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Utilization of *Bahera* Fruits for Production of Tannase and Gallic Acid by *Aspergillus heteromorphus* MTCC 5466 and Synthesis of Propyl Gallate Thereof

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Abstract: Bahera fruit (*Terminalia belerica*) was used as a novel substrate for production of tannase and gallic acid (GA) from a new isolate of *Aspergillus heteromorphus* MTCC 5466. Solid-state fermentation was carried out using wattle, quebracho, myrobalan and *bahera* fruit powder (BFP). BFP resulted in higher yield of tannase and GA (528±12 Unit/g dry substrate (gds) and 35±2 mg/gds, respectively) at 84h and increased by 2 fold when mixed with wheat bran (WB). Moreover, 4.8 and 2.4 fold increase in production of tannase and GA was observed under the optimized conditions (60% v/w moisture, 4% v/w inoculum; 1.5% w/w corn steep liquor, 3:7 w/w BFP and WB, 72h incubation at 32°C) respectively. Further, alginate microencapsulated and free tannase were compared for the esterification of GA into propyl gallate and the former was found to have more potential for the same. The GA and propyl gallate were analyzed by HPLC and FT-IR.

Key words: Bahera fruit · Tannase · Gallic acid · Propyl gallate

INTRODUCTION

Tannases (tannin acyl hydrolase E.C. 3.1.1.20) are hydrolytic enzymes that catalyze the hydrolysis of ester and depside bonds in hydrolysable tannins, releasing glucose and GA [1]. Mostly produced by microorganisms viz. fungi [2, 3], yeast [4] and bacteria [5], but they have also been described in plants [6]. Tannases are industrially important enzymes used in the production of GA [7-9], manufacture of instant tea [5], vegetable tanning effluent treatment and in determination of the structure of naturally occurring GA esters [10]. Other applications of tannases include removal of chill haze formation of beer and wines [1, 5] and detannification of poultry feed to improve the feed efficiency [11].

GA with worldwide demand of around 8000 tonnes is extensively used as an ingredient of developer in photography and printing inks [7]. It also serves as a precursor for the commercial production of an anti-microbial drug trimethoprim, some dyestuffs and synthesis of propyl gallate (PG) a food preservative [1]. Besides this, GA possesses wide range of biological activities, such as antioxidant, antibacterial, antiviral and analgesic [7]. As antioxidant GA acts as an antiapoptotic agent and helps to protect human cells against oxidative damage [8]. The price of tannase and GA is quite high at present to consider for many industrial applications and hence, it is necessary to devise strategies to produce it at cheaper rates. One such viable option is the use of SSF alongwith easily available cheap source of substrates.

Tannase production by SSF using agro-industrial residues is more advantageous over submerged fermentation (SmF) or liquid surface fermentation (LSF) [3, 12]. Recent literature on SSF for tannase production claim advantages such as high-production titers (3 to 6 times) than SmF and extracellular nature of the enzymes [2, 3]. Also, the enzyme produced in SSF exhibits good stability parameters including higher tolerance for a wide range of pH and temperature [13]. Some of the agro-industrial residues that have been used as substrates for tannase production under SSF includes palm kernel cake (PKC), tamarind seed powder (TSP), wheat bran, coffee husk [14], jamun leaves (*Syzygium cumini*) [2, 15], jamoa leaves (*Schleichera trijuga*) and keekar leaves (*Capparis decidua*) [2]. *Terminalia chebula*, pod cover of *Caesalpinia digyna* [10] and

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leaves of gobernadora (*Larrea tridentata*) [8], are the substrate that have been used for the production of both, tannase as well as GA. *Terminalia bellerica* ('Bahera' in hindi) belonging to the family '*Combretaceae'* and commonly known as belleric myrobalan, contains β-sitosterol, tannic acid (TA), ethyle gallate, galloyl glucose, triterpene and belleric acid [16], is a potential substrate for tannase production.

Approximately 30–40% of the production cost in industrial enzyme production is due to media components, hence selecting low-cost substrates and optimizing the composition is especially vital. In this study we explored the scope for simultaneous tannase and GA production with *A. heteromorphus* by using cost effective substrate BFP, for the first time, which is quite cheap and a readily available substrate in nearly all parts of the world.

MATERIALS AND METHODS

Microorganism and Inoculum Development: The organism used in the present study was isolated from soil sample of 'Assam' tea garden and identified as *Aspergillus heteromorphus* at Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India and deposited as *A. heteromorphus* MTCC 5466 under Budapest Treaty. The fungal culture was maintained on Czapek Dox agar slants containing 0.5% (w/v) of TA and stored at 4°C. The spore suspension for inoculation was prepared by adding 3 ml of sterile Tween-80 (0.01% v/v) solution to the well grown culture slant and the spores were dislodged gently using an inoculation needle under aseptic condition. Spore suspension containing about 2.32 x 10^6 colony forming unit (CFU)/ml was used as inoculum.

Materials: All the reagents used in the present study were of AR grade and purchased from Sigma Germany, Hi-Media Limited and S.D. Fine Chemicals Limited, Mumbai, India. Wheat bran, Bahera fruit (*Terminalia belerica*), myrobalan (*Terminalia chebula*), were purchased from local market, Chennai, India. Wattle (*Acacia decurrens*) powder (WP) and quebracho (*Schinopsis lorentzii*) powder (QP) were sourced from tannery division, CLRI, Chennai, India.

Processing of Substrates and Estimation of Tannin Content: Bahera and myrobalan fruits were cleaned and dried overnight at 40°C in hot air oven. Dried fruits were peeled to remove the kernel and ground in heavy-duty mixer grinder (Venus Enterprises, India). The comminuted material was passed through a 40 mesh sieve; retentate coarse material was ground again to get a powder of uniform particle size. The powder was stored in a dry and dark plastic container at room temperature. The estimation of tannin content was done for all the substrates following the protein precipitation method of Haggerman and Butler, [17].

Fermentation Conditions and Evaluation of Substrates for Tannase and GA: Tannase production was carried out in 250 ml Erlenmeyer flasks (Borosil, India) containing 10g of each individual substrate (BFP, MFP, WP and QP). In another set of experiment different ratios of each substrate to WB (total 10g) were studied. Mixed combinations of selected substrates were also tried for enhanced tannase and GA production. The initial moisture content was adjusted to 65% (v/w) with distilled water containing 1% (w/v) of CSL and sterilized at 121°C for 20 min. The flasks were inoculated with 400 µl of spore suspension and incubated at 30°C in a BOD incubator (SEMCO, India) for 5 days. Samples were taken at 24 h intervals starting from 36 h of incubation and enzyme extraction was carried out in 0.02 M acetate buffer (pH 5.5). All the experiments were carried out in triplicates and the results were expressed as the mean ± SD of triplicates.

Optimization Studies: Different types of physico-chemical parameters viz., incubation period (24-120 h), BFP: WB (0:10 -10:0 w/w), inoculum size (1-10% v/w), relative humidity (50-70% v/w), temperature (28-37°C), pH (4.5-7.0) and nutritional supplements (CSL/CDB 0.5-5.0% w/w) were studied to determine the optimum parameters for high yield of tannase and GA by the classical 'one-at-a-time' optimization method [18]. Also, the effect of addition of various nitrogen sources (urea, ammonium nitrate and ammonium dihydrogen orthophosphate, 3% w/w), carbon sources (glucose, sucrose and starch, 3% w/w) and inducers (TA, 3% w/w) to the substrate was studied for simultaneous tannase and GA production.

Extraction and Estimation of Tannase and GA: One gram of the fermented substrate was homogenized with 10 ml of 0.02M (pH 5.5) acetate buffer and the supernatant was passed through a double layered muslin cloth followed by centrifugation (Biofuge, Thermo Scientific, USA) at 8,000 rpm for 10 min. The supernatant obtained was used for estimation of GA and enzyme. The supernatant was further ultrafiltered (Millipore, USA) using a 30 kDa

cut-off membrane for partial separation and concentration of enzyme (retentate) and GA (permeate). Assay was carried out by the spectrophotometric (JASCO V-630, UK) method as described by Lekha *et al*. [12] with slight modification. To state briefly 3 ml of substrate solution (0.006% w/v of TA in 0.02M acetate buffer, pH 5.5), pre-incubated at 40°C was mixed with 100 µl of enzyme sample and the decrease in absorbance was recorded for five minutes at 310 nm. One unit of tannase activity was defined as the decrease in absorbance of TA by 0.01 due to hydrolysis of ester bonds. GA was estimated by the method of Bajpai and Patil [9], where absorbance of GA was recorded at two wavelengths (254.6 and 293.8 nm), simultaneously and expressed as:

$$GA\ (mg/ml) = 21.77\ (A_{254.6}) - 17.17\ (A_{293.8})$$

The resulting values were converted to units per gram dry substrate (U/gds) and milligram per gram of dry substrates (mg/gds) for tannase and GA respectively, after estimating the moisture correction.

HPLC and FTIR Analysis of GA: Extraction of GA was carried out according to the method described by Bajpai and Patil, [9]. For recovery of GA, the pH of permeate was adjusted to 2.0 with HCl and cooled to 0°C. GA shows instability at low pH and precipitates out from the broth at 0°C. The precipitate obtained was filtered under vacuum, then dissolved in a minimum amount of acidified hot water and re-crystallized at 4°C. The characterization of GA was carried out by HPLC (JASCO PU-2080, UK) and FT-IR (Nicoleit, Madison-20 DXB, WIS, USA) analysis in comparison with the standard GA. HPLC was performed with a gradient of solvent A (water/trifluroacetic acid, 98:1 v/v) and solvent B (water/acetonitrile, 80:20 v/v), applied to a reversed-phase Nova-pack C_{18} cartridge (25 cm x 4.0 mm i.d. and 4.6 ìm particle size) with a flow rate of 1.0 ml/min. Further for FT-IR, standard and sample GA (vacuum dried) was pelleted after proper mixing with potassium bromide and analysis was carried out by using the Attenuated Total Reflection (ATR) technique. An infrared spectrum of GA was recorded from 4000 to 400 cm^{-1} and different peaks were analysed.

Tannase Catalyzed Esterification of GA to PG: Produced GA and partially purified tannase (free and sodium alginate microencapsulated) was used for esterification studies to synthesize PG, in presence of 1-propanol as proton donor. The substrate (2.0 ml of 0.2M GA dissolved in 1-propanol) was added to the 10 ml of reaction mixture, composed of 2 ml acetate buffer (0.02M, pH 5.5) and 8 ml hexane. One gram of microencapsulated and equivalent unit of free tannase (300 U) was added to the above reaction mixture separately in each flask and incubated at 40°C in an orbital shaker (Scigenics, India) at 180 rpm. Samples (200µl) were withdrawn from the system at every 2h, vacuum evaporated (Buchi, Switzerland) and dissolved in methanol. Synthesized PG was estimated by the HPLC method described by Xiaowei *et al.* [19] from a calibration curve plotted (Y = 0.03144X - 0.00194, R = 0.9999) as PG concentration (X, g/ml) versus the ratio (Y) between PG peak area and internal standard (ethyl p-hydroxybenzoate) substance peak area. As for synthesis of PG, the values were determined by the disappearance of GA in reaction mixture and expressed in terms of percent molar conversion of GA to PG. Further, PG was characterized by HPLC and FT-IR analysis by the method explained in section 2.7 in order to check the esterification process.

RESULTS AND DISCUSSIONS

Evaluation of Substrates: Selection of a substrate for production of enzymes and other microbial products depends on several factors like cost, availability and suitability of the substrate for obtaining the desired product and thus requires screening of several agro-industrial residues [13]. Accordingly, substrate screening was carried out to evaluate tannin containing materials for simultaneous production of tannase and GA. Tannin content in various substrates viz., BFP, MFP, WP and QP was found to be 25, 32, 45 and 42% (w/w), respectively. BFP was found to be the best substrate for tannase and GA production (528 U/gds, 35 mg/gds 1060 U/gds, 68 mg/gds) followed by MFP (420 U/gds, 30 mg/gds) WP (162 U/gds, 5 mg/gds) and QP (184 U/gds, 6 mg/gds). Addition of WB to the substrates resulted in nearly two fold increase in production of tannase as well as GA (Table 1). Although, MFP, WP and QP contain higher amounts of tannins but, they yielded less tannase production. This may be due to the higher ratio of condensed tannins [20] which are not easily hydrolysable. In a similar study myrobalan and pod cover powder were used as substrates for tannase and GA production, but tannase yield was comparatively low [10]. Kumar *et al*. [15] have reported the use of some tannin containing plant leaves as solid substrate for the production of tannase by *A. ruber* and concluded jamun leaves (*Syzygium cumini*) and amla (*Emblica officinalis*) leaves as good substrates for tannase production, but yield was

Table 1: Evaluation of various tannin containing substrates for tannase and GA from *A. heteromorphus.* Results are average of triplicate experiments±standard error

	Production of tannase (U/gds) and GA (mg/gds)									
	36 h		60 h		84 h		108 h		120 h	
Substrates	U/gds	mg/gds	U/gds	mg/gds	U/gds	mg/gds	U/gds	mg/gds	U/gds	mg/gds
BFP	218±12	25±1	248±12	25±1	528±12	35±2	410±12	32±1	248±12	30±1
MFP	182±6	20±1	212±6	22±2	420±10	30±2	322±8	28±2	212±6	25±1
QP	42±4	0±0	62±4	2.0±0	184±6	6±0.1	144±6	5±0.1	82±4	2±0
WP	36±4	0±0	54±4	2.0±0	162±6	5±0.1	116±4	5±0.1	64±4	0±0
BFP:WB										
2: 8	226±4	26±0.1	582±6	48±0.4	880±6	60±0.6	812±8	58±0.3	578±6	50±0.4
3: 7	348±8	28±0.2	622±12	50±0.5	1060±12	65±0.8	920±10	63±0.4	612±12	52±0.5
4: 6	382±6	30±0.2	630±9	52±0.6	1020±6	68±0.8	910±10	63±0.4	610±9	50±0.6
5: 5	344±8	28±0.6	548±8	46±0.6	846±8	54±0.6	650±8	52±0.6	442±8	46±0.6
MFP:WB										
2: 8	136±2	16±0.2	348±8	30±0.4	620±8	40±0.5	580±10	44±0.2	334±8	28±0.4
3: 7	250±3	24±0.2	414±7	36±0.4	724±10	47±0.6	692±12	46±0.3	416±7	30±0.4
4: 6	288±5	26±0.2	462±7	38±0.4	780±10	50±0.6	588±11	44±0.4	456±8	38±0.4
5: 5	266±4	25±0.5	342±4	34±0.5	456±6	44±0.5	434±6	42±0.5	402±6	34±0.5
QP: WB										
2: 8	96±2	4±0.1	220±6	8±0.2	226±4	8±0.2	212±6	6±0.2	116±2	2±0.1
3: 7	112±4	4±0.1	258±8	8±0.2	340±4	13±0.4	248±6	8±0.2	180±8	5±0.1
4: 6	140±4	6±0.1	290±6	10±0.3	380±8	14±0.4	282±8	8±0.3	272±6	7±0.2
5: 5	144±6	6±0.2	274±8	9±0.3	352±8	10±0.3	272±6	7±0.2	258±8	5±0.2
WP: WB										
2: 8	110±4	0±0	292±7	2±0.1	342±4	3±0.1	322±7	2±0.1	180±6	0±0
3: 7	158±4	2±0	382±8	4±0.2	398±8	5±0.2	390±8	4±0.2	222±8	2±0
4: 6	192±5	2±0	380±6	3±0.1	388±6	5±0.2	380±6	3±0.2	120±5	2±0
5: 5	178±5	2±0.1	224±5	2±0.1	256±5	3±0.1	244±6	2±0.1	112±5	0±0

very low (69 U/gds). In another report also jamun leaves were established as the best substrate for tannase production (141.34 U/gds) followed by jamoa (*Schleichera trijuga*) (128 U/gds) and keekar leaves (*Capparis decidua*) (108 U/gds) [2]. Solid state culture of a tannin-rich desert plant gobernadora (*Larrea tridentata*) results in comparatively higher accumulation of GA (0.33 g/L) and tannase (1043 U/L) in comparison to other reports [8]. Moreover, in the view of easy availability and productivity, BFP dominates over all other substrates and produced a higher amount of tannase and GA in comparison to other agro-industrial substrates reported till date.

Parametric Optimization: Tannase synthesis and GA production are directly proportional i.e. maximum synthesis of tannase results in higher yield of GA [6]. Therefore, for maximum yield of tannase physicochemical parameters were optimized which could also be correlated for GA production.

Effect of Time of Incubation: This is one of the most essential parameters for fermentation and enzyme production from a commercial point of view to assess the economic feasibility of the process. Tannase production was studied in relation to the time of incubation and was found to be maximum (1224 U/gds) at 72 h of incubation at 30°C with 65% (v/w) initial moisture content and remained nearly constant till 96 h followed by a significant decline at 108 h (Fig. 1a), may be due to accumulation of end product and toxic metabolites secreted during fermentation. This is similar to the report of Sabu *et al*. [14], where 72 h reported as optimum when SSF was carried out using coffee husk with *Lactobacillus* sp. ASR-S1. In another report of Sabu *et al*. [21], comparatively higher incubation time of 96 h reported as optimum when SSF was carried out using PKC (13.03 U/gds) and TSP (6.44 U/gds) as substrates. Tannase production by *A. fumigatus* reached the maximum (129.9 U/gds) in the case of SSF with jamun leaves after 96 h of incubation [2]. Chatterjee *et al*. [22] have also reported

higher incubation period of 120 h as optimum for tannase production by *R. oryzae* using WB and TA as substrate, but in the case of Raaman *et al*. [3] relatively short incubation period of 60 h was reported for SSF in comparison to SmF (72 h) and LSF (96 h). The optimum incubation period was found to be 60 h in the case of *R. oryzae* but *A. foetidus* took 72 h for maximum enzyme and GA yield and however gave higher values than that of *R. oryzae* [23]. Even a shorter incubation period of 43 h was reported optimum for GA and tannase production by fungal SSF of a tannin-rich desert plant gobernadora [8] and 48 h reported as optimum under modified solid state fermentation (MSSF) by co-culture of *Rhizopus oryzae* and *Aspergillus foetidus* [10].

Substrates Concentration (BFP:WB) and Cumulative Effect of Substrates (BFP:MFB:WB): Tannase production is not a constitutive process; it is inducible in nature and induced by various substrates containing tannins [1]. BFP is a good source of hydrolysable tannins so different concentrations of BFP in terms of WB ratio were studied and 3:7 was found optimum for maximum production of tannase and GA (Fig. 1b). Further increase in BFP concentration resulted in reduced productivity, which may be due to higher accumulation of substrate and also intermediate hydrolysate of TA such as 1,2,3,4,6 pentagalloyl glucose, 2,3,4,6 tetragalloyl glucose and monogalloyl glucose produced during hydrolysis could be binding to the enzyme site [6]. However, no significant effect of different concentration of combined substrates (BFP:MFP:WB) was observed (Fig. 1c) in contrast to BFP and WB. That may be again because of accumulation of higher substrate concentration and its inhibitory effect on tannase and GA production. But in the case of Mukherjee and Banerjee [23], the effect of substrate ratios (*Terminalia chebula* and *Caesalpinia digyna*) on tannase and GA production using mixed substrates was studied and a ratio of 3:7 was reported as optimum.

Effect of Inoculum Concentration: Tannase being an adaptive enzyme, optimum concentration of induced inoculum is required for maximum tannase production [1]. In the case of present study less (< 4% v/w) amount of inoculum was insufficient for complete utilization of the total available substrate. The optimum concentration of induced inoculum (2.32 x 10^6 CFU/ml) required for the process was 4% (v/w) (Fig. 2a) and further increase till 7% (v/w), induces faster sporulation but not reflect any significant improvement in overall productivity.

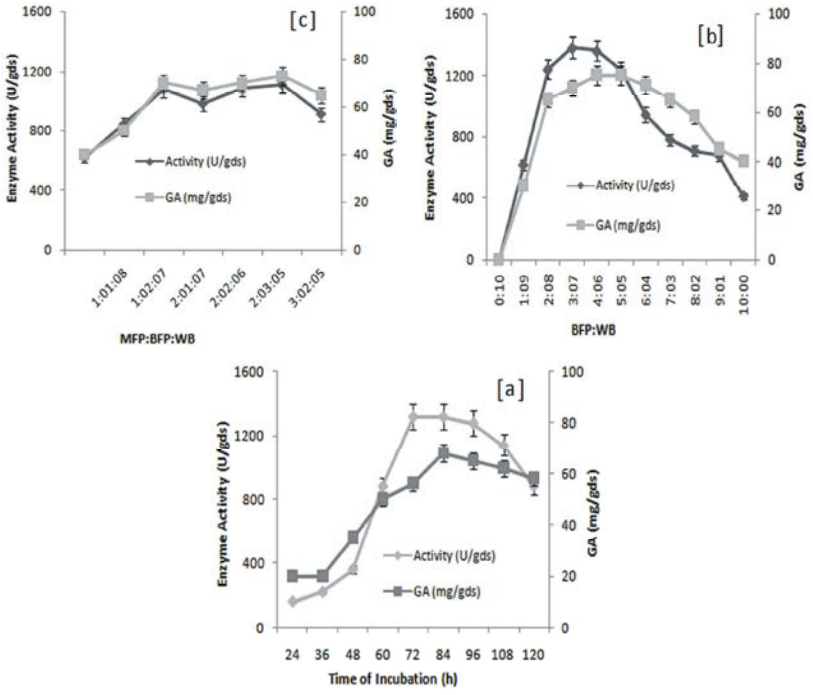

Fig. 1: Effect of incubation time [a], BFP:WB ratio [b] and combined effect of different substrates [c] on production of tannase and GA from BFP using A. heteromorphus. Error bars represent the standard error determined in the triplicate experiments

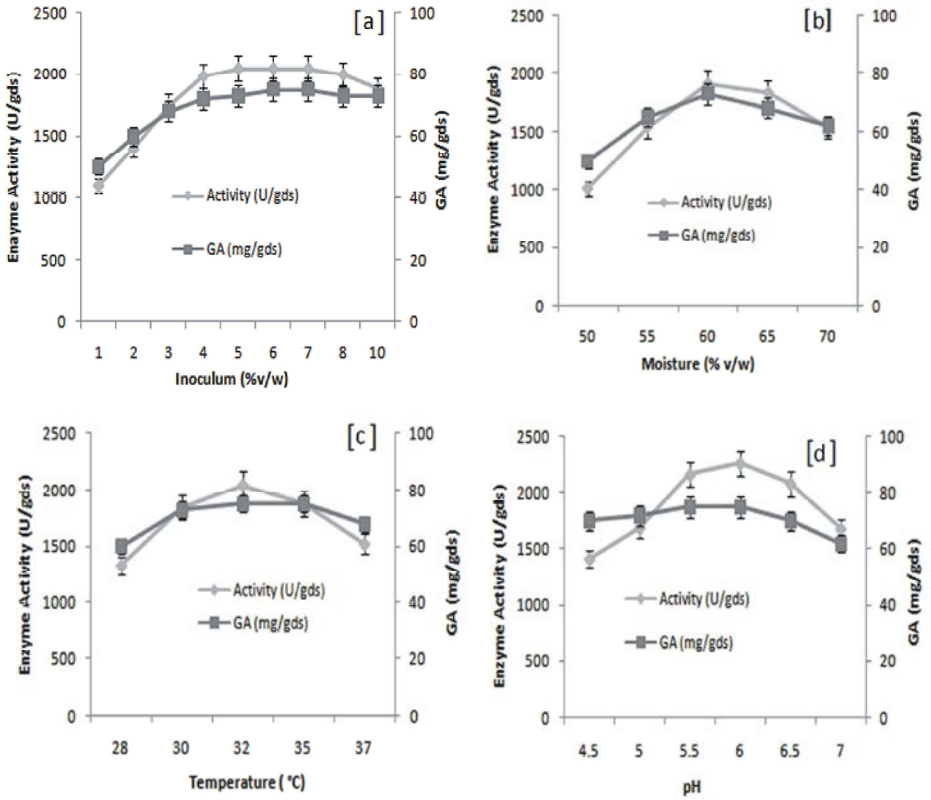

Fig. 2: Effect of inoculum concentration [a], moisture level [b], incubation temperature [c] and pH [d] on production of tannase and GA from BFP by A. heteromorphus

Effect of Moisture: Moisture level is a very important parameter in SSF because microorganisms have unique water activities [24]. Therefore, conditions should be such, that the substrate should not be too dry or too wet but provide just the amount of moisture equivalent to the organism's optimal water activity that allows the entry of nutrients easily through the cell wall, which favours biomass and enzyme production. Here out of a range (50, 55, 60, 65 and 70% v/w) of moisture 60% (v/w) was found to be the best for tannase production (Fig. 2b) further increase in humidity resulted in decreased production, which may be due to osmotic imbalance inside the cell resulting in cell lysis. Initial moisture content of the solid substrate is an important factor which dictates the growth of the organism and enzyme production and in the case of fungi a wider moisture range (20-70%) supports better growth and metabolic activities [24]. So in SSF, a particular optimum moisture range should be maintained for tannase production [21]. Comparatively higher moisture level (80% v/w) was reported as optimum for tannase (36.4 U/ml) and GA (93.25%) production by co-culture method of *R. oryzae* and *A. foetidus* [10]. Similarly, Chatterjee *et al*. [22] have reported 72% (v/w) moisture level as the most favourable for SSF of tannase. A moisture level of 70% (v/w) was reported optimum for GA and tannase production by fungal SSF of gobernadora [8]. In contrast, a lesser substrate to moisture ratio of 1:1 (50% v/w) was reported as the best for SSF of tannase from jamun leaves [2]. Microbes generally grow near the outer surface of the substrate particle and water uptake in new biomass and evaporation are thus localized at the surface of the substrate particles [10]. This causes the development of intra-particle solute and moisture gradients during fermentation, which disturb the physiology of the microorganisms and thus, overall process productivity.

Effect of Temperature: Microorganisms have unique temperature optima for proper growth and in turn enzyme production because fermentation temperature affects sporulation, germination, microbial cellular growth and thus product formation. In this study, tannase and GA yield increased initially with increase in temperature up to 32°C (Fig. 2c); further increase in temperature to 37°C hampers the mycelial growth that resulted in poor productivity. In a similar report, maximum tannase activity

was reported at 32°C when pod cover (*Caesalpinia digyna*) was used as substrate for SSF with *R. oryzae* [6], but 30 °C was reported as optimum for production of tannase (40 U/ml) and GA (93%), using mixed substrate of *Terminalia chebula* and *Caesalpinia digyna* by co-culture with *R. oryzae* and *A. foetidus* [10]. A temperature of 30°C was found optimum for GA and tannase production during fungal SSF of a tannin-rich desert plant gobernadora [8]. Comparatively higher temperature optimum of 35°C was reported for extracellular tannase from *Paecilomyces variotii* [3]. In fact it is favourable to have higher temperature optima, as the requirement for heat removal, normally very difficult in SSF, can be minimized [24]. In few reports relatively low temperature optima such as 25°C for *A. fumigatus* [2] and 28°C for *A. niger* [12], were also recorded. But it was noticed that an optimum temperature of around 30°C has been reported favourable by many workers [7, 21, 25].

Effect of pH: SSF was carried out using different pH buffers (4.5 to 7.0) as moisturizing agent but maximum production was observed at pH 6.0 (Fig. 2d**)**. Further increase in pH results in decreased production because tannases are acidic proteins in nature with an optimum pH around 5.5 [1]. In a similar report, optimum pH of 6.0 was recorded for tannase production with *P. variotii* [3]. Sabu *et al*. [21] reported optimum pH of 5.5 for tannase production by *A. niger* ATCC 16620 but in the case of Banerjee *et al*. [10] and Chatterjee *et al*. [22] optimum pH was reported at 5.0. The maximum production of tannase (174.32 U/gds) was observed at a pH 5.0 when SSF of jamun leaves was carried out with and *A. fumigatus* [2]. Kar *et al*. [25] have reported comparatively lower pH optima of 4.5 for maximum tannase production by *R. oryzae* under SSF and MSSF whereas it was 5.0 in case of *A. foetidus* for biosynthesis of tannase and gallic acid by MSSF [23]. But, in contrast, change in pH did not show any important influence on the tannase and gallic acid production during fungal SSF of a tannin-rich desert plant gobernadora [8].

Nutritional Supplement: CSL is a by-product of corn industry, consisting of water-soluble extracts of corn steeped in water, composed entirely of natural amino acids, minerals, vitamins, reducing sugars, organic acids and other elemental nutrients which are excellent source of nutrients for microorganisms [26]. Supplementation of medium with CSL and CDB enhances the growth of *A. heteromorphus* that resulted in higher production of tannase as well as GA. Hence, different concentrations of

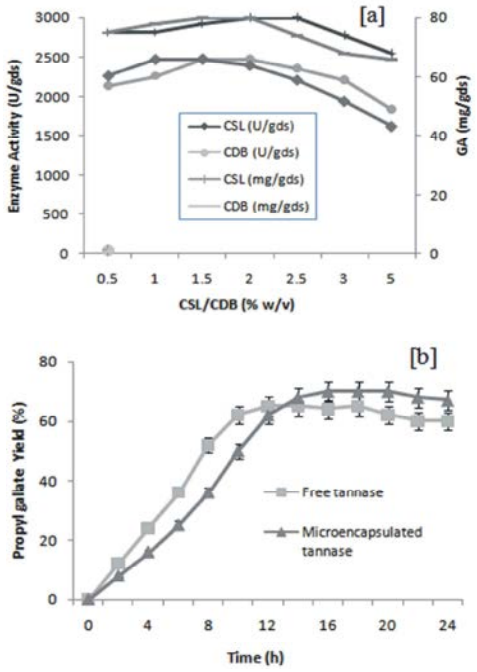

Fig. 3: Effect of nutritional supplements (CSL/CDB) [a] on production of tannase and GA from BFP by *A. heteromorphus*. Time course of tannase (free and microencapsulated) catalyzed synthesis of PG from GA [b]

CSL and CDB were tried from which 1.5% (w/w) of CSL and 1% (w/w) CDB was found to be the best as individual nutritional supplement (Fig 3a). Additional supplementation of carbon and nitrogen sources and inducer did not show any positive effect on production of tannase and GA (data not shown). In contrast maximum tannase production was reported when medium was supplemented with 1.0% glucose and sodium nitrate as carbon and nitrogen source, respectively [3]. Influence of two minimal media containing nitrogen source on tannase and GA production from gobernadora powder was evaluated in SSF and it was found that medium containing higher amount of nitrogen resulted in higher accumulation of GA [8]. Supplementation of 0.6% (w/w) of TA was found useful for synthesis of extracellular tannase under SSF using TSP, WB, PKC and coffee husk [14].

Esterification of GA to PG: The time course study of the esterification of GA to PG for free and microencapsulated tannase indicates the product synthesis in both cases but, the reaction equilibrium reached more quickly for free enzyme (12 h) than the immobilized enzyme (16 h) (Fig. 3b). However, the synthetic yield with microencapsulated tannase (72%) was nearly 12% higher

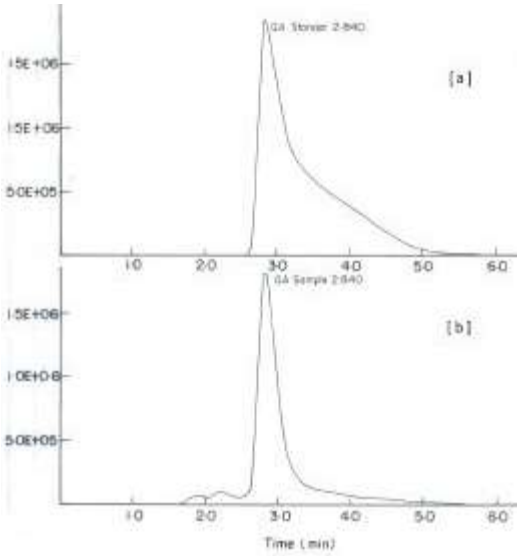

Fig. 4: HPLC analysis of purified sample GA [a] with standard GA [b]

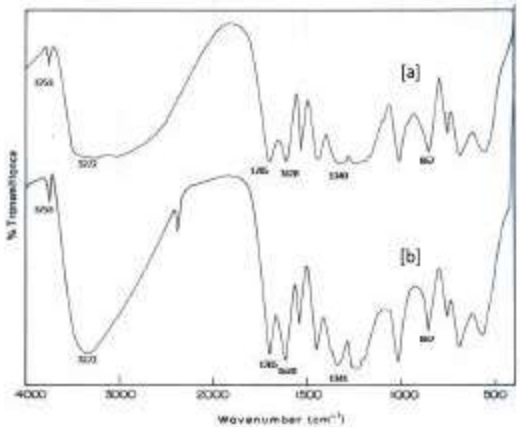

Fig. 5: FT-IR analysis of purified sample GA [a] with standard GA [b]

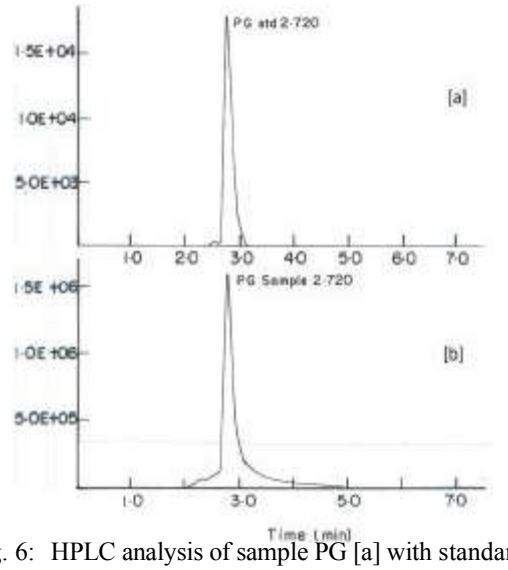

Fig. 6: HPLC analysis of sample PG [a] with standard PG [b]

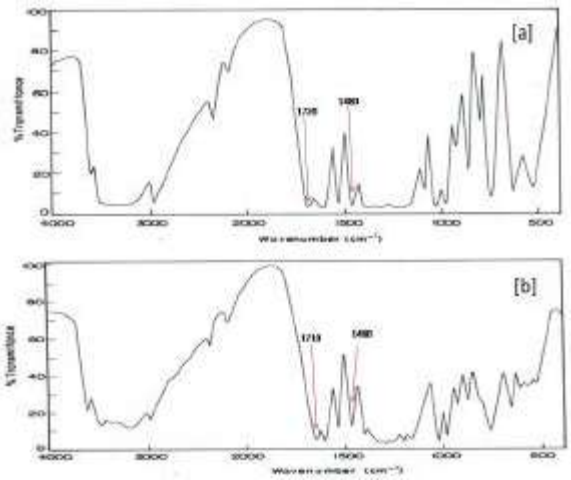

Fig. 7: FT-IR analysis of sample PG [a] with standard PG [b]

than that with free enzyme (65%). Xiaowei *et al*. [19] have also reported the higher synthesis of GA esters by immobilized tannase in comparison to free enzyme but, the yield (44.3%) was comparatively less in comparison to the yield (75%) obtained in the present study. Tannase microencapsulated within sodium-alginate complex coacervate membrane, dissolved in the aqueous solution of vesicles that results in enhance mass transfer rates of substrates and products and also prevent direct enzyme contact with the solvent, therefore avoiding solvent toxicity. This may be one of the motives for higher esterification by immobilized tannase and the low yield with free enzyme may be the result of inactivation by contact with the phase interface in biphasic system.

HPLC and FT-IR analysis of GA and PG: Purified GA was analyzed by HPLC (JASCO model, UK) and results confirmed the sample as GA since the retention time of both the sample (Fig. 4a) and standard (Fig. 4b) was found to be 2.84 min, also single peak of the GA reflects the purity of sample. Further the FT-IR analysis of the purified GA was carried out for characterization using the spectrum of standard GA. The FT-IR spectrum of the sample GA was obtained and the effective peaks were compared with that of the standard GA that contains main characteristic peaks in the range of 3753 to 574 cm^{-1} (Fig. 5a, b). The spectrum of the sample GA also contains nearly equivalent peaks. The absorbance nearing 3753.27, 3272.12, 1705.86, 1620.15, 1340.03 and 867.10 cm^{-1}

represents specific groups found in GA, respectively O-H, Aromatic C-H, C=O, C=C, -C-O and aromatic C-H bend [27].

The HPLC analysis of the synthesized PG (Fig. 6a) showed the presence of a single peak emerging at the same time (2.72 min) as the standard propyl gallate (Fig. 6b), used as reference. The chemical structure of PG was confirmed by FT-IR by analyzing the synthesized PG (Fig. 7a) with respect to the standard (Fig. 7b). Apart from the other respective peaks, typical stretches at 1720cm^{l-} and 1480cm^{-1} corresponds to the carbonyl ester and the aromatic groups [28], respectively in both, sample and standard PG. This result confirms the potential of produced tannase and GA for synthesis of PG, a potent antioxidant used in food industry.

CONCLUSIONS

This is the first attempt on tannase and GA production by using BFP. The yield achieved under optimized condition is quite high in comparison to the other reports so far using agro-industrial residues by SSF and also process is economical. Tannase catalyzed esterification of GA in 1-propanol results in synthesis of PG, a potent antioxidant used in food and cosmetic industries. Thorough work on esterification of GA needs to be carried out to produce newer compound of interest. Work on utilization of BFP for SmF and its induction mechanism on tannase production are in progress.

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