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Implementation of Different Fermentation Techniques For Induction of Tannase and Gallic Acid Using Agro-residues Substrates

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TANNASE is an inducible enzyme which hydrolysate tannin to gallic acid and used to in many industries such as food and pharmaceutical. This enzyme has scooped more attention in recent times. A maximum production of tannase (ranged from 0.30 to 0.93 enzyme index and from1.05 to 1.87U/mg specific tannase activity (STA)) and gallic acid (from 0.07 to 0.76mg/ml gallic acid concentration (GAC)) was achieved by 8 fungal strains out of 24 fungal and yeast strains belonged to genera; *Aspergillus*, *Rhizopus*, *Trichoderma*, *Fusarium, Penicillium, Candida* and *Saccharomyces*. The selected fungi were grown on tannin-rich substrates (eucalyptus leaves, pomegranate peel, banana peel, guava leaves and wheat bran) and by-products (corn steep liquor (CSL) and soybean extract) as sole carbon and nitrogen sources for tannase and GA production at 28°C for 6 days under liquid-surface (LSF), submerged (SmF) and solid-state (SSF) fermentation. Results indicated that *A. niger* A8 and *T. viride* with 10% (v/v) of inoculum size gave a high STA (from 8.08 to 10.95U/mg) and GAC (from 2.62 to 4.00mg/ml) on pomegranate and banana peels supplemented with CSL after 4 days incubation at 28°C under SSF compared to SmF and LSF. STA (ranged from 10.68 to 12.93U/mg) and GAC (ranged from 3.56 to 4.16mg/ml) were increased when inoculated the medium with both *A. niger* A8+*T. viride* (with 5:5% v/v of inoculum size) more than when inoculated with each of them separately.

Keywords: Agricultural wastes; *Aspergillus* sp., Biosynthesis, Gallic acid production, Tannase production, *Trichoderma* sp.

Introduction

Tannase (EC 3.1.1.20) is an inducible enzyme that hydrolyzes depside bonds (two or more monocyclic aromatic units linked by an ester bond) of tannins like tannic acid and release gallic acid and glucose (Beena et al., 2011). Microorganisms play a vital role to produce tannase. They contain the bacteria: *Bacillus sp*. PAB2, *B. licheniformis* KBR6 and *Klebsiella pneumoniae* KP715242 (Jana et al., 2012 and Kumar et al., 2015); yeast: *Kluyveromyces marxianus* NRRL Y-8281 and *Saccharomyces cerevisiae* CCMB 520 (Fathy et al., 2017 and Morgana de Melo Lopes et al., 2017) and fungi: *Aspergillus niger, A. japonicus, A. tubingensis, A. carbonarius*, *A. tamarii, A. ochraceus, A. foetidus* (da Costa et al., 2013; Valera et al., 2015 and Nandi & Chatterjee, 2016), *Penicillium funiculosum*, *P. oxalicum*, *P. corylophilum*, *P. citrinum* and *P. montanense* (da Costa et al., 2013 and Lima et al., 2014). Both genera of *Aspergillus* and *Penicillium* are commonly used for tannase production than other microorganisms (Belur & Mugeraya, 2011) The production process involving different methods containing liquid-surface (LSF), submerged (SmF), and solid-state fermentation (SSF) (Yadav et al., 2008). Bacteria and yeast prefer SmF process in shaker flasks than SSF and LSF as they need a large

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amounts of water is required to dissolve the nutrient contents for microbial growth (Jana et al., 2014). Meanwhile, filamentous fungi are suitable for the SSF process compared to other fermentation processes (Lekha & Lonsane, 1994 and Aguilar et al., 2002). SSF can be used in case of nearly or completely absence or near absent of free liquid when using a solid substrate as raw materials. The advantages of this method are the use low cost raw materials, little energy consumption and small bioreactor dimensions because of the concentrated media (Couto & Toca-Herrera, 2007 and Wu et al., 2018).

The total amount of farm wastes of plant or animal origin in Egypt were ranging between 30 and 35 million tons/year, of which 21 million tons of these agricultural wastes accumulate in the environment and lead to environmental pollution (Hassan et al., 2014). Microorganisms can be utilized using SSF for conversion of` plant materials and agro-industrial wastes that contain tannin-rich substrates, while using SSF to produce economic products such as tannase and gallic acid production, while overcome their accumulation problems in the environment (Paranthaman et al., 2009). Tannin-rich substrates contain coffee wastes, grape, pomegranate peels, pine bark, black cumin seeds, oak, eucalyptus, cinnamon bark, wheat bran, cajá" (*Spondias lutea* L.) and manga (*Mangifera indica* L.) residues (Paranthaman et al., 2009; Muslim et al., 2015 and Cruz et al., 2017).

Tannase has received a great deal of attention from the discovery, and it is widely used in food, animal feed, pharmaceutical, beverage, brewing, tannery and chemical industry (Govindarajan et al., 2016). As well as gallic acid, the major hydrolytic product of tannic acid is used in food, cosmetics and adhesive in the synthesis of a potent antioxidant and propylgallate (Aithal & Belur, 2013) .

This study was set out to convert tannin-rich substrates to tannase and gallic acid using different fermentation strategies by single and mixed fungal cultures.

Materials and Methods

Substrates

Commercial five tannin-rich raw materials powders of Eucalyptus leaves, pomegranate peel, banana peel, guava leaves and wheat bran were collected from open markets in Cairo, Egypt. The total tannin content of each of the materials was determined. These raw materials were used as carbon sources and were added equivalent to the original tannin percentage in the basal medium.

Corn steep liquor (CSL) and soybean protein (SBP) were obtained from a starch and glucose factory, Mostorod, Cairo and Food Technology Research Institute (FTRI), Agricultural Research Center, Giza, Egypt, respectively. These by-products were found containing 0.94 and 1.3% of total sugars and 4.64 and 8.00% of total nitrogen, respectively (Abou-Taleb et al., 2012). They were used as nitrogen sources and/or medium.

Fungal strains and cultural maintenance

Twenty-four fungal strains were used to investigate tannase production. Among them, 21 fungal strains were found belonging to the genus *Aspergillus* (*Aspergillus* sp. ASP 1, ASP2, ASP 4, ASP 21, ASP 11, ASP3m, *A. flavus*, *A. niger* A8, *A. terrus* and *A. nidulans*), *Rhizopus* (*Rhizopus* sp. R6, R10, *R. stolonifera* and *R. nigricans*), *Trichoderma* (*Trichoderma* sp. N13, T3 and *T. viride*), *Fusarium* (*Fusarium* sp. FUS1 and *F. oxysporum*) and *Penicillium* (*Penicillium* sp. P1 and P5) and the 3 yeast strains: *Candida* sp. C1 and M2 and *Saccharomyces cerevisiae*. Theses strains were obtained from the Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The fungal and yeast strains were cultured on slants of potato dextrose agar (Difco Manual, 1998) and Sabouraud dextrose agar slants (Bacteriological Analytical Manual (BAM), 1998) for 24-48hr and then kept at 4°C for further use, respectively.

Media used for tannase production

Medium (1): Modified Czapek-Dox agar medium was used for a qualitative assay of tannase using a plate assay technique (Muslim et al., 2015). The medium composited of (g/L): 10, tannic acid; 2, $NaNO_3$; 1, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 0.01, $FeSO_4.7H_2O$; 0.5, KCl and 20, agar. The pH of the medium was adjusted to 5.7. The tannic acid was sterilized separately using 0.2ml pore-size Millipore sterile filter and added to the medium under aseptic conditions according to Bradoo et al. (1996).

Medium (2): Is also the modified Czapek-Dox broth medium but without agar. It was used for quantitative tannase determination and for fungal cultivation under LSF and SmF.

Medium (3): Was used for SSF. Its consisted of tannin-rich raw material (10g) and mineral salt Czapek-Dox (10ml). The medium was adjusted pH to 5.7 and the moisture content of 50%.

Screening for tannase producing fungi and yeast on solid and in broth media

The microbial strains were tested for tannase production by using the plate assay technique. The fungi and yeast were cultivated on plates containing medium (1) and then incubated at 28°C for 48-72hr. Diameter (mm) of the clear zone was appeared around the fungal colony was taken as an enzymatic index using formula and the following equation described by Ferbiyanto et al. (2015):

Enzymatic Index (EI)= (Diameter of the clear zone- diameter of the microbial colony)/diameter of the colony.

Fermentation techniques

Submerged fermentation (SmF)

Submerged fermentation was performed in plugged Erlenmeyer flasks (250ml), containing 100 ml of medium (2) inoculated with 10ml (v/v) of spore suspensions containing with $2.5x10^7$ fungal spores/ml. The flasks were incubated for 6 days at 28°C with agitation at 150rpm using a rotary shaker. The culture was filtrated through Whatman no. 1 filter paper. The produced pellicles were dried and their weights were determined. The filtrate that contain the crude enzyme was used for enzyme activity and protein assessments. The specific enzyme activity (U/mg) was calculated using the Foustoukos (2014) equation:

Specific tannase activity (STA)= Enzyme activity (U)/protein content (mg/ml).

Liquid-surface fermentation (LSF)

The cultivation technique and the determinations were the same as for the SmF one but performed under static conditions (no agitation).

Solid state fermentation (SSF)

It was carried out in 500 ml plugged Erlenmeyer flasks containing the medium (3) inoculated with 10ml (v/v) of spore suspensions (with $2.5x10^7$ spore of each fungal strain/ ml). The flasks were incubated at 28°C under static conditions for 6 days. At the end of fermentation course, the mat was used for biomass determination and the enzyme was extracted by addition 50ml of acetate buffer (0.05M, pH 5) and then agitated in a rotary shaker for 1hr at 200rpm (Pinto et al., 2001). The solution was filtered through a Whatman no. 1 filter paper and assayed for enzyme activity and protein content. The produced mat was dried and their weights were determined.

Effect of incubation period

After inoculation of medium (3) with the selected strains and incubation at 28°C for different fermentation ranged from 0 to 6 days using SSF.

Effect of single and mixed cultures

Under SSF, the medium (3) in flasks was inoculated with a single culture [10% (v/v) inoculum size] of *A. niger* A8 or *T. viride* and co-cultures of both organisms with different inoculum sizes (v/v) of 3:5 , 5:3 , 5:5 , 5:10 and 10:5, respectively.

Analytical methods

Tannin content was determined according to the method of proposed by Folin-Denis described by Makkar et al. (1993). The mg tannin was assayed using a tannic acid standard.

Cell dry weight was determined by separation of the fungal mycelia from the fermentation medium using Whatman no. 1 filter paper and then washing 3 times with distilled water and drying at 80°C until constant weight.

Tannase activity was determined using a UV spectrophotometric method as described by Iibuchi et al. (1967). 0.5ml of crude enzyme (filtrate) was added to 2ml solution contained 0.35g tannic acid dissolved in 100ml of 0.05M citrate buffer (pH 5.5) and incubated in a water bath at 37°C. 0.2ml of the reacting compound was withdrawn at zero time (t1) and after 10min of incubation time (t2), then, the enzyme reaction was stopped by addition of 2ml ethanol (90%). The absorbance of the t1 and t2 were figured out at 310nm using a UV spectrophotometer (Chrom Tech CT-2200 UV/Vis). One unit (U) of tannase activity was considered the amount of enzyme needed to hydrolyze 1µmol of ester per 1min per ml. The enzyme activity was calculated according to the following formula:

Enzyme activity (U/ml)= 114 x [(At1- At2)/(t2-t1)].

where; A is the absorbance and t was the time in minutes.

Protein concentration was determined following the method of Bradford (1976) using standard of a bovine serum albumin.

Gallic acid concentration (GAC) was estimated according to the method of Bajpai & Patil (2008). The GA was measured in culture filtrate after dilution to 100-fold in acetate buffer (0.2M at pH 5.0) then recorded at 255nm and at 294nm using a UV spectrophotometer (Chrom Tech CT-2200 UV/Vis). The GAC (mg/ml) was calculated using specific extinction coefficient according to the following formula:

GAC (mg/ml)= 21.77 (A255) – 17.17 (A294).

Statistical analysis

The obtained data were analyzed using IBM® SPSS® Statistics Server Version 23.0. (2015) as suggested by Duncan (1955) at the 5% confidence level.

Results and Discussion

Screening of tannase and gallic acid producing fungal strains

Results in Table 1 show that the tested strains gave clear zone diameters ranging from 5 to 27mm with an enzymatic index ranging from 0.04 to 0.93. The higher significant diameter of clear zone ($P \leq 0.05$) was recorded for the *Aspergillus niger* A8 (27mm) and followed by the *Trichoderma viride* (23mm), *Aspergillus nidulans* (20mm), *Rhizopus stolonifera* (19mm), *Aspergillus flavus* (16mm), *Fusarium oxysporum* (13mm) and *Penicillium* sp. P5 (13mm), respectively, while the highest value of the enzymatic index was also recorded for the *A. niger* A8 (0.93).

TABLE 1. Tannase activity index determined in solid medium and production of tannase and gallic acid in broth medium for the tested fungal strains.

Fungal strains	CZD (mm)	EI	CDW (g/L)	STA (U/mg protein)	GAC (mg/ml)
Aspergillus flavus	16^{d}	0.47	1.40^{e}	1.08^{f}	0.08^{d}
Aspergillus niger A8	27^{a}	0.93	1.95^{a}	1.87^{a}	0.76^{a}
Aspergillus nidulans	20^{c}	0.54	1.69^{c}	1.45^{c}	0.54^{c}
Aspergillus terrus	14^{e}	0.40	1.38^{e}	1.25^{d}	0.14^{c}
Aspergillus sp. ASP 1	5^{h}	0.28	0.74^{j}	0.56^{j}	0.005^{g}
Aspergillus sp. ASP 2	5^{h}	0.16	0.77^{j}	0.67^{i}	0.006^{g}
Aspergillus sp. ASP3m	7^{fg}	0.08	0.82^{j}	0.63^{j}	0.005^{g}
Aspergillus sp. ASP 4	9^{f}	0.20	1.13^{g}	0.95^{g}	0.009^{f}
Aspergillus sp. ASP 11	10^{f}	0.25	1.04^{h}	0.87^{hi}	0.007^{f}
Aspergillus sp. ASP 21	8^{fg}	0.18	1.03^{h}	0.94^{g}	0.13^{c}
Fusarium oxysporum	13^{e}	0.30	1.38^{e}	1.60^{d}	0.65^{b}
Fusarium sp. FUS1	5^{h}	0.04	0.68^{j}	0.42^{k}	0.004^{g}
Penicillium sp. P1	9^{f}	0.17	1.14^{g}	0.90^{g}	0.05^{e}
Penicillium sp. P5	13^{e}	0.37	1.26^{f}	1.05^{f}	0.07^{d}
Rhizopus nigricans	10^{f}	0.11	1.04^{h}	0.85^{hi}	0.05^{e}
Rhizopus stolonifera	19^{c}	0.52	1.56^{d}	1.16^{e}	0.12^{c}
Rhizopus sp. R6	12^{f}	0.09	1.18^{g}	0.92^{g}	0.07^{d}
Rhizopus sp. R10	9^{f}	0.13	1.05^{h}	0.93^{g}	0.06^{e}
Trichoderma viride	23^{b}	0.77	1.87^{b}	1.68^{b}	0.71^{a}
Trichoderma sp. T3	8^{fg}	0.27	0.89^{i}	0.61^{j}	0.005^{g}
Trichoderma sp. N13	11^{f}	0.17	1.02^{h}	0.94^{g}	0.06^{e}
Candida sp. C1	10^{f}	0.16	1.00^{h}	0.80^{i}	0.09^{d}
Candida sp. M2	9^{f}	0.22	0.93^{i}	0.77^{i}	0.008^{f}
Saccharomyces cerevisiae	6^{g}	0.05	0.78^{j}	0.45^{k}	0.004^{g}

- CZD= Clear zone diameter, EI= Enzymatic index, CDW= Cell dry weight, STA= Specific tannase activity, GAC= Gallic acid concentration.
- Means followed by different letters are significantly different at $P< 0.05$ level (Duncan, 1955).

Screening of tannase and gallic acid producing strains was performed in broth medium during submerged fermentation are presented in Table 1. The 24 strains gave specific enzyme activities and gallic acid concentrations ranging from 0.42 to 1.87U/mg protein and 0.004 to 0.76mg/ml, respectively with cell dry weight ranging from 0.68 to 1.95g/L, respectively.

Data presented in Table 1 also showed that among the 24 fungal strains, 8 strains belonging to *A. flavus*, *R. stolonifera*, *A. niger* A8, *A. terrus*, *A. nidulans*, *T. viride*, *F. oxysporum* and *Pencillium* sp. P5 produced the highest EI of 0.47, 0.52, 0.93,0.40, 0.54, 0.77, 0.30 and 0.37, STA of 1.08, 1.16, 1.87, 1.25, 1.45, 1.68, 1.60 and 1.05U/mg protein, and GAC of 0.08, 0.12, 0.76, 0.14, 0.54, 0.71, 0.65 and 0.07mg/ml which were highly significant at P≤ 0.05, respectively. The lowest production of tannase and gallic acid were recorded for the *Fusarium* sp. FUS1 and *S. cerevisiae*. According to the results of these primary screening, the above 8 fungal strains were selected for further studies.

However the data here came in accordance with those of Banerjee & Mahapatra (2012) who reported that fungi belonging to *Aspergillus* sp. and *Penicillium* sp. are good producers of tannase, whereas others belonging to yeast are low tannase producers (Banerjee & Pati, 2007).

Bioconversion of tannin substrates into tannase and gallic acid by selected fungi by various fermentation techniques

Five tannin-rich raw materials were used being eucalyptus leaves (15.2mg/g), pomegranate peel (40.4mg/g), banana peel (20.5mg/g), guava leaves (8.7mg/g), wheat bran (2.6mg/g). These substrates were tried as a sole carbon source and compared to tannic acid in respect of their tannase and gallic acid production by eight fungal species *A. flavus*, *R. stolonifera*, *A. niger* A8, *A. terrus*, *A. nidulans*, *T. viride*, *F. oxysporum* and *Pencillium* sp. P5 using solid state (SSF), liquid-surface (LSF) and submerged fermentation (SmF).

Results in Table 2 indicating that all the selected fungi were found capable of grow on pomegranate and banana peels, and their degradation using the three fermentation methods for tannase and gallic acid production had high efficiency than in case of degradation of other tannin-rich raw materials and tannic acid which was used as a positive control. Moreover, both the fungi *A. niger* A8 and *T. viride* can be preferred for degradation of pomegranate and banana peels as compared to the other tested fungal species. The two fungi recoded significant increases ranged from 1.9 to 2.9 fold and from 1.2 to 2.1 fold as compared to fermentation of the tannic acid, respectively. Muslim et al. (2015) also found that the pomegranate peels extract followed by pine bark were among the tested raw material (grape and black cumin seeds and oak, eucalyptus and cinnamon barks extract) giving the highest tannase yield by *Erwinia carotovora* Et3.

The results here indicating that the highest production of enzyme and gallic acid were recorded with using SSF technique followed by SmF one. This is most likely due to that the enzyme was wholly secreted extracellularly in SSF while it was partially intercellularly in the SmF. This confirms the results recorded by Lekha & Lonsane (1994) who found that *Aspergillus* sp. gave the highest tannase productivity in SSF because of tannases (exo and endo-enzymes) production compared to SmF (the exotannase was secreted only). Moreover, Cunha et al. (2012) and Ranganathan (2015) reported that SSF technique can improve the interaction between the fungal cells and the raw materials and also the lower foam formation which increase the biosynthesis of the enzyme. However, SmF lead to accumulation of some intermediate metabolites that can decrease the enzyme production, as stated by (Subramaniyam & Vimala, 2012). Anyway, the LSF technique showed the lowest values of biomass production, enzyme activity and GAC.

The collected data also indicated that SSF significantly increased tannin degradation by both the selected fungi compared to the LSF and SmF. SSF of pomegranate peel led to the maximum production of STA (2.8 fold, ranging from 1.1 to 1.3- fold over increase) and GA of 4.4 to 5.8 fold, 1.1 to 1.4 -fold over increase by both *A. niger* A8 and *T. viride* compared to the LSF and SmF, respectively. However, SSF of banana peel by both *A. niger* A8 and *T. viride* showed the highest activity of enzyme, ranged from 1.8 to 2.4 fold representing 1.1 to 1.3 fold over increase and GAC ranged from 3.0 to 6.4 fold, from 1.5 to 2.5 fold over increase compared to the LSF and SmF fermentation methods, respectively.

TABLE 2. Biomass, tannase and gallic acid production by fungi using agricultural wastes and by-product as a carbon sources by different fermentation methods at 28°C for 6 days of incubation period.

Fermentation methods	Agricultural wastes*	Parameters	Fungal strains							
			1	2	3	4	5	6	7	8
		CDW (g/L)	**1.05±0.16**	**1.71±0.09**	**1.29±2.61**	**1.09±0.34**	**1.58±0.69**	**1.10±1.30**	**1.85±2.31**	**1.62±1.10**
	Tannic acid (control)	STA (U/mg)	1.18±1.34	2.16±1.22	1.64±1.17	1.17±1.53	1.84±1.10	1.43±0.06	2.51±0.67	1.52±0.04
		GAC (mg/ ml)	0.05±0.45	0.19±0.81	0.13±0.13	0.02±1.20	0.22±0.12	0.14±0.74	0.28±0.43	0.07±1.12
		CDW (g/L)	1.14±0.08	1.68±0.15	1.46±1.24	1.25±1.11	1.69±0.05	1.13±2.44	1.94±1.12	1.85±2.11
	Eucalyptus leaves	STA (U/mg)	1.04±2.45	2.89±0.08	1.44±3.10	1.06±0.68	2.06±1.72	1.38±0.13	2.78±0.07	1.66±0.19
		GAC (mg/ ml)	0.14±1.12	0.21±1.34	0.17±0.55	0.15±0.65	0.21±0.52	0.05±1.30	0.23±0.09	0.067±1.23
		CDW (g/L)	1.84±0.67	2.16±0.02	1.98±1.11	1.65±0.27	2.10±0.14	1.69±1.56	3.08±0.12	1.79±1.82
	Pomegranate peel	STA (U/mg)	2.93±2.33	5.36±0.12	3.48±3.62	1.85±1.52	4.63±1.66	2.82±1.11	6.38±0.20	2.39±1.21
		GAC (mg/ ml)	1.19±0.71	1.34±1.30	1.25±1.11	1.01±1.45	1.27±0.06	1.04±1.07	1.63±0.11	0.97±0.11
Solid state (SSF)		CDW (g/L)	1.93±1.27	2.86±0.11	1.76±1.38	1.39±1.00	2.53±1.38	1.75±0.09	2.43±0.08	1.92±2.10
	Banana peel	STA (U/mg)	1.48±0.81	4.49±0.09	2.67±1.66	1.59±0.08	3.04±1.15	1.77±1.11	3.13±0.11	2.13±1.23
		GAC (mg/ ml)	0.21±1.22	1.08±0.12	0.34±0.92	0.12±1.22	0.40±1.11	0.09±0.71	0.42±0.06	0.36±1.61
		CDW (g/L)	1.02±1.45	1.89±1.19	1.08±1.72	1.09±4.15	1.46±1.64	1.06±2.71	1.64±1.17	1.88±1.82
	Guava leaves	STA (U/mg)	0.96±0.63	2.24±0.09	1.05±2.63	0.84±1.10	1.35±1.10	1.29±0.10	2.96±1.02	1.44±1.16
		GAC (mg/ ml)	0.08±1.12	0.93±0.65	0.17±1.12	0.004±1.21	0.16±0.82	0.12±1.22	0.33±1.54	0.21±1.22
		CDW (g/L)	1.87±0.07	2.38±1.27	2.11±2.81	1.68±1.73	2.15±2.12	1.75±1.10	2.27±0.09	1.97±2.21
	Wheat bran	STA (U/mg)	1.36±1.52	3.37±1.05	2.45±1.10	1.38±1.21	2.83±0.07	1.65±1.64	3.02±1.15	2.05±0.06
		GAC (mg/ ml)	0.08±0.55	0.39±1.10	0.20±2.13	0.17±1.73	0.31±0.33	0.22±0.90	0.34±0.88	0.19±1.45

TABLE 2. Cont.

Fermentation methods	Agricultural wastes*	Parameters	Fungal strains							
			1	2	3	4	5	6	7	8
Liquid-surface (LSF)	Tannic acid (control)	CDW (g/L)	1.04±0.18	1.18±0.07	1.20±1.20	1.08±2.11	1.15±1.55	1.00±0.73	1.22±1.34	1.06±0.82
		STA (U/mg)	0.69±1.54	1.03±1.11	1.09±2.56	0.85±1.61	1.06±1.02	0.66±1.47	1.12±1.11	1.02±0.09
		GAC (mg/ ml)	0.03±1.62	0.08±0.60	0.08±1.10	0.04±0.80	0.07±1.12	0.006±1.10	0.09±0.55	0.08±1.15
	Eucalyptus leaves	CDW (g/L)	1.12±2.76	1.44±1.51	1.19±1.92	1.14±1.16	1.25±2.15	1.18±0.55	1.08±1.22	1.02±1.23
		STA (U/mg)	0.88±1.33	1.52±0.16	1.05±3.24	0.97±0.84	1.36±2.04	1.02±1.96	0.91±1.14	0.86±1.06
		GAC (mg/ ml)	0.005±1.41	0.14±0.88	0.07±0.54	0.007±1.64	0.12±0.77	0.08±1.05	0.005±1.34	0.007±0.92
	Pomegranate peel	CDW (g/L)	1.32±3.51	1.77±0.12	1.51±2.81	1.24±1.11	1.34±2.22	1.28±1.54	1.89±0.35	1.30±1.17
		STA (U/mg)	1.40±1.87	1.94±0.34	1.49±1.12	1.20±2.15	1.27±0.41	1.14±1.22	2.31±0.14	1.24±1.03
		GAC (mg/ ml)	0.14±0.11	0.23±0.52	1.10±0.77	0.07±1.41	0.09±0.56	0.09±0.91	0.37±0.15	0.12±0.71
	Banana peel	CDW (g/L)	1.37±2.15	1.68±0.05	1.28±0.07	1.21±1.66	1.31±0.23	1.24±1.27	1.46±0.24	1.19±0.92
		STA (U/mg)	1.72±1.14	1.88±0.17	1.53±3.26	1.16±0.72	1.62±1.22	1.20±1.10	1.78±0.47	1.07±1.63
		GAC (mg/ ml)	0.13±0.73	0.17±1.5	0.12±2.24	0.06±0.01	0.12±0.61	0.09±0.83	0.14±1.11	0.08±0.65
	Guava leaves	CDW (g/L)	1.08±5.32	1.23±0.18	1.07±2.71	1.05±1.91	1.00±1.62	1.05±0.45	1.19±1.10	0.94±2.21
		STA (U/mg)	0.78±1.56	1.18±1.21	0.77±0.07	0.71±0.23	0.57±0.07	0.78±1.20	1.10±0.61	0.26±1.12
		GAC (mg/ ml)	0.008±1.10	0.09±0.45	0.004±1.10	0.001±0.55	0.002±1.33	0.003±1.62	0.08±1.10	0.002±0.22
	Wheat bran	CDW (g/L)	1.16±0.07	1.60±1.76	1.22±0.19	1.18±1.52	1.24±1.23	1.20±0.09	1.44±4.22	1.31±0.16
		STA (U/mg)	0.91±0.18	1.69±1.12	1.47±1.42	1.05±0.09	1.35±2.35	1.27±0.12	1.67±1.11	1.20±0.07
		GAC (mg/ ml)	0.06±2.33	0.17±1.15	0.13±0.61	0.08±0.09	0.12±1.12	0.11±0.55	0.12±0.32	0.09±1.22

TABLE 2. Cont.

Fermentation methods	Agricultural wastes*	Parameters	Fungal strains							
			1	2	3	4	5	6	7	8
Submerged (SmF)	Tannic acid (control)	CDW (g/L)	1.40±1.71	1.95±1.10	1.38±2.34	1.26±0.61	1.69±3.83	1.38±1.12	1.87±2.20	1.56±0.81
		STA (U/mg)	1.08±1.04	1.87±0.62	1.25±1.81	1.05±1.11	1.45±0.74	1.60±0.42	1.68±1.22	1.16±1.17
		GAC (mg/ ml)	0.08±1.11	0.76±0.08	0.14±0.12	0.07±1..22	0.54±0.14	0.65±0.91	0.71±0.12	0.12±1.32
	Eucalyptus leaves	CDW (g/L)	1.23±0.06	2.04±2.66	1.83±0.52	1.68±0.06	1.86±0.22	1.23±2.22	2.14±1.14	1.93±1.34
		STA (U/mg)	0.95±0.72	2.01±1.23	1.27±1.10	1.01±1.63	1.64±1.32	1.53±1.71	2.15±0.75	1.58±0.63
		GAC (mg/ ml)	0.06±1.23	0.62±0.81	0.13±1.10	0.09±0.22	0.15±1.33	0.10±0.15	0.16±0.91	0.11±1.23
	Pomegranate peel	CDW (g/L)	1.96±1.82	2.36±0.11	2.18±1.16	1.93±0.23	2.18±0.92	2.07±1.34	2.58±0.23	2.13±1.22
		STA (U/mg)	1.88±2.31	4.79±0.21	2.20±1.04	1.49±0.71	3.73±1.66	2.23±0.71	4.95±0.10	2.65±2.63
		GAC (mg/ ml)	0.14±0.93	1.17±0.32	0.14±1.12	0.09±0.06	0.65±0.22	0.11±1.50	1.14±1.3	0.16±0.54
	Banana peel	CDW (g/L)	1.98±1.74	2.93±0.28	1.45±1.12	1.90±1.11	2.61±1.00	1.97±1.44	2.74±0.33	2.01±1.00
		STA (U/mg)	1.90±1.22	3.41±0.12	1.80±2.26	1.62±0.94	2.34±0.45	2.16±0.51	2.75±0.14	1.89±0.72
		GAC (mg/ ml)	0.16±0.51	0.74±0.18	0.09±0.07	0.07±1.41	0.12±0.74	0.10±1.12	0.17±0.10	0.09±0.06
	Guava leaves	CDW (g/L)	1.17±1.18	1.70±1.00	1.16±0.73	1.12±2.14	1.69±1.52	1.25±1.14	2.11±1.11	1.95±1.22
		STA (U/mg)	0.98±0.26	1.76±1.25	0.92±1.46	0.80±1.22	1.13±1.10	1.79±1.85	2.03±2.62	1.08±1.41
		GAC (mg/ ml)	0.007±0.82	0.07±0.12	0.006±1.46	0.004±0.06	0.08±1.12	0.10±0.34	0.14±1.25	0.08±0.34
	Wheat bran	CDW (g/L)	1.98±3.13	2.35±0.51	2.23±1.66	1.98±1.10	2.24±3.66	1.93±0.74	2.46±1.23	2.00±0.07
		STA (U/mg)	0.97±0.37	3.03±0.92	2.17±2.12	1.24±0.83	2.19±0.09	1.84±1.10	2.38±0.80	1.87±1.00
		GAC (mg/ ml)	0.007±1.42	0.56±1.12	0.15±0.02	0.09±1.10	0.16±0.28	0.12±0.71	0.15±1.26	0.14±0.15

- Agricultural wastes* was used as carbon sources, CDW= Cell dry weight, STA= Specific tannase activity, GAC= Gallic acid concentration, 1= *Aspergillus flavus,* 2= *Aspergillus niger,* 3= *Aspergillus terrus,* 4= *Pencillium* sp., 5= *Aspergillus nidulans,* 6= *Fusarium oxysporium,* 7= *Trichoderma viride,* 8= *Rhizopus stolonifera.*
- ±= Standard error.

A maximum production of tannase was achieved by the filamentous fungi *A. niger* when using the SSF process was used, which increased about 2.5 and 4.8 folds as compared to SmF and LSF processes (Lekha & Lonsane, 1994 and Aguilar et al., 2002). Moreover, Pandey (1992) and Lekha & Lonsane (1994) confirmed that the LSF method was proved to be not appropriate for tannase biosynthesis as it needed a longer period of fermentation and that the enzyme is produced intracellularly.

The best fungal strains were proved to be the *A. niger* A8 and *T. viride*, which indicated the highest capabilities of degradation of tannin-rich materials, especially the pomegranate peel and banana peel under solid state fermentation at 30°C for 6 days. Both strains and both peels of pomegranate and banana were chosen for further investigation.

Effect of additive by-products as nitrogen source on tannase and gallic acid production

Results in Table 3 show superiority of the CSL than the SBE as nitrogen sources for fungal growth and production of tannase and gallic acid by both the tested fungal strains, using the pomegranate peel or banana peel using the different fermentation techniques when compared to $NaNO_3$ as a positive control.

In the case of *A. niger* A8, the SSF process gave the highest STA on CSL (8.26U/mg protein) with 19 and 54% increase over SBE and $NaNO_3$, respectively and 6.82U/mg protein (14 and 53% over increase SBE and $NaNO_3$) in the presence of pomegranate and banana peels, respectively. The maximum gallic acid accumulation on CSL was 2.70mg/ml with 1.5 and 2.0 fold increase over SBE and $NaNO_3$, respectively and 1.66mg/ml (1.1 and 1.5 fold increase over SBE and $NaNO_3$) in presence of pomegranate and banana peels, respectively.

In the case of *T. viride,* the maximum of STA and GAC were recorded on CSL over those recorded for the SBE and $NaNO_3$ and in presence of pomegranate and banana peels, respectively.

In this respect, Sabu et al. (2005) and Murad et al. (2014) reported that supplemental nitrogen is a substantial for enhancement of microbial growth and enzyme synthesis. In specific, Huang et al. (2005) and Malgireddy & Nimma (2015) revealed that *Aspergillus* SHL 6 and *A. terreus* preferred organic nitrogen rather the inorganic sources for tannase production.

The results presented here (Table 3) also indicate that the cultivation of both the fungal strains on medium supplemented with CSL under the SSF technique enhanced production of tannase than SmF and LSF techniques, suggesting that CSL is a good source of nitrogen. In addition, its high vitamin (i.e. nicotinic acid, pantothenic acid, biotin, thiamine, pyridoxine and cyanocobalamin) and amino acids as, alanine, arginine, aspartic acid, histidine, glutamic acid, glycine, leucine, lysine, serine and valine) for the growth of the microbe (such as *Penicillium* and *E. coli*) (Nascimento et al., 2011). In addition, Prasad et al. (2011) noticed that addition of CSL for nutrient supplementation, containing N and under SSF led to enhancement of growth of the *A. heteromorphus* and increased production of tannase and GA.

From the above results, it could be concluded that the highest STA and GA accumulation were achieved by the tested fungal strains when using CSL by-product as a sole nitrogen source on medium supplemented with pomegranate peel or banana peel under SSF technique.

Effect of incubation period on tannase and gallic acid production

Results in Fig. 1 A, B indicated that the cell biomass growth of *A. niger* A8 and *T. viride* and the yields enzyme and GA were gradually increased with increase of the incubation period up to 4 days, whether in the presence of pomegranate peel or banana peel supplemented with CSL under SSF technique. The increase in incubation period had resulted in decreased fungal growth and yields of tannase and GA. This might be due to that the fungal growth stage entering the decline phase of the growth, thereby reducing the production of both enzyme and GA production, confirming the results of (Haq et al., 2005). Furthermore, Sepahy et al. (2011) related this reduction process to micro and macronutrients consumption from the production medium, which change the pH of the medium. In addition, Kaur et al. (1998) confirmed a positive relationship between cell

TABLE 3. Effect of by-products as a nitrogen sources on tannase and gallic acid production by *A. niger* and *T. viride* on pomegranate and banana peels at 28°C for 6 days of incubation period using different fermentation methods.

Substrates	Strains	Nitrogen sources	Parameters	Fermentation methods		
				SSF	LS	SmF
Pomegranate peel	*A. niger*	Control	CDW (g/L)	2.16±0.02^{a}	1.77±0.12^{b}	2.36±0.11^{a}
			STA (U/mg protein)	5.36±0.12^{a}	1.94±0.34^{c}	4.79±0.21^{b}
			GAC (mg/ml)	1.34±1.30^{a}	0.23±0.52^{c}	1.17±0.32^{a}
		CSL	CDW (g/L)	3.00±0.05^{a}	2.15±0.61^{b}	3.15±0.13^{a}
			STA (U/mg protein)	8.26±0.10^{a}	3.99±0.11^{c}	6.50±0.08^{b}
			GAC (mg/ml)	2.70±0.07^{a}	0.56±1.2^{c}	1.52±0.10^{b}
		SBE	CDW (g/L)	2.55±0.01^{a}	1.92±0.22^{b}	2.71±0.17^{a}
			STA (U/mg protein)	6.94±0.08^{a}	3.24±0.15^{c}	5.13±0.25^{b}
			GAC (mg/ml)	1.83±0.11^{a}	0.48±0.23^{c}	1.27±0.40^{b}
	T. viride	Control	CDW (g/L)	3.08±0.12^{a}	1.89±0.35^{c}	2.58±0.23^{b}
			STA (U/mg protein)	6.38±0.20^{a}	2.31±0.14^{c}	4.95±0.10^{b}
			GAC (mg/ml)	1.63±0.11^{a}	0.37±0.15^{c}	1.14±1.3^{ab}
		CSL	CDW (g/L)	2.90±0.10^{b}	2.43±0.08^{c}	3.20±0.11^{ab}
			STA (U/mg protein)	7.30±0.07^{a}	4.42±0.02^{c}	6.17±0.06^{b}
			GAC (mg/ml)	2.08±0.16^{a}	1.05±0.20^{c}	1.61±0.20^{b}
		SBE	CDW (g/L)	2.51±0.17^{b}	2.31±0.25^{bc}	2.83±0.13^{a}
			STA (U/mg protein)	6.97±0.19^{a}	4.18±0.04^{c}	5.88±0.02^{b}
			GAC (mg/ml)	1.80±0.15^{a}	1.00±0.11^{c}	1.32±0.14^{b}
Mean				68.79^{a}	36.24^{c}	58.28^{b}
Banana peel	*A. niger*	Control	CDW (g/L)	2.86±0.11^{a}	1.68±0.05^{b}	2.93±0.28^{a}
			STA (U/mg protein)	4.49±0.09^{a}	1.88±0.17^{c}	3.41±0.12^{b}
			GAC (mg/ml)	1.08±0.12^{a}	0.17±1.5^{c}	0.74±0.18^{b}
		CSL	CDW (g/L)	2.52±0.17^{ab}	1.98±0.02^{b}	2.87±0.34^{a}
			STA (U/mg protein)	6.86±0.02^{a}	3.61±0.23^{c}	5.45±0.16^{b}
			GAC (mg/ml)	1.66±0.13^{a}	0.58±1.22^{c}	1.16±0.16^{b}
		SBE	CDW (g/L)	2.38±0.16^{ab}	1.91±0.53^{c}	2.88±0.02^{a}
			STA (U/mg protein)	6.04±0.08^{a}	3.09±0.12^{c}	4.98±0.09^{b}
			GAC (mg/ml)	1.54±0.34^{a}	0.35±1.21^{c}	1.14±0.22^{b}
	T. viride	Control	CDW (g/L)	2.43±0.08^{a}	1.46±0.24^{b}	2.74±0.33^{a}
			STA (U/mg protein)	3.13±0.11^{a}	1.78±0.47^{c}	2.75±0.14^{b}
			GAC (mg/ml)	0.42±0.06^{a}	0.14±1.11^{b}	0.17±0.10^{b}
		CSL	CDW (g/L)	2.83±0.12^{ab}	1.81±0.25^{c}	3.04±0.18^{a}
			STA (U/mg protein)	7.08±0.05^{a}	2.81±0.23^{c}	4.88±0.17^{b}
			GAC (mg/ml)	1.72±0.08^{a}	0.20±0.72^{c}	1.12±1.10^{b}
		SBE	CDW (g/L)	2.50±0.10^{a}	1.81±0.14^{c}	2.67±0.35^{a}
			STA (U/mg protein)	6.22±0.07^{a}	3.03±0.18^{c}	5.17±0.29^{b}
			GAC (mg/ml)	1.61±0.81^{a}	0.29±0.09^{c}	1.29±1.10^{b}
Mean				57.37^{a}	28.58^{c}	49.39^{b}

- CSL= Corn steep liquor, SBE= Soybean extract, SSF= Solid state fermentation, LS= Liquid-surface, SF= Submerged fermentation, CDW= Cell dry wWeight, STA= Specific tannase activity, GAC= Gallic acid concentration.
- ±= Standard error.
- Means in the same row followed by different letters are significantly different at P< 0.05 level (Duncan, 1955).

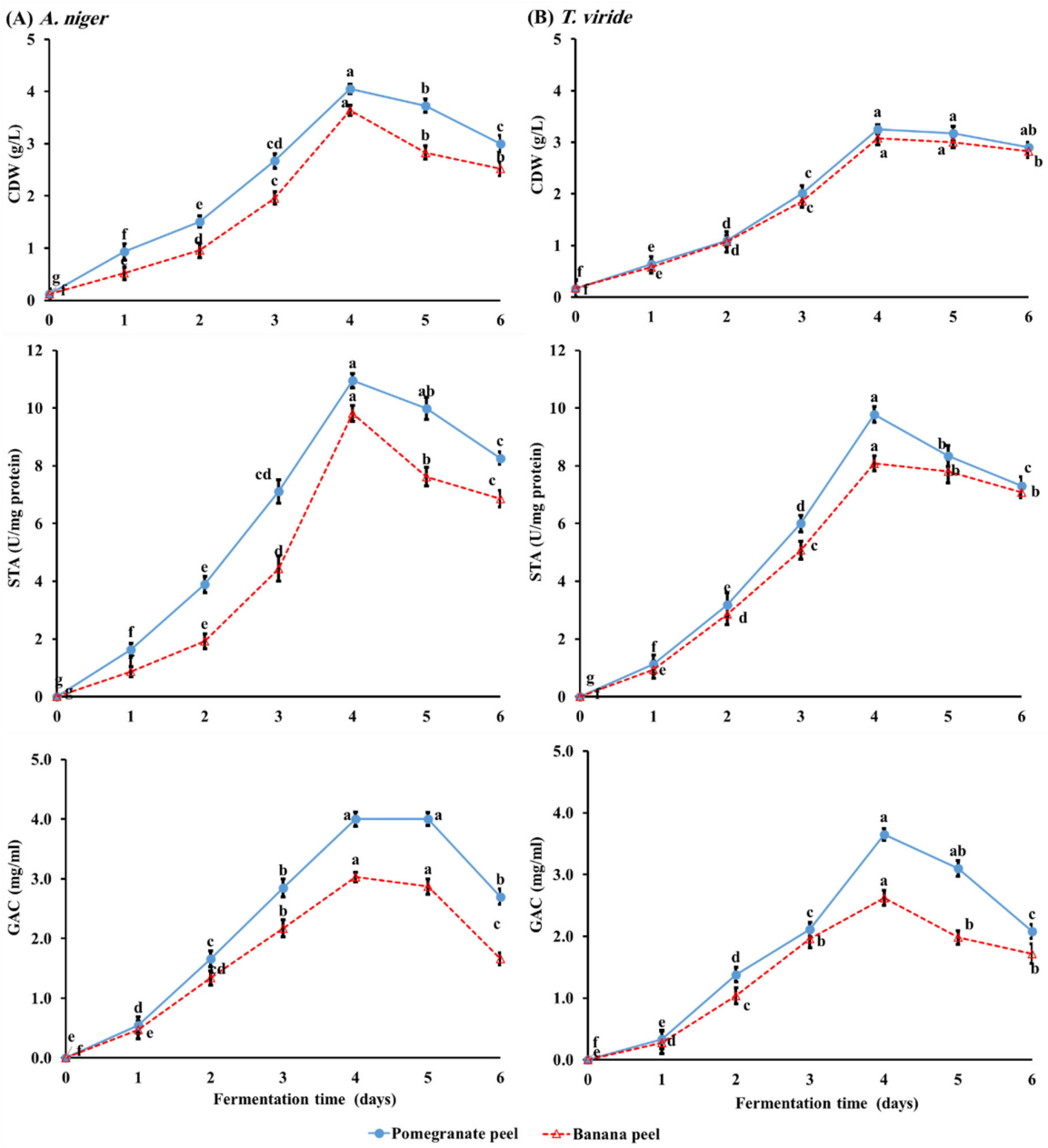


Fig. 1. Effect of fermentation time on biomass, tannase and gallic acid production by *A. niger* (A) and *T. viride* (B) on pomegranate and banana peels supplemented with corn steep liquor at 28°C during 6 days of incubation period using solid state fermentation (CDW= Cell dry weight, STA= Specific tannase activity, GAC= Gallic acid concentration; mean values with different letters in the same line are significantly different P< 0.05; error bars presented standard error).

growth and enzyme production, in one hand, and between the incubation period and enzyme biosynthesis, on the other.

The tested fungi produced the enzyme and GA during the logarithmic phase of growth and the specific growth (μG) and production of tannase (μT) and gallic acid (μGA) rates were calculated in this phase. μG was 0.49 and 0.65 d^{-1} for *A. niger* A8 and 0.54 and 0.56 d^{-1} for *T. viride* in the presence of pomegranate and banana peels, respectively. The μT and μGA of *A. niger* A8 and *T. viride* in the presence of pomegranate peel were 0.63 and 0.66d^{-1} and 0.72 and 0.80d^{-1} and in the presence of banana peel were 0.81 and 0.62d^{-1} and 0.72 and 0.75d^{-1}, respectively. so, it could be stated that the highest tannase and gallic acid production were achieved in the presence of banana peel by *A.*

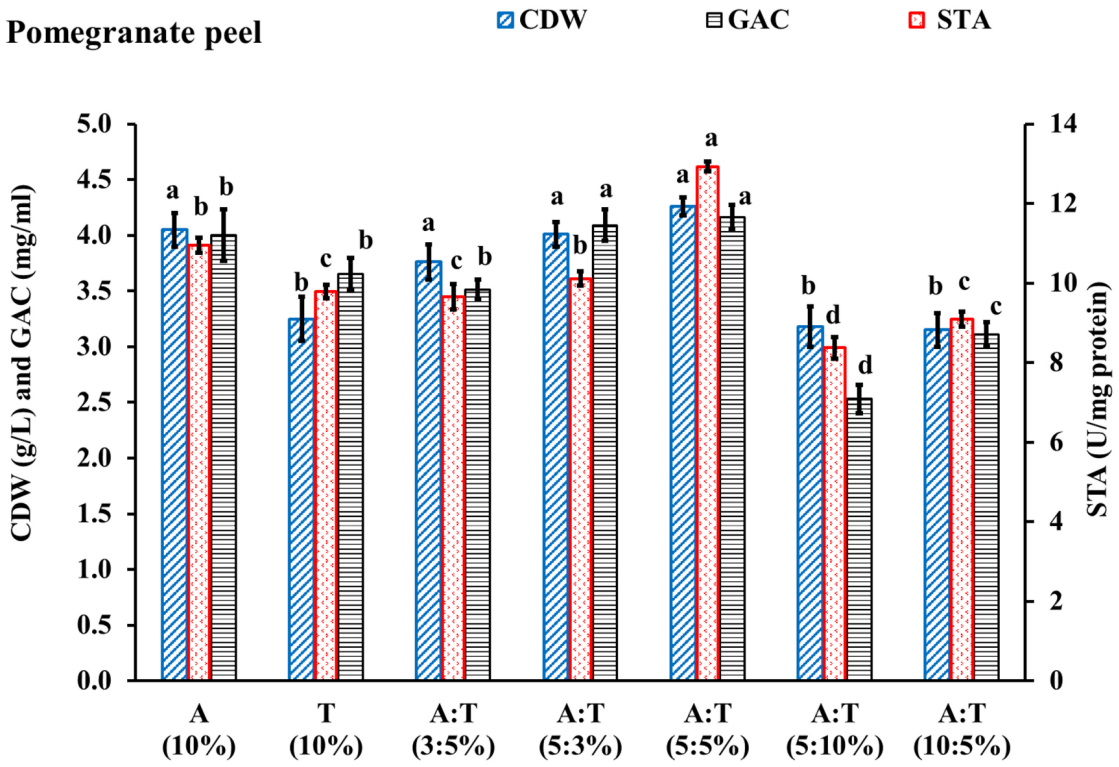
niger A8 and in the presence of pomegranate peel by *T. viride*, respectively, where recorded a high specific production rate.

In case of *A. niger* A8, the maximum values of biomass were achieved after 4th day on pomegranate peel or banana peel under SSF process, respectively (Fig. 1 A). As well, *T. viride* in the presence of pomegranate or banana peels attained the highest cell dry weight after 4 days of incubation, respectively (Fig. 1 B). In this respect, Paranthaman et al. (2009) and Murad et al. (2014) reported highest production of tannase and GA under SSF process achieved after 96hr of the incubation.

Effect of consortia of A. niger A8 and T. viride with different inocula sizes on tannase and gallic acid production

Results in Fig. 2 clearly show that co-cultivation of *A. niger* A8 and *T. viride* on pomegranate or banana peels gave the highest STA and GAC when inoculated with 5:5% (v/v) of inoculum size followed with 5:3% (v/v) of inoculum size, respectively. The data also exhibited that inoculation of pomegranate and banana peels with co-cultures of *A. niger* A8: *T. viride* (at 5:5% (v/v) inoculum size) significantly ($P \leq 0.05$) increased the production of tannase if compared to inoculation with 10% (v/v) of mono-culture of *A. niger* A8 or *T. viride*, respectively. It might be due to synergistic interactions as proposed by Shata et al. (2014). Moreover, Paranthaman et al. (2009) found that co-cultivation of *A. niger* + *P. chrysogenum*, *P. chrysogenum* + *T. viride*, and *A. niger* + *T. viride* enhanced the tannase activity as compared to their single cultures. Moreover, Hu et al. (2011) reported that mixed culture induced higher enzyme activity than monocultures. Shata et al. (2014) confirmed this result when found that co-cultivation of *A. niger* NRC 9A and *T. reesei* NRRL 6165 increased the enzyme activity by about 1.38 and 21.12-fold more than when the *A. niger* NRC 9A and *T. reesei* NRRL 6165 were used individually.

The lowest yield of STA and GA (Fig. 2) were produced with inoculation of pomegranate or banana peels with 5:10 or 10:5% (v/v) of mixture culture of the *A. niger* A8: *T. viride*, respectively. The data also indicating that no significant difference impact was observed with inoculation of banana peel with 5:10 or 10:5% (v/v) of co-culture of *A. niger* A8: *T. viride.* However, with single culture inoculation, *A. niger* A8 was preferred for the degradation of pomegranate and banana peels by secreting tannase and producing gallic



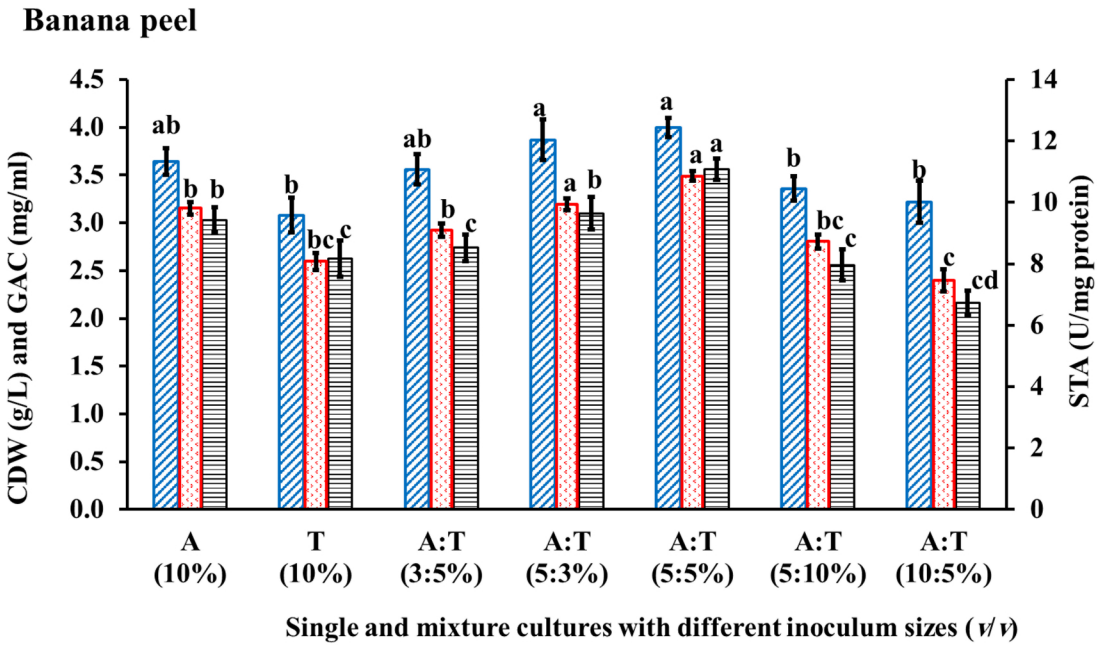


Fig. 2. Influence of inoculum size (v/v) of single and mixture cultures of *A. niger* and *T. viride* on biomass, tannase and gallic acid production on pomegranate and banana peels supplemented with corn steep liquor at 28°C during 4 days of incubation period using solid state fermentation (A= *A. niger*, T= *T. viride*, CDW= Cell dry weight, STA= Specific tannase activity, GAC= Gallic acid concentration; mean values with different letters on top of bars are significantly different (P<0.05); error bars presented standard error).

compared to *T. viride*. In this respect, Paranthaman et al. (2009) found that the tannase production by *A. niger* could be increased by about 40 and 19 % than by *P. chrysogenum* and *T. viride*, respectively.

Conclusions

In the present study, it can be concluded that some fungi were capable of producing tannase by degradation of tannin-rich substrates and produce gallic acid under various fermentation techniques after 4-6 days of incubation. Solid-state fermentation was the best technique for the maximum production of tannase and gallic acid on productive medium contained pomegranate or banana peels and corn steep liquor compared to liquid-surface and submerged fermentation. This productive medium was inoculated with 5:5% of *A. niger* A8: *T. viride* for enhanced the of tannase and gallic acid compared to inoculation with a single culture of both organisms.

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تطبيق تقنيات التخمير المختلفة لإنتاج التانيز وحمض الجاليك باستخدام ركائز المخلفات الزراعية

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التانيز هو إنزيم محفز يقوم بتحليل تانين إلى حمض الجاليك ويستخدم في العديد من الصناعات مثل الأغذية والأدوية. وقد اجتذب هذا الأنزيم المزيد من الإهتمام في الآونة الأخيرة. تم تحقيق أقصى إنتاج من التانيز (تراوحت من 0.30 إلى 0.93 مؤشر الإنزيم ومن 0.50 إلى 1.87 وحده/ ملجم نشاط التانيز المحدد (STA)) وحمض الجاليك (من 0.07 إلى 0.76 ملجم/مل من تركيز حمض الجاليك (GAC)) بواسطة 8 سلالات فطرية من أصل 24 سلالة فطرية وخميرة تنتمي إلى الأجناس; *Trichoderma* ، *Rhizopus* ، *Aspergillus* ، *Candida* ، *Penicillium* ، *Fusarium* و *Saccharomyces* . تم زراعة الفطريات المختارة على ركائز غنية بالتانين (أوراق الكافور، قشر الرمان، قشر الموز، أوراق الجوافة ونخالة القمح) ومنتجات ثانوية (مستخلص منقوع الذرة (CSL) ومستخلص الفول الصويا) كمصدر وحيد للكربون والنيتروجين لإنتاج التانيز و حمض الجاليك GA عند 28 درجة مئوية لمدة 6 أيام بتقنية التخمير بالمزرعة السائلة الثابتة (LSF)، السائلة المغمورة (SmF) ومزرعة السطح الصلب (SSF). أشارت النتائج إلى أن A8 *A. niger* و *T. viride* بنسبة 10٪ (v/v) من حجم اللقاح أعطى إنتاج عالي من نشاط التانيز المحدد (STA) (يتراوح من 8.08 إلى 10.95 وحده/ملجم (وتركيز حمض الجاليك (يتراوح من 2.62 إلى 4.00 ملجم/مللي)على قشور الرمان والموز المدعمة بمستخلص منقوع الذرة بعد 4 أيام من فتره التحضين عند 28 درجة مئوية بتقنية مزرعة السطح الصلب مقارنة بـالمزارع السائلة المغمورة و السائلة الثابتة. لوحظ زيادة كبيرة في نشاط التانيز المحدد (تراوحت من 10.68 إلى 12.93 وحده/ملجم (وتركيز حمض الجاليك (تراوحت بين 3.56 إلى 4.16 ملجم/مل) عند تلقيح البيئة الغذائية بخليط من فطري *A. niger* A8 + *T. viride* بحجم لقاح 5: 5% (حجم: حجم) مقارنا بالتلقيح كل فطر علي حدة.