using a linear gradient from 0% to 100% with 50 mM sodium acetate buffer containing 1 M NaCl at 1 mL/min for 60 min. Obtained fractions were analyzed for tannase activity. Fractions containing tannase activity were pooled and dialyzed against 50 mM sodium acetate pH 5, prior to gel filtration chromatography.

Gel Filtration Chromatography

Samples containing tannase activity were loaded into Superdex G-200 (1.5×30 cm) gel filtration column (Amersham Pharmacia, Piscataway, NJ, USA) pre-equilibrated with 50 mM sodium acetate pH 5 containing 150 mM NaCl and eluted with the same buffer at 0.5 mL/min. Fractions were analyzed for tannase activity and those containing tannase activity were used for enzyme characterization.

Enzyme Characterization

SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed in a vertical X cell Sure Lock System (Invitrogen, Carlsbad, CA, USA) connected to a 1,000×500 power supply. A 20-μL aliquot sample was mixed with 10 μL of 2× Laemmli buffer (Sigma Aldrich, St Louis, MO, USA) and boiled at 95 °C for 5 min before loading onto the gel. Protein samples were resolved on 12% Bis-Tris precast gel (Invitrogen, Carlsbad, CA, USA). Running buffer was the 1× MOPS SDS buffer (Invitrogen, Carlsbad, CA, USA). Silver stain SDS-PAGE molecular weight standards, low range (Biorad, Hercules, CA, USA), were used. After electrophoresis, proteins were visualized by staining with silver nitrate [17]. Location of tannase activity within the gel was determined as described by Ramirez-Coronel et al. [39].

Enzyme Deglycosylation

The purified enzyme was deglycosylated using endoglycosidase H and *N*-glycosidase F. For deglycosylation with endoglycosidase H, the samples were prepared by adding 10 μL of purified tannase in 10 μL of incubation buffer (0.1% SDS, 1% Tween 20, 25 mM EDTA dissolved in 50 mM sodium acetate buffer pH 5.5). Endoglycosidase H was added at 10 mU final concentration. The mixture was incubated at 37 °C for 18 h. The deglycosylation with *N*-glycosidase F was done by adding 10 μL of the purified tannase, 10 μL 100 mM phosphate buffer pH 7, 1 μL denaturation solution (2% SDS, 1 M β-mercaptoetanol), and 1 μL detergent solution (15% Tween 20). *N*-glycosidase F was added at 10 mU final concentration. The mixture was incubated at 37 °C for 18 h. Samples were resolved on a SDS-PAGE.

Enzyme Stability

The optimum temperature was determined by incubating the reaction mixture for 15 min at different temperatures (30, 40, 50, 60, 70, 80, and 90 °C). The substrate solution (0.01 M methyl gallate in 50 mM phosphate buffer pH 7) and 50 mM phosphate buffer were pre-incubated at the different temperatures before beginning the enzymatic reaction. The tannase activity was assayed as described above. The stability temperature assay was done by incubating for 1 h the purified enzyme at the different temperatures mentioned previously and determining the residual tannase activity. The obtained tannase activity at 30 °C was taken as control. Experiments were done in triplicate.

The optimum pH for tannase activity was determined at 30 °C by incubating the enzyme with substrate at different pH (3 to 8) for 15 min. The tannase activity was assayed as

described above. The stability pH was determined incubating the enzyme for 24 h in buffer at different pH values. Residual tannase activity was determined. The control was done by incubating the enzyme in water for 24 h. Experiments were done in triplicate.

Enzyme Catalysis

The Michaelis–Menten constant (K_m) was calculated for two substrates (tannic acid and methyl gallate) by monitoring tannase activity at different substrate concentrations in 50 mM phosphate buffer pH 7 (0.015 mM–2.4 mM methyl gallate concentrations and 0.015 mM–0.3 mM tannic acid concentrations). The effect of substrate concentration on tannase activity was determined as described above. K_m was calculated by the method of Lineweaver–Burk.

Effect of Metal Ions and Chelator on Tannase Activity

The effect of seven salts $MgSO_4$, $CuSO_4$, $FeSO_4$, KCl, $CaCl_2$, $ZnSO_4$, and $MnCl_2$ and the influence of the chelator EDTA on tannase activity were studied. Tannase activity was assayed by adding the compound at 1 mM final concentration. The enzyme activity was assayed as described above.

Data Analysis

The significance value used to reject the null hypothesis was $P<0.05$. Where it was needed a Tukey's range test was used for mean treatments separation.

Results

Tannase Production in SSF and SmF

Tannase was produced extracellularly by *A. niger* under SmF and SSF using tannic acid as sole carbon source. At the end of incubation crude extracts with 1,861.64 and 14.76 U/mL of culture broth were obtained from SSF and SmF. This represents a production and productivity 126 times higher in SSF than SmF. Also, the specific activity of SSF crude tannase extract was 5.5 times greater than that produced under SmF.

Purification

Crude tannase extracts were fractionated employing isoelectric focusing. The tannase fractionation profiles were into acid region (pH 3–6). Fraction containing maximum SSF tannase was at pH 3.3 and the fraction containing maximum SmF tannase activity was at pH 4.6. Therefore the tannase isoelectric points are different.

After isoelectrofocusing fractionation, the fractions containing tannase activity were applied to ionic-exchange chromatography. Anionic-exchange chromatography allowed the fractionation of tannase extract produced in SSF; whereas for the tannase, extract produced in SmF only allowed the protein concentration. An efficient fractionation of SmF tannase extract was obtained using high S cationic-exchange chromatography column.

Gel filtration chromatography using Superdex G-200 column allowed the resolution of a single tannase band. SSF and SmF pure tannase obtained after gel chromatography had a specific activity of 264.45 and 2,066.67 U/mg, respectively. This represents a 153- and 477-fold purification of tannase produced under SSF and SmF, respectively. The recovery of SSF enzyme was 11% while SmF enzyme recovery was only 3.4%.

Characterization

The obtained pure tannase fractions were run under SDS-PAGE (Fig. 1). Silver staining revealed bands with relative molecular masses of 102 kDa for SSF tannase and 105 kDa for SmF tannase. A second polyacrylamide gel was run for detection of tannase activity containing SSF and SmF pure tannase fraction (Fig. 2). The detection of one band with tannase activity coincided with the 102 and 105 kDa silver staining band for SSF and SmF tannase, respectively.

Native and deglycosylated tannases are shown in Fig. 3. Deglycosidase enzymes used were *N*-glycosidase F (N-G-F) and endoglycosidase H (Endo H), both enzymes act on *N*-glycosylations. N-G-F enzyme acts on high mannose N-linked glycosylation (HM-N-Gly) and Endo H enzyme acts breaking bonds between *N*-acetylglucosamine and asparagine. Lanes 1 and 2 correspond to SSF and SmF tannases, respectively. The relative molecular weights were slightly different (102 and 105 kDa, respectively). Treatment with Endo H glycosidase produced similar effect to both tannases (they lost about 18 kDa in weight), the SSF enzyme migrated 84.5 kDa and SmF tannase at 86 kDa. On the other hand, treatment with N-G-F demonstrated differences in *N*-glycosylation levels: the deglycosylated SSF tannase migrated at 78 kDa, whereas SmF tannase did not show lost in weight.

Tannase assay was performed at different pH (from 3 to 8) to obtain optimum and stability pH's values, results are shown in Fig. 4. It is known that the optimum pH is determined for protonation and deprotonation of the active site amino acids and by the changes induced by ionization of other amino acids. The enzymes were active over the wide range studied. SSF and SmF tannases activity were found to increase as pH increase. Optimum pH to SSF tannase was at pH 6 and 6–7 for SmF tannase. A minor secondary peak at pH 4 was found in both enzymes with the 70% of tannase activation.

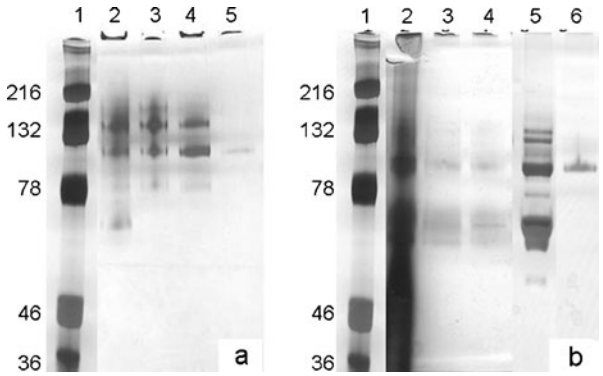

Fig. 1 SDS-PAGE of *A. niger* GH1 tannase silver stained. **a** SSF tannase and **b** SmF tannase. Lane *1* (**a**, **b**) molecular mass marker. Lane *2* (**a**, **b**) isoelectrofocusing fractions contain tannase activity. Lane *3* (**a**, **b**) high-Q chromatography fractions contain tannase activity. Lane *4* (**a**, **b**) high-trap Q chromatography fractions contain tannase activity. Lane *5* (**b**), high S chromatography fraction contain tannase activity. Lanes *5* (**a**) and *6* (*b*), Sephadex 200 gel filtration fractions contain tannase activity

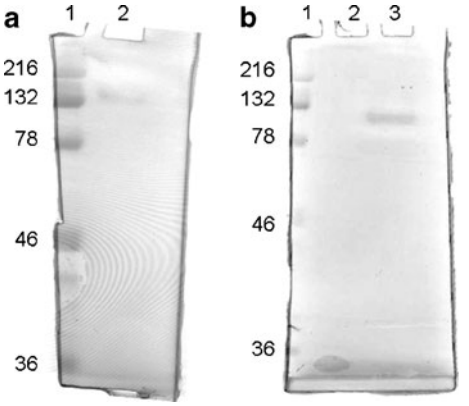

Fig. 2 Localization in gel of tannase activity. **a** SSF tannase and **b** SmF tannase. Lane *1* (**a**, **b**) molecular mass marker; lanes *2* (**a**) and *3* (**b**), tannase activity band

The stability pH was 6 at 4 °C for 24 h for both purified tannases maintaining 100% of enzymatic activity. SmF tannase supported 90–100% enzymatic activity at pH 5–7. Whereas the SSF tannase at pH values of 5 and 6, supported 85–100% activity. The SmF tannase was most stable at different pH than SSF tannase.

The temperature for optimum activity of SSF and SmF tannases were 60 °C. The SSF and SmF tannases of *A. niger* were stable at 60 °C for 1 h incubation retaining around 90% of activity. Both purified tannases lost the activity at 80 °C, nevertheless, the SmF tannase only lost 20% of activity at 70 °C for 1 h incubation and SSF tannase retaining 30% of activity at the same conditions (Fig. 5).

The analysis of substrate concentration on SSF tannase activity yielded K_m=0.78 mM and V_{max}=5.09 mM/min; and SmF tannase yielded K_m=7.41 mM and V_{max}=5.27 mM/min for methyl gallate substrate. For tannic acid substrate, SSF tannase yielded K_m=1.4 mM and V_{max}=11.03 mM/min; SmF tannase K_m=0.49 mM and V_{max}=0.63 mM/min. SSF tannase showed higher affinity for methyl gallate substrate, whereas SmF tannase for tannic acid substrate.

The effect of metal ions on tannase activity was evaluated. None of the studied metal ions increased the enzymatic activity. An inhibition pattern was not observed when SSF and

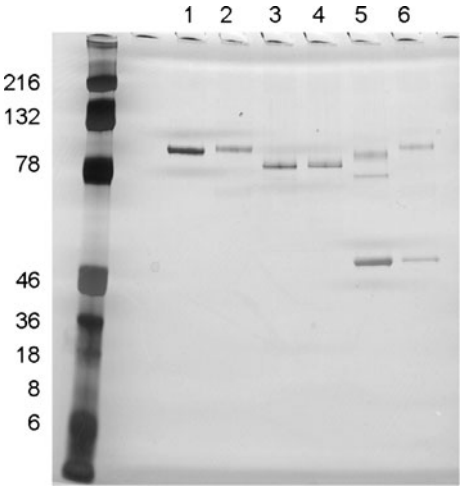

Fig. 3 SDS-PAGE analysis of purified tannase (reduced, silver stained). Lanes *1* and *2*, SSF and SmF native pure tannases, respectively. Lanes *3* and *4*, SSF and SmF deglycosylated tannases with Endo H, respectively. Lanes *5* and *6*, SSF and SmF deglycosylated tannases with *N*-glycosidase F, respectively

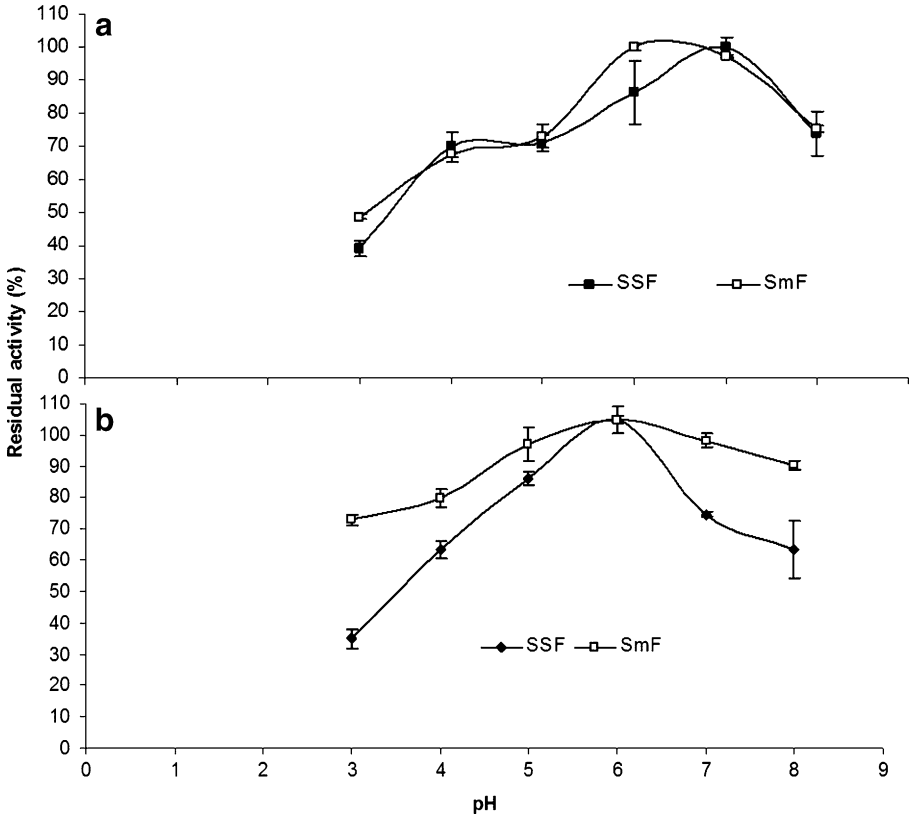

Fig. 4 Effect of pH on **a** activity and **b** stability on *A. niger* tannases produced in SSF and SmF

SmF tannases were compared. All metals ions had an inhibition activity of 30–46% on SSF tannase. On SmF tannase, $FeSO_4$ had the maximum inhibition activity (63%) and $CaCl_2$ the minimum with 7%. The majority of metal ions inhibited 12–33% SmF tannase activity (except $CuSO_4$, 41%). SSF tannase was more susceptible to the presence of metal ions. There are no reports that compared the influence on tannases produced under different culture conditions.

Discussion

Tannase is an enzyme with several interesting applications in industry. However, its potential has been limited mainly due to its high production cost [4]. Several authors have proposed the application of SSF for improving the production of tannase and other industrial enzymes [5]. Several advantages of SSF process over traditional SmF have been reported. Among them, it is often cited that enzyme titers are higher than in SmF, when comparing the same strain and fermentation broth [48]. There are a few papers reporting higher stability of SSF tannase at a broad range of pH and temperature [29, 30, 40]. But there is no evidence about structural differences of both enzymes that explain such observations. In this paper, we report a comparative study of structure, stability, and catalytic properties of *A. niger* tannase produced under SSF and SmF.

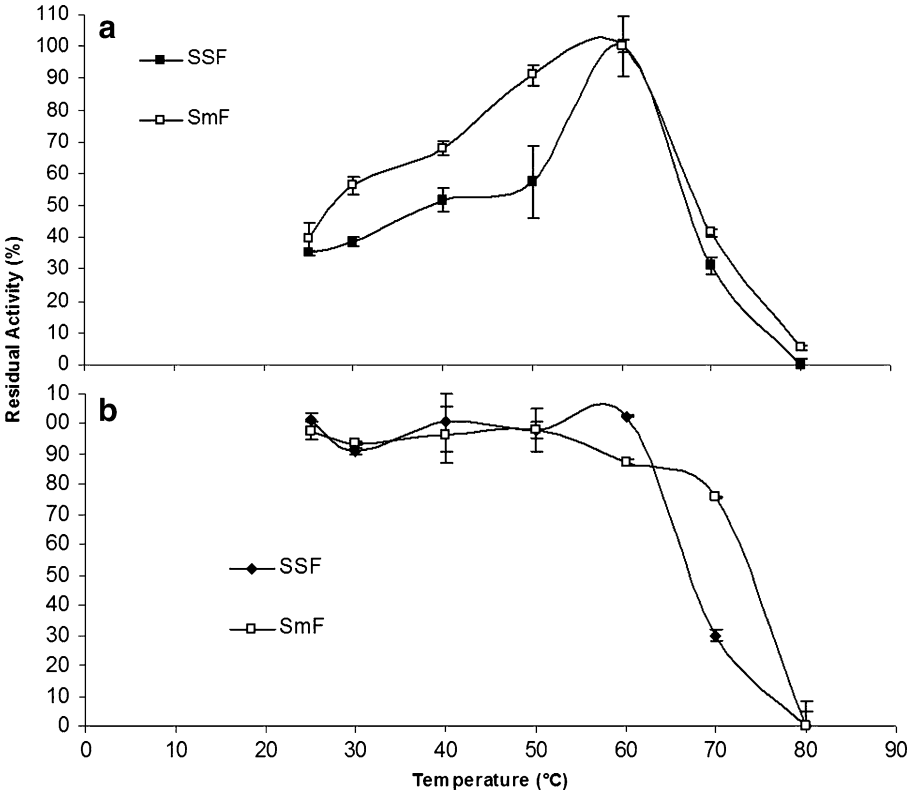

Fig. 5 Effect of temperature on **a** activity and **b** stability on *A. niger* tannases produced in SSF and SmF

Higher productivity of tannase was obtained in SSF than SmF. Aguilar and co-workers [2, 3] reported similar results; they found the tannase productivity five times higher in SSF than SmF. According to Viniegra et al. [48], this behavior is generally found and it is due to the influence of different diffusion parameters of solutes and oxygen on the matrix of SSF.

Tannase produced by *A. niger* in SSF showed 5.5 times higher specific activity than SmF tannase. Similar results were found for lipase and polygalacturonase production by *Aspergillus* species. In those cases, specific activity of enzymes was 1.4–5 times greater when it was produced under SSF [34, 42, 46].

Isoelectrofocusing of tannase produced by SSF and SmF evidenced different isoelectric points of enzymes. Isoelectric point of characterized tannases is between 3.5 and 4.4. For example, Ramirez-Coronel et al. [39] reported an isoelectric point of 3.8 for the extracellular tannase produced in SSF by *A. niger*. More recently, Beena et al. [11] characterized an *Aspergillus awamori* tannase with isoelectric point of 4.4. This is the first article that reports differential profiles of isoelectrofocusing points of extracellular tannases produced under SmF and SSF.

During protein purification, the use of different chromatography columns indicates differences on electrostatic properties of tannase produced under the two different fermentation systems. At the moment, the use of cationic-exchange chromatography column for tannase purification has not been reported.

A slight, but significant difference was found in molecular weight of both tannases (102 and 105 kDa for SSF and SmF, respectively). Also an important difference on the degree of

glycosylation was observed when both enzymes were hydrolyzed by *N*-glycosidase F. This is the first report on structural differences between tannases produced under SSF and SmF. These structural disparities could explain their different behavior.

It has long been known that glycosylation often improve the stability and solubility of proteins. N-linked glycosides bind aromatic residues and reduce solvent access to regions of the peptide backbone. Additionally, glycoside residues could stabilize the disulfide bonds limiting the peptide backbone flexibility [13]. In the case of *A. niger* tannase, the enzyme produced in SSF has an apparent higher content of glycoside residues, but showed a lower thermal and chemical stability. This could contradict the general observation of protein stabilization by glycosylation, but in order to confirm this hypothesis it is necessary to investigate if the tannase produced by *A. niger* under SSF is the same protein that produced under SmF.

It has been observed that culture conditions of SSF can significantly modify the molecular mechanisms that regulate gene expression. For example, the main glucoamylases of *Aspergillus oryzae* are encoded by *glaA* and *glaB* genes and expression of these genes strongly depends on the culture system. In solid-state culture, the level of *glaB* expression is 200-fold higher than that of *glaA*, and in submerged culture, the level of *glaA* expression is 10-fold higher than that of *glaB* [24]. On the other hand, differences in post-translational modifications of protein when they are produced under SSF have been found. For example, the three principal enzymes responsible of the β-glucosidase activity of *Aspergillus kawachii* are coded by the same gene and subsequently modified at different degrees of glycosylation (M_W=120, 130, and 145 kDa). Two of these enzymes are secreted extracellularly, and the other one remains bound to the cell wall. When *A. kawachii* is grown in SSF, the ratio of production between these enzymes (coded by the same gene) is modified, and as consequence, the extracellular/intracellular ratio for β-glucosidase is 16-fold enhanced with respect at the SmF system [26]. In the case of *A. niger* tannase, this is the first report on structural differences between the enzyme produced in SmF and SSF, and the molecular mechanism of the tannase production are still unknown.

Most of fungal tannases has been reported to be multimeric proteins formed by two to eight subunits, e.g., Ramírez-Coronel [39] purified and characterized an *A. niger*

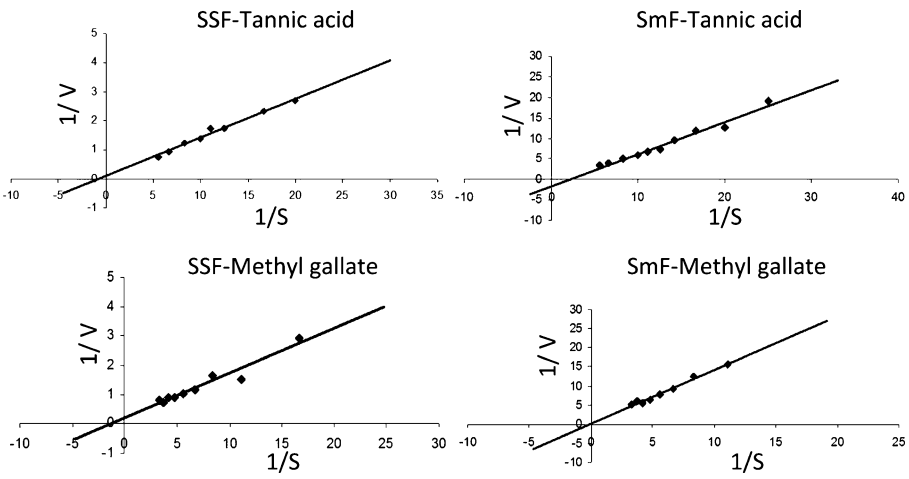

Fig. 6 K_m and V_{max} of *A. niger* tannase produced in **a** SSF and **b** SmF with (1) methyl gallate and (2) tannin acid as substrate were calculated by Lineweaver–Burk plot

tannase which is active in monomeric and dimeric iso-forms of 90 and 180 kDa, respectively; Böer and co-workers found that tannase from the dimorphic yeast *Arxula adeninivorans* is composed of homo-tetramer with subunits of 80 kDa [18]; Beena et al. reported a tannase of *A. awamori* formed by six identical subunits of 37.8 kDa; meanwhile, 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 [25].

All fungal and yeast tannases are glycoprotein with a variable content of carbohydrates ranging from 5.4% to 64% [1, 8, 10, 11, 14, 18, 27, 31]. Tannase glycosylations consist primarily of neutral sugars like mannose, galactose, and hexosamines [7]. The biological function of this high carbohydrate content is unknown, but may be related to ability to tolerate the denaturing action of tannin [30]. This hypothesis is supported by the observation that tannase and other tannin-resistant proteins are glycoprotein with a high content of carbohydrate [45].

Two peaks were found for optimal pH of SSF and SmF tannases. Sharma et al. [43] described similar results; they reported two pH optima peaks of 6.0 and 4.5. According to those authors, the presence of two optimal pH values of activity may are related to different galloyl-esterases activities, gallic acid esterase, and depsidase. This characteristic allows multiple tannase applications.

Tannase from *A. niger* showed a high thermotolerant activity. This property has been reported by several authors for other *Aspergillus* tannases [29, 43, 49]. However, there are few reports of tannases that show activity at 70 °C [12, 39]. The thermotolerant enzymes are preferred for industrial applications.

Apparently, the glycosylation level may affect the interaction between the enzyme and substrate. K_m and V_{max} were calculated by Lineweaver–Burk plot (Fig. 6).

All metal ions tested had a negative effect on tannase activity. Bhardwaj et al. [15] reported $CaCl_2$, $CdCl_2$, $MnCl_2$, $MgSO_4$, and $ZnCl_2$ had inhibition effect on *A. niger* intracellular tannase activity (40–58%), EDTA did not show inhibitor effect. Rajakumar and Nandy [38] found inhibition effect of Cu^{+2}, Zn^{+2}, Fe^{+2}, and Mg^{+2} on *A. oryzae* tannase activity.

This study revealed important differences in stability and catalysis of tannase produced under SmF and SSF by a wild *A. niger*, which may evidence the effect of fermentation system on enzyme functionality and specificity. These disparities in behavior could be related with differences in glycosylation. Finally, tannase can be produced as a specialty depending of the desired industrial characteristics.

Acknowledgments Jaqueline Renovato and Luis Rodríguez-Durán thank the National Council on Science and Technology (CONACYT), Mexico for the post-graduate scholarship.

References

1. Adachi, O., Watanabe, M., & Yamada, H. (1968). Studies on fungal tannase. Part II. Physicochemical properties of tannase of *Aspergillus flavus*. *J Agric Food Chem, 32*, 1079–1085.
2. Aguilar, C. N., Augur, C., Favela-Torres, E., & Viniegra-González, G. (2001). Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid-state fermentation: Influence of glucose and tannic acid. *J Ind Microbiol Biotechnol, 26*, 296–302.

3. Aguilar, C. N., Augur, C., Favela-Torres, E., & Viniegra-González, G. (2001). Induction and repression patterns of fungal tannase in solid-state and submerged cultures. *Process Biochem, 36*, 565–570.
4. Aguilar, C. N., & Gutiérrez-Sánchez, G. (2001). Review: Sources, properties, applications and potential uses of tannin acyl hydrolase. *Food Sci Technol Int, 7*, 373–382.
5. Aguilar, C. N., Gutiérrez-Sánchez, G., Prado-Barragán, L. A., Rodríguez-Herrera, R., Martínez-Hernandez, J. L., & Contreras-Esquivel, J. C. (2010). Perspectives of solid state fermentation for production of food enzymes. *Am J Biochem Biotechnol, 4*, 354–366.
6. Aguilar, C. N., Rodríguez, R., Gutiérrez-Sánchez, G., Augur, C., Favela-Torres, E., Prado-Barragan, L. A., et al. (2007). Microbial tannases: Advances and perspectives. *Appl Microbiol Biotechnol, 76*, 47–59.
7. Albertse EH (2002) Cloning, expression and characterization of tannase from *Aspergillus* species. Ms Thesis, University of the Free State, Bloemfontein, South Africa
8. Aoki, K., Shinke, R., & Nishira, H. (1976). Chemical composition and molecular weight of yeast tannase. *Agric Biol Chem, 40*, 297–302.
9. Ayed, L., & Hamdi, M. (2002). Culture conditions of tannase production by *Lactobacillus plantarum*. *Biotechnol Lett, 24*, 1763–1765.
10. Barthomeuf, C., Regerat, F., & Pourrat, H. (1994). Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. *J Ferment Bioeng, 77*, 320–323.
11. Beena, P. S., Soorej, M. B., Elyas, K. K., Bhat Sarita, G., & Chandrasekaran, M. (2010). Acidophilic tannase from marine *Aspergillus awamori* BTMFW032. *J Microbiol Biotechnol, 20*, 1403–1414.
12. Belur, P. D., Gopal, M., Nirmala, K. R., & Basavaraj, N. (2010). Production of novel cell-associated tannase from newly isolated *Serratia ficaria* DTC. *J Microbiol Biotechnol, 20*, 732–736.
13. Benoit, I., Asther, M., Sulzenbacher, G., Record, E., Marmuse, L., Parsiegla, G., et al. (2006). Respective importance of protein folding and glycosylation in the thermal stability of recombinant feruloyl esterase A. *FEBS Lett, 580*, 5815–5821.
14. Beverini, M., & Metche, M. (1990). Identification, purification, physiochemical properties of tannase from *Aspergillus oryzae*. *Sci Aliments, 10*, 807–816.
15. Bhardwaj, R., Singh, B., & Bhat, T. K. (2003). Purification and characterization of tannin acyl hydrolase from *Aspergillus niger* MTCC 2425. *J Basic Microbiol, 43*, 449–461.
16. Bhat, T. K., Singh, B., & Sharma, O. P. (1998). Microbial degradation of tannins—A current perspective. *Biodegradation, 9*, 343–357.
17. Blum, H., Beier, H., & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis, 8*, 93–99.
18. Böer, E., Bode, R., Mock, H. P., Piontek, M., & Kunze, G. (2009). Atan1p—An extracellular tannase from the dimorphic yeast *Arxula adeninivorans*: Molecular cloning of the ATAN1 gene and characterization of the recombinant enzyme. *Yeast, 26*, 323–337.
19. Bradoo, S., Gupta, R., & Saxena, R. K. (1997). Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus*. *Process Biochem, 32*, 135–139.
20. Costa, A. M., Ribeiro, W. X., Kato, E., Monteiro, A. R. G., & Peralta, R. M. (2008). Production of tannase by *Aspergillus tamarii* in submerged cultures. *Braz Arch Biol Technol, 51*, 399–404.
21. Curiel, J. A., Rodríguez, H., Acebrón, I., Mancheño, J. M., De Blanca Rivas, L., & Muñoz, R. (2009). Production and physicochemical properties of recombinant *Lactobacillus plantarum* tannase. *J Agric Food Chem, 57*, 6224–6230.
22. Das Mohapatra, P. K., Maity, C., Rao, R. S., Pati, B. R., & Mondal, K. C. (2009). Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology. *Food Res Int, 42*, 430–435.
23. Enemuor, S. C., & Odibo, F. J. C. (2009). Culture conditions for the production of a tannase of *Aspergillus tamarii* IMI388810. *Afr J Biotechnol, 8*, 2554–2557.
24. Hata, Y., Ishida, H., Ichikawa, E., Kawato, A., Suginami, K., & Imayasu, S. (1998). Nucleotide sequence of an alternative glucoamylase-encoding gene (*glaB*) expressed in solid-state culture of *Aspergillus oryzae*. *Gene, 207*, 127–134.
25. Hatamoto, O., Watarai, T., Kikuchi, M., Mizusawa, K., & Sekine, H. (1996). Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*. *Gene, 175*, 215–221.
26. Iwashita, K. (2002). Recent studies of protein secretion by filamentous fungi. *J Biosci Bioeng, 94*, 530–535.
27. Kasieczka-Burnecka, M., Kuc, K., Kalinowska, H., Knap, M., & Turkiewicz, M. (2007). Purification and characterization of two cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain *Verticillium* sp. P9. *Appl Microbiol Biotechnol, 77*, 77–89.
28. Kumar, R. A., Gunasekaran, P., & Lakshmanan, M. (1999). Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent. *J Basic Microbiol, 39*, 161–168.

29. Lekha, P. K., & Lonsane, B. K. (1994). Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface an submerged fermentations. *Process Biochem, 29*, 497–503.
30. Lekha, P. K., & Lonsane, B. K. (1997). In S. Neidleman & A. Laskin (Eds.), *Advances in Applied Microbiology vol. 44* (pp. 215–260). San Diego: Academic.
31. Mahapatra, K., Nanda, R. K., Bag, S. S., Banerjee, R., Pandey, A., & Szakacs, G. (2005). Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori* nakazawa. *Process Biochem, 40*, 3251–3254.
32. Manjit, Yadav, A., Aggarwal, N. K., Kumar, K., & Kumar, A. (2008). Tannase production by *Aspergillus fumigatus* MA under solid-state fermentation. *World J Microbiol Biotechnol, 24*, 3023–3030.
33. Mata-Gomez, M. A., Rodríguez, L. V., Ramos, E. L., Renovato, J., Cruz-Hernández, M. A., Rodríguez, R., et al. (2009). A novel tannase from the xerophilic fungus *Aspergillus niger* GH1. *J Microbiol Biotechnol, 19*, 987–996.
34. Mohanasrinivasan, V., Dhrisya, P., Dipinsha, K. P., Unnithan, C. M., Viswanath, K. M., & Devi, C. S. (2009). A comparative study of the lipase yield by solid state and submerged fermentations using fungal species from biopharmaceutical oil waste. *Afr J Biotechnol, 8*, 73–76.
35. Mondal, K. C., & Pati, B. R. (2000). Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. *J Basic Microbiol, 40*, 223–232.
36. Natarajan, K., & Rajendran, A. (2009). Effect of fermentation parameters on extra cellular tannase production by *Lactobacillus plantarum* MTCC 1407. *E J Chem, 6*, 979–984.
37. Prasad D, Gupta RK, Naidu RB, Kamini NR, Gowthaman MK (2008) Solid state fermentation of tannase from a new *Aspergillus* isolate. 49th Annual Conference of Association of Microbiologists of India. New Delhi, India.
38. Rajakumar, G., & Nandy, S. C. (1983). Isolation, purification, and some properties of *Penicillium chrysogenum* tannase. *Appl Environ Microbiol, 46*, 525–527.
39. Ramírez-Coronel, M. A., Viniegra-González, G., Darvill, A., & Augur, C. (2003). A novel tannase from *Aspergillus niger* with β-glucosidase activity. *Microbiology, 149*, 2941–2946.
40. Rana, N. K., & Bhat, T. K. (2005). Effect of fermentation system on the production and properties of tannase of *Aspergillus niger* van Tieghem MTCC 2425. *J Gen Appl Microbiol, 51*, 203–212.
41. Rodríguez, H., de las Rivas, B., Gómez-Cordovés, C., & Muñoz, R. (2008). Characterization of tannase activity in cell-free extracts of *Lactobacillus plantarum* CECT 748T. *Int J Food Microbiol, 121*, 92–98.
42. Saqib, A. A. N., Hassan, M., Khan, N. F., & Baig, S. (2010). Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). *Process Biochem, 45*, 641–646.
43. Sharma, S., Bhat, T. K., & Dawra, R. K. (1999). Isolation, purification and properties of tannase from *Aspergillus niger* van Tieghem. *World J Microbiol Biotechnol, 15*, 673–677.
44. Sharma, S., & Gupta, M. N. (2003). Synthesis of antioxidant propyl gallate using tannase from *Aspergillus niger* van Teighem in nonaqueous media. *Bioorg Med Chem Lett, 13*, 395–397.
45. Strumeyer, D. H., & Malin, M. J. (1970). Resistance of extracellular yeast invertase and other glycoproteins to denaturation by tannins. *Biochem J, 118*, 899–900.
46. Taşkın, E., & Stratilová, E. (2008). Polygalacturonases produced under solid state and submerged fermentation conditions by two strains of *Aspergillus foetidus*. *Turk J Biochem, 33*, 190–196.
47. Vaquero, I., Marcobal, A., & Muñoz, R. (2004). Tannase activity by lactic acid bacteria isolated from grape must and wine. *Int J Food Microbiol, 96*, 199–204.
48. Viniegra-González, G., Favela-Torres, E., Aguilar, C., Romero-Gomez, S. D. J., Díaz-Godínez, G., & Augur, C. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem Eng J, 13*, 157–167.
49. Yamada, K., Iibuchi, S., & Minoda, Y. (1968). Studies on tannin acyl hydrolase of microorganisms. Isolation and identification of producing molds and studies on the conditions of cultivation. *Agric Biol Chem, 45*, 233–240.
50. Yu, X. W., & Li, Y. Q. (2006). Kinetics and thermodynamics of synthesis of propyl gallate by mycelium-bound tannase from *Aspergillus niger* in organic solvent. *J Mol Catal B Enzym, 40*, 44–50.