Appl Biochem Biotechnol (2011) 164:612–628
DOI 10.1007/s12010-011-9162-x

Propyl Gallate Synthesis Using Acidophilic Tannase and Simultaneous Production of Tannase and Gallic Acid by Marine *Aspergillus awamori* BTMFW032

P. S. Beena · Soorej M. Basheer · Sarita G. Bhat · Ali H. Bahkali · M. Chandrasekaran

Received: 11 November 2010 / Accepted: 9 January 2011 /
Published online: 29 January 2011

Abstract Marine *Aspergillus awamori* BTMFW032, recently reported by us, produce acidophilic tannase as extracellular enzyme. Here, we report the application of this enzyme for synthesis of propyl gallate by direct transesterification of tannic acid and in tea cream solubilisation besides the simultaneous production of gallic acid along with tannase under submerged fermentation by this fungus. This acidophilic tannase enabled synthesis of propyl gallate by direct transesterification of tannic acid using propanol as organic reaction media under low water conditions. The identity of the product was confirmed with thin layer chromatography and Fourier transform infrared spectroscopy. It was noted that 699 U/ml of enzyme could give 60% solubilisation of tea cream within 1 h. Enzyme production medium was optimized adopting Box–Behnken design for simultaneous synthesis of tannase and gallic acid. Process variables including tannic acid, sodium chloride, ferrous sulphate, dipotassium hydrogen phosphate, incubation period and agitation were recognized as the critical factors that influenced tannase and gallic acid production. The model obtained predicted 4,824.61 U/ml of tannase and 136.206 μg/ml gallic acid after 48 h of incubation, whereas optimized medium supported 5,085 U/ml tannase and 372.6 μg/ml of gallic acid production after 36 and 84 h of incubation, respectively, with a 15-fold increase in both enzyme and gallic acid production. Results indicated scope for utilization of this acidophilic tannase for transesterification of tannic acid into propyl gallate, tea cream solubilisation and simultaneous production of gallic acid along with tannase.

Keywords *Aspergillus awamori* · Acidophilic tannase · Propyl gallate synthesis · Gallic acid

P. S. Beena (✉) · S. M. Basheer · S. G. Bhat · M. Chandrasekaran
Department of Biotechnology, Microbial Technology Laboratory,
Cochin University of Science and Technology, Cochin 682022 Kerala, India
e-mail: beena.santhosh@gmail.com

A. H. Bahkali · M. Chandrasekaran
Department of Botany and Microbiology, College of Science, King Saud University, PB No. 2455,
Riyadh 11451, Kingdom of Saudi Arabia

Introduction

Tannase or tannin acyl hydrolase (E.C.3.1.1.20), an industrially important enzyme, has potential application in the clarification of beer and fruit juices, manufacture of coffee-flavoured soft drinks, manufacture of instant tea, improvement in flavour of grape wine and as an analytical probe for determining the structure of naturally occurring gallic acid esters [1]. Tannase is used for the cleavage of polyphenolics such as dehydrodimer cross-links present in the cell walls of plants which are necessary for plant cell wall digestibility [2] and for the degradation of tannins present in the effluents of tanneries which represent serious environmental problems [3].

Tannase catalyses the hydrolysis of ester bond and depside bond present in hydrolysable tannins to form glucose and gallic acid. Industrial production of gallic acid (3,4,5-trihydroxybenzoic acid) is generally accomplished by the bioconversion of tannic acid by tannase. Gallic acid is mostly used in the pharmaceutical industry for production of anti-bacterial drug trimethoprim [4–6]. It is also used in the manufacturing of gallic acid esters such as propyl gallate, which is widely used as food antioxidant in the manufacture of pyrogallol, in leather industry and as a photosensitive resin in semiconductor production [5, 7–9]. Pyrogallol is used in staining fur, leather and hair, and also as a photographic developer [5]. Further, gallic acid possesses wide range of biological activities such as antibacterial, antiviral and analgesic. As an antioxidant, gallic acid acts as an anti-apoptotic agent and helps to protect human cells against oxidative damage [10]. Gallic acid is also found to show cytotoxic activity against cancer cells without harming normal cells [9]. Because of its several interesting properties and commercial applications, gallic acid is a compound of great interest to both pharmaceutical and chemical industries.

Industrial production of tannase is achieved mostly by submerged fermentation (SmF) using microbes, as intracellular form, implying additional costs in its production [11]. Tannase is recently commercialised by Biocon (India), Kikkoman (Japan) ASA Specila enzyme GmbH (Germany) and JFC GmbH (Germany), with different catalytic units depending on the product presentation.

Conventional media optimization for tannase production handles single parameter per trial, and this approach frequently fails to consider the effects of possible interactions between factors [12, 13]. Response surface methodology (RSM), a collection of mathematical and statistical techniques, is a powerful method that enables testing of multiple process variables and identification and quantification of significant interactions between the variables. Hence, this technique is widely used to optimize different biotechnological processes [14, 15]. It is stated that statistical design methods for media manipulation may be considered a better strategy for overproduction of enzymes [16]. Statistical design has already been reported for production of intracellular tannase by terrestrial *Aspergillus awamori* [1], and extracellular tannase by *Aspergillus niger* [17] and *Aspergillus foetidus* [15]. In this communication, we report the propyl gallate synthesis using acidophilic tannase and simultaneous production of acidophilic tannase extracellularly along with gallic acid in the medium by a marine *A. awamori* BTMFW032 [18].

Materials and Methods

Fungal Strain

A. awamori BTMFW032 isolated from seawater and available as stock culture at Microbial Technology Lab, Department of Biotechnology, Cochin University of

Science and Technology was used. The strain was maintained on Czapek-Dox minimal agar slants supplemented with 1% tannic acid. Since tannase is an inducible enzyme, pre-induced inoculum was prepared and used. Spore inoculum was prepared with 10-day-old culture grown in the same medium at 30 °C. Spores were harvested in a 0.01% (*v*/*v*) solution of sterile Tween 80 in distilled water and were counted in a Neubauer chamber [19].

Tannase Production

Tannase production using SmF was performed with Czapek-Dox minimal medium prepared with 50% aged seawater and supplemented with 1% tannic acid (final concentration 1%), unless otherwise specified. Prepared medium was inoculated with spore inoculum (7.6×10^7 cfu/ml) at a concentration of 1% (*v*/*v*) and incubated for 2 days at room temperature (RT, 28±2 °C) at 100 rpm in a rotary shaker. After incubation, the biomass was separated by centrifugation at 10,000 rpm for 15 min at 4 °C. The mycelia-free fermented broth was collected and assayed for tannase activity and gallic acid.

Purification of Tannase

Tannase was purified as previously reported by Beena et al. [18]. To 100 ml of the crude culture filtrate taken in a conical flask, 50 g of aluminium oxide was added, mixed well for 30 min and incubated at 4 °C for 2 h with intermittent shaking. The clear solution obtained was then filtered through Whatman No. 1 filter paper. The aluminium oxide was later recovered by several washes in 1 N NaOH followed by a wash in 1 N HCl and in distilled water before reuse. Ultrafiltration was conducted using membrane cartridges (Amicon Ultra, Millipore, USA) of different molecular mass cut-offs (30 and 100 kDa). An aliquot of 1.5 ml of concentrated enzyme extract, obtained after ultrafiltration, was subjected to gel filtration chromatography with Sephadex G-200 (Sigma-Aldrich), and the gel filtration column was equilibrated with a 0.05-M HCl–KCl buffer (pH 2; selected after initial standardization). Elution was carried out with the same buffer at a flow rate of 1 ml/min, and fractions (1.5 ml) were collected. The column was maintained at 4 °C throughout the experiment. The purified enzyme was used for further studies.

Synthesis of Propyl Gallate

Propyl gallate was synthesized by transesterification of tannic acid [20] in the presence of purified acidophilic tannase, produced by marine *A. awamori* BTMFW032 [18]. Two millilitres of the tannase enzyme (699 U/ml) was added to 10 ml of 10 mM tannic acid in *n*-propanol and shaken at 100 rpm for 72 h. After incubation, the content was extracted with double-fold volume of ethyl acetate and separated using a separating funnel. Ethyl acetate was evaporated to recover propyl gallate in powder form. This powder was further analyzed for the presence of propyl gallate by thin layer chromatography (TLC) with silica gel G-60 F_{254} (E. Merck, Mumbai, India). The solvent system consisted of ethyl acetate, chloroform and formic acid (4:4:1, *v*/*v*/*v*). After drying, the plates were developed by spraying a solution of $FeCl_3$ [15]. Standard propyl gallate (Merck, Germany) and samples were run in the same plate. R_f value was calculated. Sample was scrapped from the TLC plate, eluted from the silica gel, and Fourier transform infrared spectroscopy (FTIR) analysis was performed. The propyl gallate was quantified spectrophotomerically at 560 nm following the method of Prasad et al. [21].

Tea Cream Solubilisation

Purified acidophilic tannase produced by marine *A. awamori* BTMFW032 [18] was used for the tea cream solubilisation studies. A standard brew commercially available was prepared following the method outlined by Rutter and Stainsby [22]. Eight grammes of tea procured from the market (commercially available Kannan Devan tea, India) was infused into 100 ml of distilled water at 90 °C for 6 min and filtered immediately through cotton fabric. Total solids were determined by incorporating an aliquot of infusion in pre-weighed Petri plates, dried in an oven at 80 °C overnight [22], and final weight was calculated. The brew was cooled overnight at 4 °C for the cream formation and centrifuged at 8,000 rpm for 15 min at 4 °C. The clarified supernatant was then decanted from the cream layer and analyzed for the total solid content. The weight of the cream solids was obtained by the difference from the total solid content of the original brew [22]. Ten millilitres of tea extract was treated with 1 ml of tannase (699 U/ml) and incubated at 30 °C for 1 h. The solid contents were determined in an aliquot of tannase-treated infusion and the control which included 1 ml of distilled water instead of enzyme. The samples were kept at 4 °C overnight to allow cream formation. The infusion was centrifuged, and the solid contents were determined in the supernatant of the tannase-treated tea infusion and in the control samples. The difference in weight of solid contents was reported as cream solubilised from the extract.

$$\text{Cream content}(\%) = \frac{(\text{Total solids in control} - \text{Total solids in control after centrifugation})}{(\text{Total solids in control})} \times 100$$

$$\%\text{Cream solubilized} = \frac{(\text{Cream content in control} - \text{Cream content in enzyme-treated sample})}{(\text{Cream content in control})} \times 100$$

Statistical Optimization of Enzyme Production Medium

During the course of our earlier studies, it was observed that the marine *A. awamori* BTMFW032 produced simultaneously tannase and gallic acid under SmF. Hence, statistical optimization of the fermentation medium for maximal production of acidophilic tannase and gallic acid was carried out in two stages employing Plackett–Burman (PB) design followed by RSM with Box–Behnken design.

Plackett–Burman design Eleven variables which showed considerable influence on tannase production during the course of optimization studies conducted earlier employing "one factor per trial" (data not shown) were used for the study. The experiments were designed according to PB design [23]. The variables and their experimental levels studied are presented in Table 1.

Box–Behnken design and response surface methodology The variables selected by the PB method were applied to RSM in order to evaluate the influence of each variable as well as the effect of their interactions on tannase and gallic acid production [24]. Table 2 shows the variables and the experimental levels studied with Box–Behnken method of RSM. The experiments were carried out in triplicate. The effect of variables and their interactions and all the coefficients were calculated by the software package Design Expert Version 6.0. The validation of the response surface was carried out through solutions obtained, and a time course study was conducted with the optimized parameters in shaken flasks in triplicate.

Table 1 Values of independent variables and levels used in Plackett–Burman experimental design employed for selection of variables that influence tannase production by *Aspergillus awamori* BTMFW032 under submerged fermentation

Independent variables	Different levels		Units
	Upper level	Lower level	
Tannic acid	4	0.5	%
Sodium chloride	1	0	M
Sodium nitrate	50	10	mM
Potassium chloride	10	0	mM
Magnesium sulphate	5	0	mM
Ferrous sulphate	0.5	0	mM
Dipotassium hydrogen phosphate	15	1	mM
pH	7	2	
Incubation hours	48	12	h
Inoculum	4	0.5	%
Agitation	150	0	rpm

Gallic Acid Extraction

The mycelia-free crude extract obtained after fermentation was equilibrated with different matrices like diatomaceous earth, silica gel and activated charcoal. NaCl was used for eluting the gallic acid from the equilibrated culture filtrate. Initially, the concentration of NaCl was standardized for the maximal recovery of gallic acid. Elute obtained was mixed well with two volumes of ethyl acetate and separated using a separating funnel. Later, ethyl acetate was evaporated to recover gallic acid in powder form and analyzed as mentioned elsewhere in this paper. The gallic acid extracted was also analyzed by TLC with silica gel G-60 F_{254} (E. Merck, Mumbai, India) using the solvent system ethyl acetate, chloroform and formic acid (4:4:1, *v*/*v*/*v*). After drying, the plates were developed by spraying a solution of $FeCl_3$ [15]. Standard gallic acid and sample were run in the same plate. Retention factor (R_f) value was calculated according to the following equation from the chromatogram:

$$R_f = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

Table 2 Variables and their experimental levels adopted in Box–Behnken method for selection of variables that influence tannase and gallic acid production by *Aspergillus awamori* BTMFW032 under submerged fermentation

Variables	Low level (−1)	Medium level (0)	High level (+1)
Tannic acid	0.5%	2.25%	4%
Sodium chloride	0	0.5 M	1 M
Ferrous sulphate	0	0.25 mM	0.5 mM
Di potassium hydrogen phosphate	1 mM	8 mM	15 mM
Incubation hours	12	30	48
Agitation	0 rpm	75 rpm	150 rpm

Sample was scrapped from the TLC plate, eluted from the silica gel, and FTIR analysis was performed.

Analytical Procedures

Tannase and protein assay Tannase was assayed according to a modified method of Sharma et al. [25] based on the formation of chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4-ketothiazolidine) using 0.01 M methyl gallate as substrate in 0.05 M citrate buffer. Protein was estimated according to the method of Bradford [26]. Tannase activity was expressed in international units, and 1 U of tannase activity was defined as the amount of enzyme required for the release of 1 μmol of gallic acid per minute under the assay conditions.

Gallic acid Gallic acid accumulation was assayed by spectrophotometric method of methanolic rhodanine [25].

Results and Discussion

The acidophilic tannase produced by marine *A. awamori* BTMFW032 was noted to have a molecular mass of 230 and 37.8 kDa, respectively, estimated based on sodium dodecyl sulphate polyacrylamide gel electrophoresis analyses under non-reducing and reducing conditions, indicating presence of six identical monomers [18]. Lekha and Lonsane [27] reported a molecular mass of 160,000 Da for the native enzyme produced by *A. niger* PKL104 as estimated by gel filtration, and under reduced conditions, they concluded that the native form of the enzyme is a tetramer consisting of four subunits of about 42,000 Da. Marco et al. [19] isolated tannase with three subunits of 50, 75 and 100 kDa from *A. niger* GH1. Tannases from *Aspergillus oryzae* [28] and *Aspergillus flavus* [29], produced by SmF, were reported to have a molecular mass of around 200 kDa. *A. awamori* tannase was noted to be a glycoprotein in nature, with 8.02% carbohydrate and a pI of 4.4 [18]. Tannase was reported earlier as a glycoprotein and a typical serine esterase containing a serine in the active site of the enzyme [30] with a molecular mass of 186,000 Da and containing 43% sugars and a pI of 4.3. [31]. Marine *A. awamori* tannase was observed to be active and stable over a wide range of temperatures [18], between 5 °C and 60 °C with a temperature optimum of 30 °C. An interesting observation is that unlike many previous tannases reported, it is active even at 5 °C. A terrestrial *A. awamori* Nakazawa tannase was reported to exhibit optimum activity at 35 °C [32]. This difference in temperature optima between the two *A. awamori* strains could be attributed to the fact the marine *A. awamori* normally experiences temperatures of around 30 °C in its natural environment and hence prefers that temperature for maximal activity, whereas the terrestrial strain is commonly exposed to high temperatures of above 30 °C. Two pH optima were observed for maximal activity, one at an extreme pH of 2.0 and the other at a pH of 8.0. Stability at pH 2.0 was maintained for 24 h, whereas a relatively rapid decrease in activity at the more neutral pH of 8.0 strongly supports a hypothesis for an acidophilic nature of the enzyme. Fungal tannase is an acidic protein [32] and hence requires acidic conditions in the environment for maximal activity. Most tannases reported in previous literature were found to be active only at around a pH of 5.0, although recently, it was reported that tannase isolated from *A. niger* GH1 was active at a pH of 2.0. Very rarely, enzymes are active at very low pH with high acidic nature [33].

Considering the differential properties of our acidophilic tannase compared to other reported tannases, herein, we tested the suitability of our tannase to be used as a catalyst for propyl gallate synthesis by transesterification and for tea cream solubilisation.

Propyl Gallate Synthesis by Transesterification

Propyl gallate is an antioxidant used in the food industry and has nutraceutical importance. In this study, we explored the prospects of producing this compound through transesterification of tannic acid using acidophilic tannase obtained from marine *A. awamori* BTMFW032. Results obtained for the FTIR analysis of the propyl gallate obtained after the transesterification reaction (Fig. 1) showed bands at 2,963.43 and 2,925.19 cm^{-1}, which indicated presence of the alkane group having CH_2. This correlates with the bonding pattern of commercially available propyl gallate standard. All other peaks responsible for C–O, C–H, C=C and O–H were visible at their appropriate range confirming the presence of propyl gallate. These results indicated the suitability of acidophilic enzyme obtained from marine *A. awamori* BTMFW032 for the production of antioxidant propyl gallate by transesterification. The use of immobilized tannase derived from *A. niger* for the synthesis of propyl gallate by direct transesterification of tannic acid using propanol as organic reaction media under low water conditions has been established earlier by Sharma and Gupta [20]. They recorded 15% to 86% conversion of tannic acid by transesterification after optimizing different variables one by one. Whereas, in our study with acidophilic tannase from marine *A. awamori* BTMFW032, we recorded 3.2% conversion of tannic acid into propyl gallate under unoptimized conditions. Our results corroborate with their observations with tannase of *A. niger*. Maybe the tannase produced by *Aspergillus* sp. irrespective of their source of environment has the property for transesterification in non-aqueous reaction media, which is preferred in recent times for organic synthesis of valuable products.

Tea Cream Solubilisation

Tannase has been recognized as a potential agent for the manufacture of instant tea. Hence, we evaluated the potential of acidophilic tannase obtained from marine *A. awamori* BTMFW032 for its potential for application in tea cream solubilisation, which is an important step in the manufacture of instant tea. From the results obtained in the present study, it was observed that 60.01% of cream was solubilised by 1 ml of purified enzyme having an activity of 699 U/ml within 1 h. The present study indicated that the acidophilic tannase has potential for application in the tea industry. Efficiency of tannase produced by *A. niger* PKL104 for solubilisation of tea cream solids was reported [11]. Partially purified enzyme with an activity of 600 U could solubilise 30.4% tea cream, and after optimization of temperature and enzyme concentration, 92% of solubilisation was obtained with 3,000 U of enzyme at 35 °C with an incubation of 2 h [11]. In our study, optimization of solubilisation was not conducted, and only the efficiency of partially purified enzyme on solubilisation was tested. Of course, detailed studies may be required for improving the process of tea cream solubilisation in large scale.

Simultaneous Production of Tannase and Gallic Acid

Marine *A. awamori* BTMFW032 was observed to produce simultaneously acidophilic tannase and gallic acid during SmF. Tannase and gallic acid yields were, respectively, 340.9 U/ml and 24.6 μg/ml under unoptimized conditions. PB design and response surface

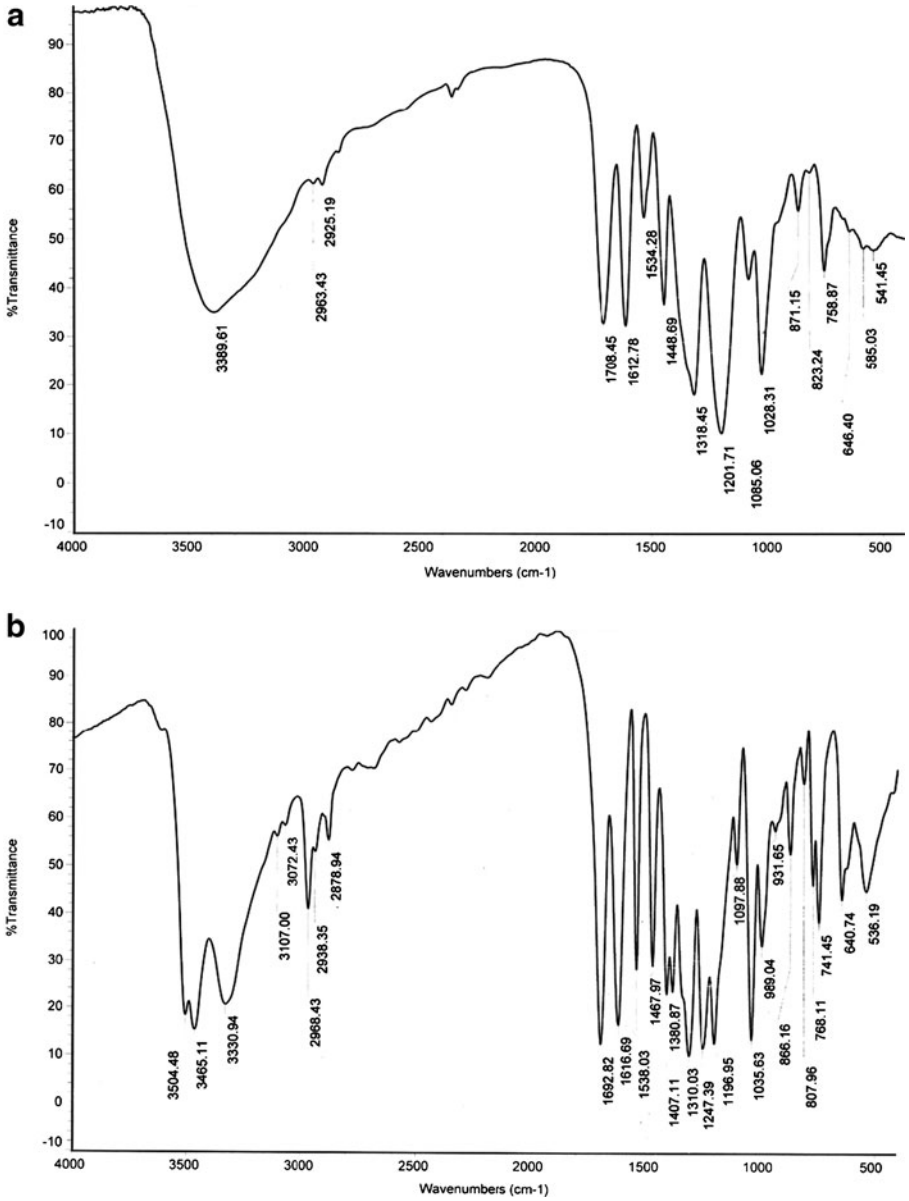

Fig. 1 FTIR analysis of **a** extracted propyl gallate and **b** standard propyl gallate

method (RSM) were employed for identifying the variables that influence maximal production of acidophilic tannase and gallic acid. From the results presented in Fig. 2 for the studies with PB design, it was noted that tannic acid, incubation period, $FeSO_4$, K_2HPO_4 and agitation effected an enhancement in enzyme production, along with the increase in their concentrations, whereas NaCl, pH and inoculum concentrations recorded a negative effect on enzyme production when there was an increase in the value of the variable. Further, it was noted that among the 11 variables which were selected based on the

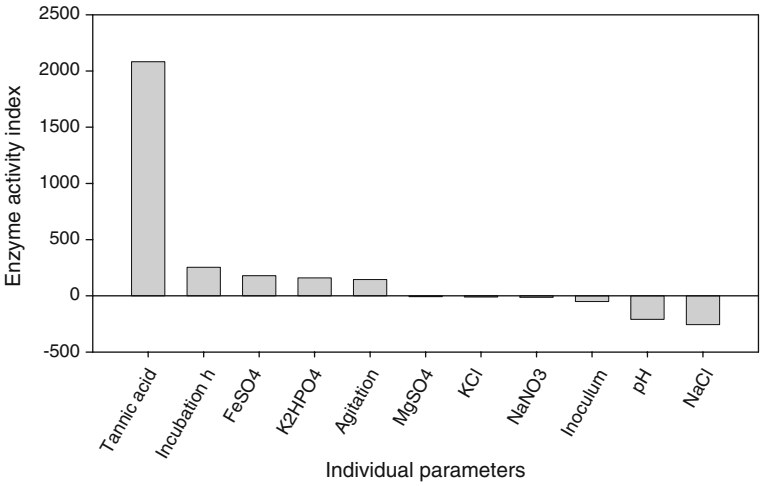

Fig. 2 Pareto chart showing effect of individual factors on production of tannase production by *A. awamori* BTMFW032 under SmF

results obtained for the studies on one factor per trial (data not shown), only six factors, namely, tannic acid, incubation period, $FeSO_4$, K_2HPO_4, agitation and NaCl, were identified as the most significant variables that influenced simultaneous production of tannase and gallic acid. It was also noted that at low pH and higher concentration of tannic acid at the commencement of SmF, the organism could not survive, and consequently, pH 5.6 (selected based on PB design studies) was used as a constant value during the course of the study. The statistical significance of the model was evaluated using ANOVA, and the results were used to fit a first-order polynomial equation; the model equation for tannase activity (Y) could be written as

$$\begin{aligned}\text{Tannase activity } (Y)\ \text{U/ml} = {} & -542.93 + 1,190.26\text{*Tannic acid} - 512.84\text{*NaCl} \\ & + 718.02\text{*}FeSO_4 + 22.77\text{*}K_2HPO_4 - 83.73\text{*pH} \\ & + 14.09\text{*Incubation h.}\end{aligned}$$

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with F value of 158.60 and P value of 0.0001. The value of Prob>F less than 0.05 indicates the significance of this model. The coefficient of determination R^2 was calculated as 0.9948, which indicated that approximately 99.5% of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of variables, was 0.9885. The coefficient of variance was found to be 10.50, and the adequate precision that measures the signal-to-noise ratio was 29.514. A ratio greater than 4 is desirable as it indicates an adequate signal. Application of the PB method [23] enables selection of variables which have the largest influence on the desired product yield. In the present study, this fact could be experienced in the rapid recognition of six most important variables from amongst the 11 factors that was originally studied.

Tannic acid, incubation period, $FeSO_4$, K_2HPO_4, agitation and NaCl, identified as significant variables based on PB design, were further optimized employing RSM using

Box–Behnken design. The design evaluated six independent variables each one at three levels for tannase and gallic acid production.

The quadratic model chosen to represent the relationship fitted for the six variables is

$$Y = \beta o + {}^{6}\sum \beta_i X_i + {}^{6}\sum \beta_{ii} X_i^2 + {}^{6}\sum {}^{6}\sum \beta_{ij} X_i X_j;\ i = 1\ \ i = 1\ \ i = 1\ \ j = 1$$

where Y is the production of tannase and gallic acid, and X_1, X_2, X_3, X_4, X_5 and X_6 are the six independent variables for tannic acid, NaCl, $FeSO_4$, K_2HPO_4, incubation period and agitation and βo is the intercept term. The results obtained for Box–Behnken design experiments were analyzed by ANOVA, which yielded the following regression equation for the level of (1) tannase production:

$$\begin{aligned}
\text{Tannase activity, } Y\ (\text{U/ml}) &= -5,665.96 + 3,162.11\text{*Tannic acid} + 4,303.68\text{*NaCl} \\
&+ 11,546.33\text{*FeSO}_4 + 249.27\text{*K}_2\text{HPO}_4 + 134.10\text{*Incubation h} + 32.04\text{*Agitation} \\
&- 73.66\text{*Tannicacid*NaCl} - 380.12\text{*Tannic acid*FeSO}_4 + 7.60\text{*Tannic acid*K}_2\text{HPO}_4 \\
&+ 3.52\text{*Tannicacid*Incubation h} - 0.08\text{*Tannic acid*Agitation} - 1,933.97\text{*NaCl*FeSO}_4 \\
&- 27.51\text{*NaCl*K}_2\text{HPO}_4 + 10.92\text{*NaCl*Incubation h} + 6.05\text{*NaCl*Agitation} \\
&- 115.85\text{*FeSO}_4\text{*K}_2\text{HPO}_4 - 74.71\text{*FeSO}_4\text{*Incubation h} + 7.72\text{*FeSO}_4\text{*Agitation} \\
&+ 3.46\text{*K}_2\text{HPO}_4\text{*Incubationh} + 0.21\text{*K}_2\text{HPO}_4\text{*Agitation} + 0.07\text{*Incubation h*Agitation} \\
&- 673.78\text{*Tannicacid}^2 - 4,722.99\text{*NaCl}^2 - 12,683.84\text{*FeSO}_4{}^2 \\
&- 20.19\text{*K}_2\text{HPO}_4{}^2 - {}^{-}2.97\text{*Incubation h}^2 - 0.23\text{*Agitation}^2
\end{aligned}$$

and (2) for the level of gallic acid production:

$$\begin{aligned}
\text{Gallic acid, } Y\ (\mu\text{g/ml}) &= -39.14 + 51.64\text{*Tannic acid} + 1.42\text{*NaCl} - 8.62\text{*FeSO}_4 \\
&+ 8.58\text{*K}_2\text{HPO}_4 + 2.30\text{*Incubation h} + 0.37\text{*Agitation} - 3.19\text{*Tannic acid*NaCl} \\
&+ 10.05\text{*Tannic acid*FeSO}_4 - 0.25\text{*Tannic acid*K}_2\text{HPO}_4 \\
&+ 0.64\text{*Tannic acid*Incubation h} + 0.049\text{*Tannic acid*Agitation} + 31.07\text{*NaCl*FeSO}_4 \\
&+ 0.76\text{*NaCl*K}_2\text{HPO}_4 - 0.07\text{*NaCl*Incubation h} - 0.03\text{*NaCl*Agitation} \\
&+ 0.09\text{*FeSO}_4\text{*K}_2\text{HPO}_4 + 1.65\text{*FeSO}_4\text{*Incubation h} + 0.08\text{*FeSO}_4\text{*Agitation} \\
&- 0.03\text{*K}_2\text{HPO}_4\text{*Incubation h} + 0.02\text{*K}_2\text{HPO}_4\text{*Agitation} - 7.62\text{E} \\
&- 003\text{*Incubation h*Agitation} - 13.12\text{*Tannic acid}^2 - 3.27\text{*NaCl}^2 - 171.12\text{*FeSO}_4{}^2 \\
&- 0.48\text{*K}_2\text{HPO}_4{}^2 - 0.05\text{*Incubation h}^2 - 2.10\text{E} - 003\text{*Agitation}^2.
\end{aligned}$$

The ANOVA analysis of tannase production showed a model F value of 7.38 and P value of 0.0002; the values of Prob>F less than 0.05 indicated that the model terms are significant. Two linear and six quadratic terms were significant model terms for the response. The coefficient of determination R^2 was calculated as 0.8845, which ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that

approximately 88.45% of the variability in the dependent variable (response) could be explained by the model. All selected parameters were significant, and varied levels of interactions were recorded for the variables in their cumulative effect on tannase production. The coefficient of variance was found to be 45.28. The adequate precision that measures the signal-to-noise ratio was 10.184. Thus, this model could be used to navigate the design space.

The ANOVA analysis of gallic acid production yielded values of Prob>F less than 0.05, suggesting that the model terms are significant, with a P value of 0.0001 and an F value of 6.53. Two linear and six quadratic terms were significant model terms for the response. An estimated value of 0.8714 for the coefficient of determination R^2 ensured a satisfactory adjustment of the quadratic model to the experimental data, indicating that approximately 87.14% of the variability in the dependent variable (response) could be explained by the model. It was observed that all the selected parameters were significant, and varied levels of interactions were recorded for the variables in their cumulative effect on gallic acid production. The coefficient of variance was found to be 21.29, and the adequate precision that measures the signal-to-noise ratio was 9.921, which indicated that this model could be used to navigate the design space.

Experimental data obtained for the effect of six selected physicochemical factors on production of tannase and gallic acid by marine *A. awamori* BTMFW032 (Table 3) showed strong dependence on the presence and levels of selected factors since it was noted that tannase production varied between 212.99 and 4,870.4 U/ml, while gallic acid production varied between 19.77 and 136.206 μg/ml under the experimental conditions studied.

Three dimensional response surface profiles were plotted to study interaction among various physicochemical factors and determine the optimum concentration of each individual factor for maximum tannase and gallic acid production under SmF. Data obtained for the interaction of the significant variables alone are presented here. It was noted that tannic acid, which is used as an inducer, influenced both tannase and gallic acid production and its higher initial concentrations supported maximum yield. It was also observed that the individual effect of tannic acid on tannase yield was observed to increase along with increase in its concentration in PB design experiment. Whereas, in the RSM experiment, 2.56% of tannic acid was observed to be the optimum for the positive interactive effect of tannic acid with other process parameters, namely, $FeSO_4$, NaCl, agitation and incubation, towards enhanced enzyme production. It was further observed that interaction of tannic acid (2.25%) and $FeSO_4$ (0.25 mM) supported maximum enzyme production, when all other factors were maintained at their central value of concentration. Tannic acid (2.25%) and NaCl (0.5 M) interaction indicated that maximum enzyme productivity was supported at their central value of concentrations when $FeSO_4$ (0.25 mM), K_2HPO_4 (8 mM), incubation (30 h) and agitation (75 rpm) were kept at their central values tested. This resulted in a parabola (Fig. 3). In a similar pattern, the interactions of agitation (75 rpm) and tannic acid (2.25%), represented by a parabola (Fig. 4), also indicated that the maximum enzyme productivity was supported when all the tested variables were at their central values. In the case of both NaCl and agitation, maximal enzyme activity was supported by their central values (0.5 M NaCl and 75 rpm). Similarly, maximum enzyme activity at 0.5 M NaCl and 8 mM K_2HPO_4 was also noted. In the case of interactive effect between agitation (75 rpm) and $FeSO_4$ (0.25 mM), it was noted that maximum tannase production could be achieved when other factors were kept constant at their central values of concentrations. Both agitation rate and K_2HPO_4 concentration were also observed to support maximum enzyme activity at their central values. Incubation period for 36 h and 0.5 mM of $FeSO_4$ were also noted to enhance maximum enzyme activity when all other

Table 3 Optimization of medium composition and physical parameters for production of tannase and gallic acid by *Aspergillus awamori* BTMFW032 under submerged fermentation by response surface methodology (RSM)

Run	Block	Tannic acid, %	NaCl, M	$FeSO_4$, mM	K_2HPO_4, mM	Incubation, h	Agitation, rpm	Enzyme, U/ml	Gallic acid, μg/ml
1	1	−1	0	0	−1	−1	0	413.20	20.912
2	1	−1	−1	0	−1	0	0	434.50	19.771
3	1	1	−1	0	1	0	0	1,523.25	105.065
4	1	0	1	0	0	−1	1	1,268.02	136.206
5	1	−1	0	1	0	0	−1	885.41	22.560
6	1	0	0	1	−1	0	1	3,522.19	58.847
7	1	0	−1	−1	0	1	0	2,227.90	98.849
8	1	0	0	−1	−1	0	1	1,346.11	74.174
9	1	−1	1	0	−1	0	0	354.98	21.419
10	1	−1	0	1	0	0	1	647.49	35.504
11	1	0	1	0	0	1	−1	247.07	96.704
12	1	1	0	0	1	−1	0	255.59	32.403
13	1	0	0	0	0	0	0	4,870.43	125.057
14	1	0	−1	−1	0	−1	0	1,269.44	99.714
15	1	−1	0	−1	0	0	−1	52.53	62.484
16	1	−1	0	0	1	−1	0	536.74	52.002
17	1	0	−1	0	0	1	−1	846.29	81.633
18	1	1	1	0	−1	0	0	593.71	79.610
19	1	0	0	0	0	0	0	4,874.97	127.068
20	1	0	1	1	0	1	0	846.29	122.180
21	1	1	1	0	1	0	0	800.85	106.210
22	1	1	−1	0	1	0	0	212.99	46.556
23	1	0	−1	1	0	−1	0	3,441.96	100.084
24	1	0	−1	0	0	−1	1	2,690.81	131.812
25	1	0	0	0	0	0	0	4,769.44	133.443
26	1	0	1	0	0	−1	−1	840.61	79.685
27	1	1	0	1	0	0	−1	842.03	87.705
28	1	0	0	0	0	0	0	4,792.30	133.832
29	1	1	−1	0	−1	0	0	530.32	105.218
30	1	0	0	−1	1	0	1	2,891.73	102.471
31	1	0	0	1	1	0	−1	802.27	85.570
32	1	0	1	0	0	1	1	1,509.76	107.106
33	1	0	1	1	0	−1	0	2,178.20	104.520
34	1	1	0	−1	0	0	−1	852.53	88.524
35	1	0	0	1	−1	0	−1	451.54	89.757
36	1	0	0	−1	−1	0	−1	614.83	80.675
37	1	1	0	0	−1	1	0	433.08	102.522
38	1	0	−1	0	0	−1	−1	2,695.07	75.928
39	1	0	0	1	1	0	1	3,342.21	102.864
40	1	1	0	1	0	0	1	739.79	105.036
41	1	1	0	0	1	1	0	2,233.01	106.003
42	1	1	0	−1	0	0	1	839.19	106.041

Table 3 (continued)

Run	Block	Tannic acid, %	NaCl, M	$FeSO_4$, mM	K_2HPO_4, mM	Incubation, h	Agitation, rpm	Enzyme, U/ml	Gallic acid, μg/ml
43	1	−1	0	0	1	1	0	553.78	49.725
44	1	0	1	−1	0	−1	0	2,384.10	109.206
45	1	0	−1	0	0	1	1	724.17	101.352
46	1	1	0	0	−1	−1	0	1,718.14	101.519
47	1	0	0	0	0	0	0	4,727.80	128.374
48	1	0	0	1	1	0	−1	742.63	80.249
49	1	0	−1	1	0	1	0	1,644.30	108.264
50	1	0	1	−1	0	1	0	985.44	76.637
51	1	−1	1	0	1	0	0	149.09	42.813
52	1	0	0	0	0	0	0	4,814.59	133.815
53	1	−1	0	0	−1	1	0	201.63	28.559
54	1	−1	0	−1	0	0	1	259.85	32.572

variables were at central values. In general, it was inferred that all the tested variables had relatively a positive interactive effect at their central values tested in supporting maximal tannase production.

Maximum gallic acid production was supported by the interactive effect of agitation at 75 rpm and 0.25 mM $FeSO_4$ along with 2.25% tannic acid, 0.5 M NaCl, 8 mM K_2HPO_4 and 36 h of incubation (Fig. 5). It was also noted that the interactive effect of higher level of tannic acid (4%) and lower level of NaCl (0% NaCl) led to maximum gallic acid

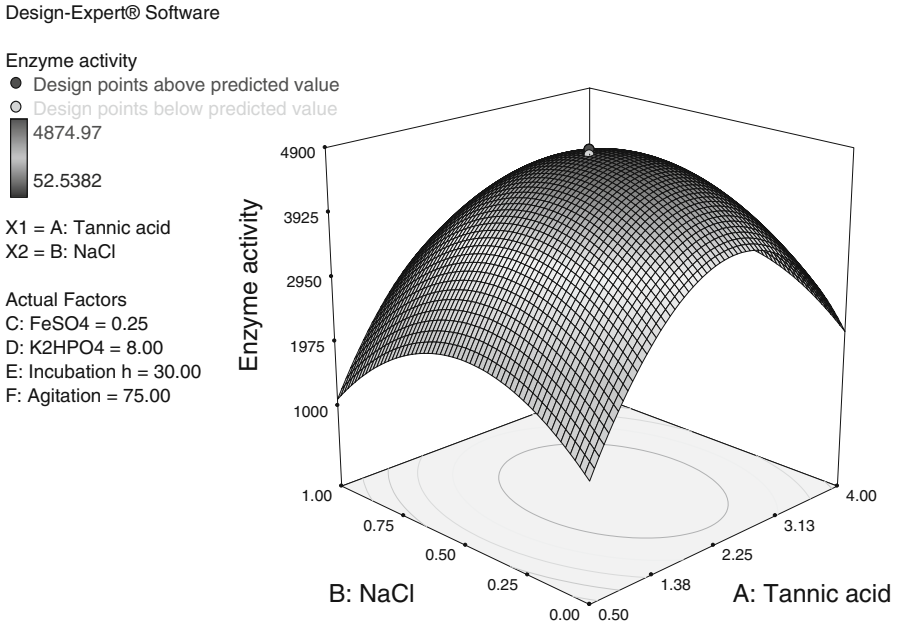

Fig. 3 Effect of tannic acid and NaCl on tannase production by *A. awamori* BTMFW032 under SmF

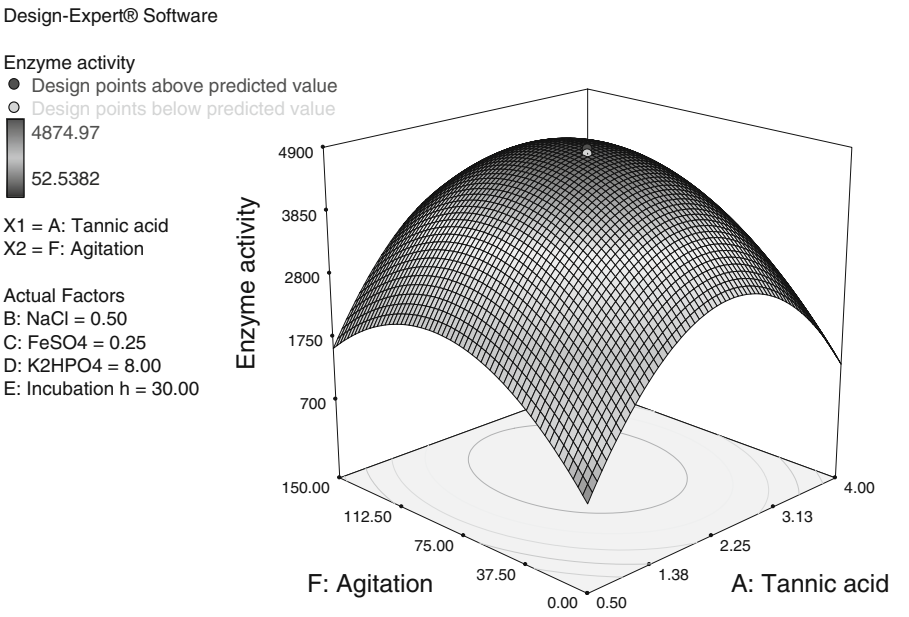

Fig. 4 Effect of tannic acid and agitation on tannase production by *A. awamori* BTMFW032 under SmF

production. Further, the maximum gallic acid production was supported by the interactive effect of 4% tannic acid and 8 mM K_2HPO_4.With respect to $FeSO_4$ (0.25 mM) and 30 h of incubation period, maximal production of gallic acid was noted at their central value tested, when all other factors were at their medium level. Similarly, agitation at 75 rpm and 8 mM K_2HPO_4 enhanced maximum gallic acid production. Interactive effect of 30 h of incubation

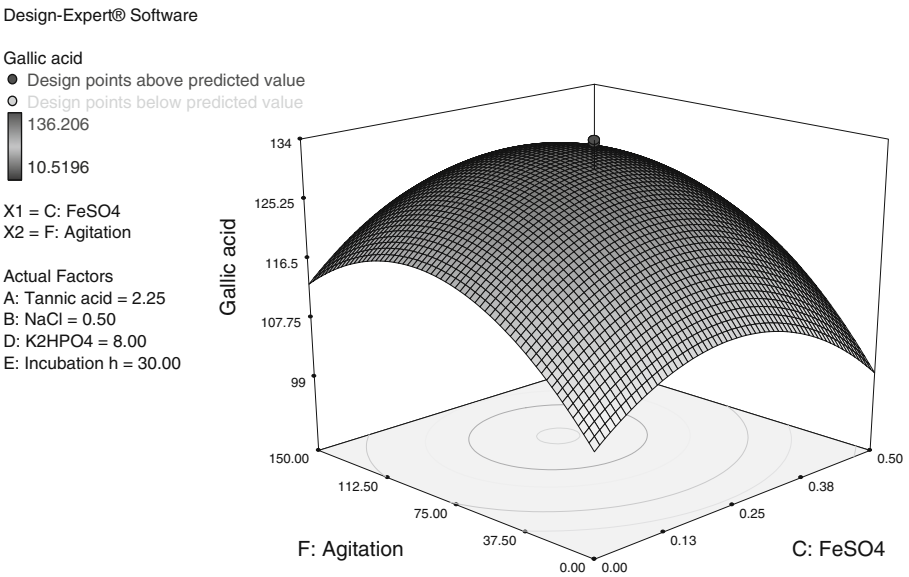

Fig. 5 Effect of agitation and $FeSO_4$ on gallic acid production by *A. awamori* BTMFW032 under SmF

and 4% tannic acid also recorded maximal gallic acid production. Further, it was also noted that interaction of 30 h incubation and 8 mM K_2HPO_4 supported maximum gallic acid production. In general, it was inferred that higher concentration (4%) of tannic acid and central values of other tested variables had relatively a positive interactive effect on enhanced gallic acid production.

Validation of the deduced response surface model based on previous experiments was carried out in shake flask under conditions predicted by the model. The experimental values were found to be very close to predicted values, and hence, model was successfully validated. The optimized conditions were as follows: tannic acid, 2.56% (*w*/*v*); inoculum, 0.5% (*v*/*v*); NaCl, 0.45 M; pH, 5.6; $FeSO_4$, 0.27 mM; incubation period, 36 h; K_2HPO_4, 9.14 mM; agitation, 93 rpm; $NaNO_3$, 47 mM; KCl, 5.1 mM; and $MgSO_4$, 4.9 mM. The RSM model obtained predicted 4,824.6 U/ml of tannase enzyme activity and 136.2 μg/ml gallic acid after 48 h of incubation, whereas after time course experiment, 5,085 U/ml tannase activity after 36 h of incubation and 372.6 μg/ml gallic acid after 84 h of incubation were noted (Fig. 6). The production of both tannase and gallic acid attained under optimized condition was nearly 15 times higher than that obtained under the unoptimized conditions (340.9 U of tannase/ml and 24.6 μg of gallic acid/ml). The experimental profiles provide a good fit to the predicted values from the model, and the system can be used either for maximizing tannase activity or gallic acid accumulation depending on the application. Tannic acid was found to have the most significant effect on tannase production. The optimization of NaCl in tannase production was not reported in literature, whereas in the present study, influence of NaCl on tannase production was recorded. This observation could be attributed to the source of *A. awamori*, which is seawater, where the organism exists in high saline environment, and hence the tannase-encoding genes could have been induced by the salt concentration. It may be also noted that in the present study, $FeSO_4$ has played a significant role in tannase synthesis and consequent gallic acid production. Hence, it is presumed that *A. awamori*, a marine fungus, required $FeSO_4$ as a key variable for

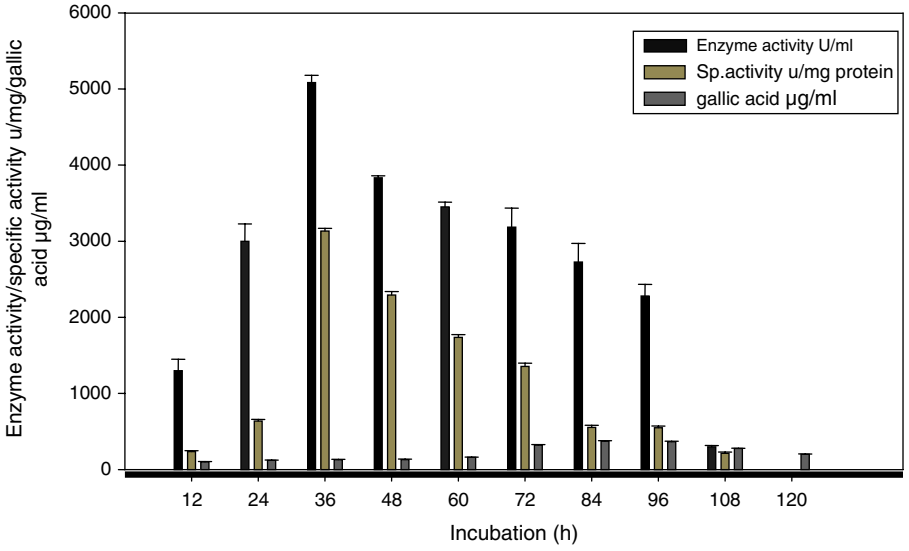

Fig. 6 Time course study under optimized condition for tannase and gallic acid production by *A. awamori* BTMFW032 under SmF

enhanced tannase synthesis. The authors have noted this with other marine bacteria and fungi during their routine growth and enzyme production in their laboratory (data not shown). It is the same case with K_2HPO_4 with respect to marine microorganisms that potassium and phosphates in seawater are stimulatory in their function in the medium towards enhanced growth and enzyme production. In the present study, tannic acid at 2.56% (*w*/*v*) was noted as optimal concentration for maximal tannase production. This observation is very much in agreement with earlier reports made for *Aspergillus japonicus* [34] and *Rhizopus oryzae* [35], which produced maximal tannase with a concentration of 2% tannic acid as optimum, whereas 5% tannic acid was reported to be suitable for tannase production by *A. niger* under solid state fermentation [36]. Seth and Chand [1] observed that terrestrial *A. awamori* produced, after optimization by RSM using Box–Behnken factorial design, 771 IU of intracellular tannase by 60 h with an initial tannic acid concentration of 35.0 gl^{-1} and maximum gallic acid accumulation (40.3 gl^{-1}) after 24 h with an initial substrate concentration of 45 gl^{-1} [1]. Kar and Banerjee [6] reported 48 h as optimum incubation period for tannase production. In the present study, maximum enzyme production was obtained after 36 h of incubation. From the time course study, it was inferred that the tannase enzyme synthesized and released into the fermentation medium during growth simultaneously converted available tannic acid in the medium into gallic acid, contributing to the gradual increase in its accumulation in the medium along with increase in the period of incubation. It was noted that in spite of a maximal tannase activity at 36 h, maximal gallic acid was recorded at 84 h. The results further indicated that the optimized medium could be used for enhanced production of tannase and gallic acid.

Gallic Acid Extraction

A method for removing impurities from fermented broth while extracting gallic acid was optimized by passing the mycelia-free extract through a column of diatomaceous earth. Gallic acid bind more easily to diatomaceous earth compared to other matrices used. Among the different concentrations of NaCl that tried to elute the gallic acid from the column, 0.4 M NaCl supported maximal elution of gallic acid, which was later extracted with ethyl acetate. Gallic acid was retained in powder form after evaporation of ethyl acetate. The gallic acid was identified by its R_f value (0.69) in TLC and FTIR. A comparative analysis was also performed with commercially available gallic acid during its identification. Presence of C=C, C–C and OH bonds identified by FTIR represented the bonds present in the gallic acid structure confirming gallic acid.

Conclusion

Based on the results obtained from the study, it is concluded that the acidophilic tannase produced by marine *A. awamori* BTMFW032 has scope for utilization in the synthesis of antioxidant propyl gallate by transesterification, tea cream solubilisation and simultaneous production of tannase and gallic acid. The medium optimization studies also indicated that productivity of both acidophilic tannase and gallic acid could be enhanced to about 15-fold under SmF. Interestingly, almost identical levels of process variables and medium requirements were noted for this marine fungus for simultaneous production of acidophilic tannase as extracellular enzyme and gallic acid. It may be noted that this is the first report on applications of acidophilic tannase as well as gallic acid production by a marine fungus.

Acknowledgement The authors wish to thank the Department of Science and Technology, India for their financial and technical support given under SERC-DST-WOS-A Project to P.S. Beena.

References

1. Seth, M., & Chand, S. (2000). *Process Biochemistry, 36*, 39–44.
2. Garcia-Conesa, M. T., Ostergaard, P., Kauppinen, S., & Williamson, G. (2001). *Carbohydrate Polymers, 44*, 319–324.
3. Van de Lagemaat, J., & Pyle, D. L. (2001). *Chemical Engineering Journal, 84*, 115–123.
4. Bajpai, B., & Patil, S. (1996). *World Journal of Microbiology & Biotechnology, 12*, 217–220.
5. Kar, B., Banerjee, R., & Bhattacharyya, B. C. (1999). *Journal of Industrial Microbiology & Biotechnology, 23*, 173–177.
6. Kar, B., & Banerjee, R. (2000). *Journal of Industrial Microbiology & Biotechnology, 25*, 29–38.
7. Mondal, K., & Pati, B. (2000). *Journal of Basic Microbiology, 40*, 223–232.
8. Banerjee, D., Mondal, K., & Bikas, R. (2001). *Journal of Basic Microbiology, 6*, 313–318.
9. Mondal, K., Banerjee, D., Banerjee, R., & Pati, B. (2001). *The Journal of General and Applied Microbiology, 47*, 263–267.
10. Treviño-Cueto, B., Luis, M., Contreras-Esquivel, J. C., Rodríguez, R., Aguilera, A., & Aguilar, C. N. (2007). *Bioresource Technology, 98*, 721–724.
11. Lekha, P. K., & Lonsane, B. K. (1994). *Process Biochemistry, 29*, 497–503.
12. Kalil, S. J., Maugeri, F., & Rodrigues, M. I. (2000). *Process Biochemistry, 35*, 539–550.
13. Egmond, M. R., & de Vlieg, J. (2000). *Biochimie, 82*, 1015–1021.
14. Saisubramanian, N., Edwinoliver, N., Nandakumar, N., Kamini, N., & Puvanakrishnan, R. (2006). *Journal of Industrial Microbiology & Biotechnology, 33*, 669–676.
15. Naidu, R. B., Saisubramanian, N., Selvakumar, D., Janardhanan, S., & Puvanakrishnan, R. (2008). *Current Trends Biotechnology and Pharmacy, 2*, 201–207.
16. Dey, G., Mitra, A., Banerjee, R., & Maiti, B. (2001). *Biochemical Engineering Journal, 7*, 227–231.
17. Sharma, S., Agarwal, L., & Saxena, R. (2007). *Indian Journal of Microbiology, 47*, 132–138.
18. Beena, P. S., Soorej, M. B., Elyas, K. K., Sarita, G. B., & Chandrasekaran, M. (2010). *Journal of Microbiology and Biotechnology, 20*, 1403–1414.
19. Marco, M. G., Rodríguez, L. V., Ramos, E. L., Renovato, J., Cruz-Hernández, M. A., Rodríguez, R., et al. (2009). *Journal of Microbiology and Biotechnology, 19*, 987–996.
20. Sharma, S., & Gupta, M. N. (2003). *Bioorganic & Medicinal Chemistry Letters, 13*, 395–397.
21. Prasad, U. V., Rao, K. E., & Sastry, C. S. P. (1985). *Food Chemistry, 17*, 209–213.
22. Rutter, P., & Stainsby, G. (1975). The solubility of tea cream. *Journal of the Science of Food and Agriculture, 26*, 455–463.
23. Plackett, R. L., & Burman, J. P. (1946). *Biometrica, 33*, 305–325.
24. Box, G. E. F., & Behnken, D. W. (1960). *Technometrics, 24*, 455–475.
25. Sharma, S., Bhat, T. K., & Dawra, R. K. (2000). *Analytical Biochemistry, 278*, 85–89.
26. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
27. Lekha, P. K., & Lonsane, B. K. (1997). *Advances in Applied Microbiology, 44*, 215–260.
28. Iibuchi, S., Minoda, Y., & Yamada, K. (1968). *Agricultural and Biological Chemistry, 32*, 803–809.
29. Adachi, O., Watanabe, M., & Yamada, H. (1968). *Agricultural and Biological Chemistry, 32*, 1079–1085.
30. Rajkumar, G. S., & Nandy, S. C. (1983). *Applied and Environmental Microbiology, 46*, 525–527.
31. Barthomeuf, C., Regerat, F., & Pourrat, H. (1994). *Journal of Fermentation Technology, 77*, 137–142.
32. Mahapatra, K., Nanda, R. K., Bag, S. S., Banerjee, R., Pandey, A., & Szakacs, G. (2005). *Process Biochemistry, 40*, 3251–3254.
33. Ikasari, L., & Mitchell, D. A. (1996). *Enzyme Microbial Technology, 19*, 171–175.
34. Bradoo, S., Gupta, R., & Saxena, R. K. (1997). *Process Biochemistry, 32*, 135–139.
35. Hadi, T. A., Banerjee, R., & Bhattarcharyya, B. C. (1994). *Bioprocess Engineering, 11*, 239–243.
36. Sabu, A., Pandey, A., Daud, M. J., & Szakacs, G. (2005). *Bioresource Technology, 96*, 1223–1228.