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Tannase and gallic acid production by *Aspergillus niger* SWP33 and *Penicillium griseoroseum* T11 using agricultural wastes under submerged and solid-state fermentation and its application

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Abstract

In this work, microbial sources can be used in the biotechnological synthesis of tannase. Microbial tannases are preferred because they are more stable and produced in higher yields than similar ones acquired from other sources. In addition, they can be exposed to genetic manipulation more easily than plants and animals. Tannase has a wide range of industrial uses, including food, and environmental biotechnology. Among 161 fungal isolates, two (SWP33 and T11) were chosen to produce more tannase and gallic acid (GA). Based on phenotypic and genotypic (18S rRNA gene sequencing) features, these isolates were identified as *Aspergillus niger* SWP33 and *Penicillium griseoroseum* T11. Maximum tannase (147.0 and 148.7 U/ml) and GA (255 and 258 mg/ml) production by *A. niger* SWP33 and *P. griseoroseum* T11: in the presence of tannic acid, were attained on the fourth and fifth days of fermentation, with specific enzyme and GA rates of 0.79 and 0.66 d^{-1} and 0.21 and 0.24 d^{-1}, respectively. When grown on tannins-rich wastes as a low-cost medium to produce tannase and GA, tested strains favored solid-state fermentation than submerged fermentation. During 6 hours of incubation, fungal tannase demonstrated activity in decolorizing reactive blue 19 and red 24 textile dyes.

Keywords: Dye decolorization; Gallic acid synthesis; Tannin agricultural wastes; Tannase; 18S rRNA sequencing

1. Introduction

Enzymes are biocatalysts that are widely used in industrial processes and have several benefits over chemical catalysts. Green biotechnology promotes the use of enzymes in the industrial manufacture of chemicals, fuels, secondary metabolites, and other products, preferably using renewable resources. Hydrolases make up the majority of industrial enzymes (about 65%) **[1].** Tannase (Tannin acyl hydrolase; E.C.3.1.1.20) is one of the hydrolases and was known to catalyze the breakdown of ester and depside bonds from hydrolyzable tannins and gallic acid esters **[2].** Also, tannase is an enzyme that aids in the extraction of phenolic chemicals by dissolving plant matrix and inducing structural changes within bioactive phenolic molecules, such as the conversion of glucosides to aglycones **[3].** Tannases are valuable industrial enzymes used in a variety of sectors, including feed, food, beverages, brewing, pharmaceutical, chemical, cosmetic, leather, and

1. Introduction

environmental biotechnology **[4]**. Tannase may be found in a variety of environments, including plants, animals, and microbes, although the microbial source is the most common because of the great output and diversity of microbial tannases. Microorganisms are also easy to cultivate and process downstream, which is why industries choose them **[5].** Fungi and yeast from the genera *Aspergillus*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Penicillium*, *Candida*, and *Saccharomyces* can hydrolyze tannin via tannase **[6,7].** The industrial process includes several techniques such as liquid surface (LSF), submerged (SmF), and solid-state fermentation (SSF). Bacteria and yeast prefer the SmF process in shaking cultures over the SSF and LSF processes because they require a high content of water to dissolve the nutrient contents for microbial development. Meanwhile, filamentous fungi are more suitable to the SSF process than other fermentation methods **[7].** When using a solid substrate as raw

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material, SSF can be implemented when there is a virtually or wholly absent or near absence of free liquid. Because of the concentrated medium, the advantages of this approach include the exploitation of low-cost raw materials, low energy usage, and small bioreactor dimensions **[8].**

The goal of this work was to bioconvert rich tannin substrates by local strains into the significant commercial products tannase and gallic acid, and then use the enzyme produced in dye decolorization.

2 Materials and Methods

2.1 Sample collection

Twelve samples were collected from various sources (4 samples of plant rhizosphere soils of bean (*Vicia faba*) obtained from Qalyubia and Giza governorates, two samples of soil down tannin-rich plants taken from Monufia governorate, 4 samples of food (sweet potato, banana, pomegranate, and tea) obtained from the local market, and two samples of seawater collected from Port Said and Alexandria governorates, Egypt. These samples were collected in sterile plastic bottles, placed in an icebox all the way to the laboratory, and kept at 4°C to be used later.

2.2 Tannin-rich raw materials and synthetic dyes collection

Eleven tannin-rich raw materials of tea, clove, pomegranate peel, thyme, mulberry leaves, guava leaves, chard leaves, banana peel, sesame, camphor, and wheat bran were collected from open markets in Cairo, Egypt. Raw materials were dried at 60°C in an oven and then finely ground to the powder form in a grinder mixer. The powder was stored in a dry place at room temperature and used as a source of crude tannins .

Commercial reactive blue 19 (disodium;1-amino-9,10-dioxo-4-[3-(2-sulfonatooxyethylsulfonyl)anilino]anthracene-2-sulfonate) and reactive red 24 (2,7-Naphthalenedisulfonic acid, 5-[[4-chloro-6-(2-chlorophenylamino)-1,3,5-triazin-2-yl]amino]-4-hydroxy-3-[(2-sulfophenyl)azo], trisodium salt) textile dyes were purchased from Nabil Tex Dyeing and Printing Company in Shubra Elkheima, Qalubyia governorate, Egypt.

2.3 Media used

2.3.1 Potato dextrose agar (PDA) and Potato dextrose broth (PDB) [9]. Potato dextrose agar (PDA) was used for the isolation and maintenance of fungal isolates. It was prepared from the following ingredients (g/L): glucose, 20; infusion of potatoes, 200; agar, 15, and pH was adjusted to 5 ± 0.2. This medium was supplemented with 0.01% tannic acid for fungal isolation and preselection.

Potato dextrose broth (PDB) is the same PDA medium without adding agar.

2.3.2 Tannic acid broth medium [10]. It was used for a quantitative assay of tannase activity and gallic acid content. It was prepared from the following components (g/L): Tannic acid, 10; $(NH_4)_2HPO_4$, 3; KH_2PO_4, 0.5; $MgSO_4$, 1; NH_4Cl, 0.5, and $CaCl_2$, 0.3. The pH of the medium was adjusted at 5±0.2.

2.4 Isolation and primary screening of tannase-producing fungi

Ten grams or milliliters of soil, food, or seawater samples were suspended in 90 milliliters of distilled water and placed in a rotary shaker (XuyMeu) at 120 rpm for 15 min then kept stable for 10 min. The samples were serially diluted (ranged from 10^{-1} to 10^{-8}) with sterile distilled water according to the method of serial dilution **[11]**. One milliliter from each dilution was plated into PDA supplemented with 0.01% tannic acid and incubated at 28º C for 96 h. At the end of the incubation period, colonies had clear zones suggest tannase production. These colonies were selected and purified using the streak plate technique **[12].** The pure fungal colonies were grown on PDA slants and kept in a refrigerator at 5°C and sub-cultured monthly for further study.

The diameter (mm) of the clear zone that appeared around the fungal colony was taken as an enzymatic index using the following equation described by **[13].**

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

2.5 Secondary screening for tannase production by the most efficient isolates

The quantitative determination of enzyme production by the pre-selected fungal isolates was carried out as a submerged fermentation technique as described by **[14].** It was performed in plugged Erlenmeyer flasks (250ml), containing 100 ml of tannic acid broth medium, and inoculated with 5% (v/v) of preselected fungal isolates. The flasks were incubated on a rotary shaker (XuyMeu) at 28°C and 150 rpm of agitation speed for 4 days. By the end of the fermentation period, the culture was filtrated using filter paper (Whatman® qualitative filter

paper, Grade 1, circles, diam. 45 mm, Sigma-Aldrich) to separate the fungal mycelia and then washed with distilled water and dried at 70°C until constant weight for cell dry weight determination. The filtrate (supernatant) that contains the crude enzyme was used for enzyme activity, gallic acid concentration, and tannin degradation assessments, as described later in sections (2.9 Extraction of crude tannins, 2.12 Tannase activity, and 2.13 Gallic acid concentration (GAC) determination).

2.6 Identification of the most efficient fungal isolates

For the phenotypic identification of isolates (SWP33 and T11), these isolates were cultivated on PDA plates at 28°C for 4 days. The isolates were identified using the morphological characteristics according to **[15,16].** For confirmation of phenotypic identification, the fungal isolate was re-identified by sequencing the 18S rRNA gene. The cultures were sent to the Sigma Company, Giza, Egypt. For molecular identification, the following procedure steps were applied: **1- DNA extraction:** The fungal genomic DNA was extracted from the cultures of the two isolates (SWP33 and T11) that cultivated on PDA by using GeneJet genomic DNA purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. **2- Amplification of 18S rDNA by PCR**: it was done using universal fungal primer F (5`- TCC GTA GGT GAA CCT GCG G -3´) and reverse R (5`- TCC TCC GCT TAT TGA TAT GC -3´), as described by **[17].** Amplification was performed in a reaction volume of 50 µl. The thermal cycle (PCR) steps were applied as follows; 10 min initial denaturation at 95°C, followed by 40 cycles of 15-sec denaturation at 95°C, 30-sec primers annealing at 60°C, 30-sec extension at 72°C, and a final 10 min extension at 72°C. The amplified DNA fragment was separated on 1.2% (w/v) agarose gel electrophoresis, eluted and purified using the Qiaquick gel extraction kit (Qiagen, Germany) following the manufacturer's protocol **[18].** The purified PCR product was sequenced using the ABI 3730xl DNA sequencer (GATC Company), the partial sequence data of 18S rDNA were aligned and analyzed for finding the closest homologous fungi. **3- Phylogenetic analysis**: The unknown query 18S rDNA nucleotide sequence was compared to nucleotide databases using http://www.ncbi.nlm.nih.gov/BLAST site **[19].** that is available from the National Center for Biotechnology Information **(NCBI)** website and retrieved from the GenBank database. The multiple-sequence alignment of these homologous sequences was then developed by using the algorithm described in CLUSTAL Omega. Then, using the neighbor method, a phylogenetic tree was drawn. The sequence of the isolates was subsequently deposited in GenBank.

2.7 Inoculum preparation

Both fungal inoculums were prepared by scratching the spores from cultivated slants in plugged Erlenmeyer flask (100 ml) containing 50 ml of sterilized PDB medium and then incubated on a rotary shaker (XuyMeu) at 150 rpm and 28 °C for 24-48 h. The number of fungal spores was counted by using a hemocytometer slide **[20].** Then inoculation 50 ml of tannic acid broth medium presentence in plugged Erlenmeyer flask (100 ml) with 5% v/v (2.9 X 10^7 and 2.7 X 10^7 spores/ml) standard inoculum of *Aspergillus niger* ok 626231 and *Penicillium griseoroseum* ok 626651, respectively.

2.8 Effect of fermentation time on tannase and gallic acid production

After inoculation of the tannic acid broth medium, samples were taken every day during 6 days of fermentation periods under aseptic conditions**.** The cell dry weight was determined, enzyme activity, and gallic acid content were determined as described later in sections (2.12 Tannase activity and 2.13 Gallic acid concentration (GAC) determination).

The relationship was plotted between time (h) and each cell dry weight, enzyme activity, and gallic acid content. During the logarithmic phase, the specific rates of growth (µG), enzyme (µE), gallic acid (µA), doubling time, and multiplication rate (MR) were calculated using the following formulas according to **[21]:**

Specific rate (μ_G, μ_E, or μ_A) (h^{-1}) = (ln X –ln X_0) / (t - t_0)

Where: X = value of growth, enzyme activity, or gallic acid content after t time (t) and X_0 = value of growth, enzyme activity, or gallic acid content at the beginning time (t_0).

Doubling time (t_d) (h) = ln2 (μ)$^{-1}$

Multiplication rate (MR) = 1 (t_d)$^{-1}$

2.9 Extraction of crude tannins

The finely ground powder of the substrates at 1% (w/v) concentration was mixed with distilled water (100 ml) and kept at room temperature for 3 days. After soaking, the mixture was boiled for 10

min and filtered using filter paper (Whatman® qualitative filter paper, Grade 1, circles, diam. 45 mm, Sigma-Aldrich). The filtered extracts were used as a source of crude natural tannin.

The total tannin content of each material was determined as a procedure described by **[22]**. Briefly, 0.5 ml of a tenfold diluted sample was mixed with 2.5 ml of tenfold diluted Folin–Ciocalteu's phenol reagent and incubated for 5 min, before 2 ml of 20 % (w/v) Na_2CO_3 was added. The mixture was incubated for 60 min at room temperature. The absorbance versus prepared blank was monitored at 760 nm. The mg tannin was assayed using a tannic acid standard. The percentage of tannin degradation was calculated according to the following formula:

$$\textbf{Percentage of tannin degradation (\%)} = \frac{(\textbf{Tannin concentration before degradation} - \textbf{Tannin concentration after degradation})}{\textbf{Tannin concentration before degradation}}$$

2.10 Fermentation techniques

The tested raw materials and their extracts were used as carbon sources and were added equivalent to the original tannin percentage in the basal medium under different fermentation techniques:

2.10.1 Submerged fermentation (SmF)

It was carried out in a tannic acid broth medium in which tannin acid was replaced by a similar amount of each extract of rich-tannin agricultural waste using a rotary shaker (XuyMeu).

2.10.2 Solid-state fermentation (SSF)

It was carried out using sterilized Petri dishes containing 5 g of each rich tannin substrates individually and inoculated with 5% (*v*/*v*) of inoculum size of fungal spores and moisturizing the inoculated substrate with 5% of basal salts in tannic acid broth medium without added tannic acid. The dishes were incubated at 28°C under static conditions for 4 days. At the end of the fermentation period, the mat was used for cell dry weight determination and the enzyme was extracted by adding 20 ml of acetate buffer (0.05M, pH 5) and the mixture was agitated using a rotary shaker (XuyMeu) for 1 h at 200 rpm **[23].** The solution was filtered using filter paper (Whatman® qualitative filter paper, Grade 1, circles, diam. 45 mm, Sigma-Aldrich) and the filtrate was used for the assessment of enzyme activity, gallic acid concentration, and tannin degradation as described later in sections (2.9 Extraction of crude tannins, 2.12 Tannase activity, and 2.13 Gallic acid concentration (GAC) determination).

2.11 Effect of various inoculum sizes of mono and co-cultures on the production of enzyme and gallic acid

Using the SSF technique, different inoculum sizes ranged from 3 to 10 % (v/v) of standard inoculum of *Aspergillus niger* ok 626231 or *Penicillium griseoroseum* ok 626651 were used as a mono-culture for enzyme and gallic acid production. As well as co-cultures of both strains with different inoculum sizes of 3 to 10% (v/v) with ratios (*v*:*v*) of 1.5:1.5 to 5.0:5.0 were tested.

2.12 Tannase activity

It was assayed by the method of UV spectrophotometric as described by **[7].** This method was performed as follows; one milliliter of the crude enzyme (filtrate) was added to a 4 ml solution (consisting of 0.35 g tannic acid dissolved in 100 ml citrate buffer (0.05M and pH 5.5) and mixed well. The mixture reaction was incubated in a water bath at 37°C and 0.2 ml of the reacting compound was withdrawn at zero time (t_0) and after 30 min of incubation time (t_1). The enzyme reaction was stopped by adding ethanol 95% (2 ml v/v). The absorbance of the t_0 and t_1 was measured using a UV spectrophotometer (Chrom Tech CT-2200 UV/Vis) at 310 nm. One unit (U) of enzyme activity was considered the amount of enzyme needed to hydrolyze one µmol of ester per one min per ml. The enzyme activity was calculated according to the following formula:

Enzyme activity (U/ml) = $114 \times [(At_0-At_1)/(t_1-t_0)]$

Where A was the absorbance, t was the time in minutes and 114 is the extinction coefficient of ester

2.13 Gallic acid concentration (GAC) determination

It was determined according to the method described by **[24].** Briefly, the culture filtrate (supernatant) was diluted to 100-fold in acetate buffer (0.2M at pH 5.0) then read at 254.6 nm and 293.8 nm 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 (A254.6) – 17.17 (A293.8). Where, A was the absorbance.

2.14 Application of co-cultures tannase as dyes decolorization

Tannase produced by co-cultures of *Aspergillus niger* ok 626231 and *Penicillium griseoroseum* ok 626651 was applied for reactive blue 19 and red 24 dyes decolorization at different concentrations (0.05 to 1.1 g) using the agar well diffusion method **[25].** These dyes at different concentrations were individually dissolved in Erlenmeyer flasks (250 ml) containing 50 ml distilled water, then 2 g agar was added, the volume was completed up to 100 ml and flasks were autoclaved at 121°C for 10 min. These flasks were poured into Petri dishes, and left to solidify at room temperature for 15 min, and the wells (5 mm in diameter) were made for each dye concentration on an individual plate. Fifty microliters of the crude enzyme were added into agar wells, and the plates left standing until the samples were fully absorbed and incubated at 37˚C for 6 h. Control plates were replaced crude enzyme with distilled water. After incubation, the clear zone formation around the well was measured which indicated the dye decolorization.

2.15 Statistical analysis

The obtained data were analyzed using IBM®SPSS® Statistics Server Version 23.0. (2015) as suggested by Duncan's Multiple Range Test at the 5% confidence level **[26].**

3 Results and Discussion

3.1 Isolation and screening of tannase producing fungi

Results in **Fig. 1** showed the number and percentage distribution of fungal isolates collected from different sources. A total of 161 fungal isolates were collected, and the widely distributed number of isolates were 75 isolates collected from soil, 44 isolates collected from seawater, and 42 isolates obtained from food (sweet potato, 2 isolates; banana, 2 isolates; tea, 37 isolates, and pomegranate, one isolate), Whereas the percentage distribution of isolates were 26.09, 27.33, and 46.58 % representing the samples collected from food, seawater, and soil, respectively**.**

3.2 Primary and secondary screening for tannaseproducing fungi on Tannic acid solid and broth medium. Results in **Table 1** showed that the 161 tested fungal isolates on solid medium supplemented with tannic acid as a sole carbon source. These isolates gave a tannase index ranging from 0.2 to 2.00 mm. These isolates were divided into 3 categories according to the tannase index. One-hundred and twenty-eight isolates were classified as low, 23 isolates were classified as moderate and 10 isolates were classified as high which tannase index ranging from 0.2 to 0.50, 0.51 to 1.00, and 1.01 to 2.00, respectively. So, the ten isolates were selected being SM4, SG29, and SQ64 were isolated from soil in Monufia, Giza, and Qalyubia governorates, and T6, T8, T11, T20, T28, and T33 were isolated from tea, and SWP33 isolated from Port Said seawater, respectively. Results in Table 2 revealed that the selected isolates gave a high tannase index, as well as clear halo zone diameter on solid medium, ranging from 1.10 to 2.00, and 19 to 75 mm respectively. The highest significant clear halo zone diameter and tannase index ($P \leq 0.05$) were recorded by T11 isolate (75 mm and 2) and followed by the SWP33 isolate (64 mm and 1.56), respectively**.** Microorganisms have the ability to break down tannic acid and form a hydrolysis zone on the screening medium to indicate the presence of tannase**[27].** Biological therapy with the organism offers a basic advantage over enzymatic methods, namely the capacity to convert a wide range of chemicals concurrently **[28].**

Tannases have been produced by fungi such as *Trichoderma* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., and *Candida* sp. **[29].** The filamentous fungi have the ability to break down the ester and depside bonds found in tannic acid to generate gallic acid and glucose as hydrolysis products according to **[30].** Furthermore, the ten isolates appeared the ability to grow and degrade tannin in broth medium, as shown in **Table 2.** The cell dry weight (CDW) ranges from 2.10 to 3.82 g/L. The tannase activity (TA), and gallic acid content (GAC), were ranging from 12.5 to 127.6 U/ml, from 33.1 to 255.4 mg/ml, with cell dry weight (CDW) ranging from 2.10 to 3.82 g/L, and tannin degradation (TD) ranging from 39.1 to 89.8%, respectively. The maximum significantly CDW (382 and 3.64 g/L), TA (127.6 and 127.2 U/ml), GAC (238.4 and 255.4 mg/ml), and TD (88.7 and 89.8%) were recorded by SWP33 and T11, respectively.

Moreover, SWP33 and T11 isolates were the most efficient isolates for tannase production using agar plates and liquid medium containing tannin as a substrate. So, these isolates were chosen for the next studies.

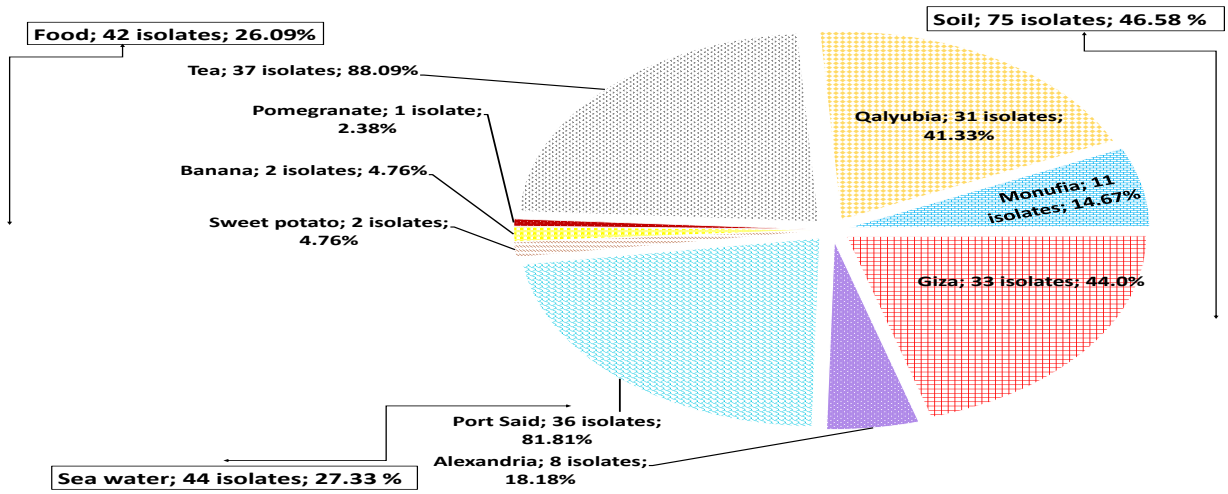

Fig.1. Number and percentage distribution of fungal isolates collected from different sources.

3.3 Phenotypic and molecular identification of the most efficient isolates

SWP33 and T11 isolates were identified based on phenotypic characteristics (morphological and cultural) at the genera level. These isolates (SWP33 and T11) belong to two different genera of *Aspergillus* and *Penicillium*, respectively following the key to **[16]**, as shown in **Fig. 2A**. For the isolate encoded SWP33, it showed conical appearance was black colonies, granular texture, and flat topography, and microscopic appearance was conidiophore of a thick-walled, smooth, large round vesicle with phialides, and metulae over entire surface and conidia of round to oval, and roughened. Furthermore, isolate encoded T11 showed conical appearance was green colonies, surface texture from granular to floccose, and microscopic appearance was hyphae septate, hyaline. Conidiophores are simple or branched. Phialides grouped in brush-like clusters (penicilli) at the ends of the conidiophores, conidia unicellular, round to ovoid, hyaline or pigmented, rough walled or smooth, in chains.

	Source of isolates	No. of isolates divided according to tannase index			
		Low ranging from 0.2-0.50	Moderate ranging from 0.51-1.00	High ranging from 1.01-2.00	Total
Food	Sweet potato	2	0	0	2
	Banana	2	0	0	2
	Pomegranate	1	0	0	1
	Tea	23	8	6	37
Soil	Qalyubia	24	6	1	31
	Monufia	10	0	1	11
	Giza	28	4	1	33
Sea water	Alexandria	7	1	0	8
	Port Said	31	4	1	36
	Total	128	23	10	161

Table 1. Number of high, moderate, and low tannase producing fungi according to tannase index on tannic acid agar medium.

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Source of isolates		On solid medium				In broth medium			
		Code of isolates	Colony growth diameter (mm)	Clear halo zone diameter (mm)	Tannase index	CDW (g/L)	TA (U/ml)	GAC (mg/ml)	TD (%)
Soil	Monufia	SM4	9	19^{h}	1.11	$2.26^{e,f}$	13.50^{g}	34.50^{g}	48.80^{h}
Soil	Giza	SG29	11	25^{f}	1.27	2.46^{d}	43.10^{e}	55.00^{f}	80.10^{f}
Soil	Qalyubia	SQ64	23	56^{e}	1.43	2.83^{c}	47.50^{d}	122.90^{d}	83.10^{e}
Food	Tea	T6	23	57^{d}	1.48	3.06^{b}	51.80^{c}	$133.70^{c,d}$	85.40^{d}
Food	Tea	T8	25	63^{c}	1.52	3.20^{b}	78.10^{b}	138.20^{c}	87.20^{c}
Food	Tea	T11	25	75^{a}	2.00	3.64^{a}	122.20^{a}	255.40^{a}	89.80^{a}
Food	Tea	T20	11	25^{f}	1.27	2.49^{d}	$44.70^{d,e}$	77.50^{e}	82.30^{e}
Food	Tea	T28	10	22^{g}	1.20	$2.43^{d,e}$	16.70^{f}	39.10^{g}	50.30^{g}
Food	Tea	T33	9	19^{h}	1.11	2.10^{f}	12.50^{g}	33.10^{g}	39.10^{i}
Sea water	Port Said	SWP33	25	64^{b}	1.56	3.82^{a}	127.60^{a}	238.40^{b}	88.70^{b}

Table 2. Screening for tannase production by the most efficient isolates on tannic acid solid and broth medium after 4 days of the fermentation period.
CDW=Cell dry weight, TA= Tannase activity, GAC= Gallic acid content, TD= Tannin degradation.
[a,b] Values with small letters above column having different superscripts are significantly different (at p≤0.05)

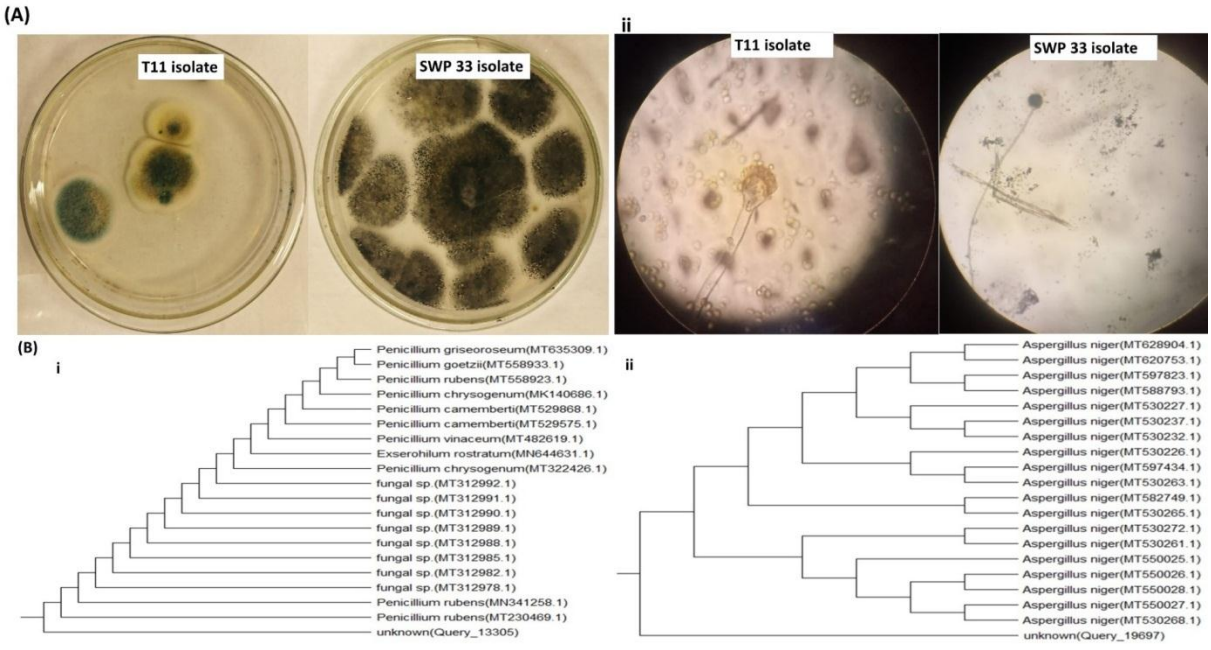

Fig. 2. Identification of the selected fungi (T11 and SWP33) based on
(A) the colonies shape on PDA medium (i) and light microscopic photos (ii) at magnification power (400X) for morphological examination.
(B) Neighbor-joining tree based on 18S rRNA sequences of the genera *Penicillium* (i) and *Aspergillus* (ii) obtained from BLAST search showing the position of isolates and related strains.

The identification of two selected fungal isolates (SWP33 and T11) was confirmed by using the 18S rRNA gene sequencing were aligned to those available in the GenBank database using the BLAST (Basic Local Alignment Search Tool) Program and phylogenetic analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi?). The phylogenetic trees were displayed using the TREEVIEW program (**Fig. 2B**). Isolate T11 had a sequence identity of 100 % with *Penicillium*

griseoroseum MT635309.1, while SWP33 isolate was close identity (99%) to *Aspergillus niger*. MT628904.1. Therefore, these strains were designated as *Penicillium griseoroseum* T11 and *Aspergillus niger* SWP33. The sequences of the tested strains were subsequently deposited in GenBank as *Penicillium griseoroseum* T11 (GenBank accession no. ok 626651), and *Aspergillus niger* SWP33 (GenBank accession no. ok 626231), respectively.

This confirms the results recorded by **[30]**, that *Aspergillus* sp. and *Penicillium* sp. were fungi that can biodegrade condensed and hydrolyzable tannins. Likewise, **[31]** found that *Aspergillus* sp. and *Penicillium* sp. were the best strains that produced tannase and gallic acid in SmF and SSF.

3.4 Fungal growth and tannase production.

The samples were taken daily during the 6 days of the cultivation period. The selected fungal strains were cultivated in tannic acid broth medium at 28 °C for 6 days. Data illustrated by **Fig. (3A-i)** showed that *A. niger* ok 626231 and *P. griseoroseum* ok 626651 grew exponentially during the first 2 and 3 days of fermentation period, respectively. The growth of both strains was found to be more constant (stationary phase) for 3-5 days and 4-5 days, then began to slightly decrease (decline phase). Whereas the maximum amount of cell dry weight (3.49 and 2.91 g/L) of *A. niger* ok 626231 and *P. griseoroseum* ok 626651 were recorded on the 4th day and 5th day, respectively Data in **Fig. (3A-ii)** also showed a high determination coefficient between the growth of *A. niger* ok 626231 and *P. griseoroseum* ok 626651 and fermentation period at log phase with R^2 values to be 0.99 and 0.98, respectively. The exponential equation ($y=0.1122e^{1.55x}$ and $y= 0.048e^{1.34x}$) represented in **Fig. (3A-ii)** expressed of the specific growth rate (μG) of both *A. niger* ok 626231 and *P. griseoroseum* ok 626651 was 1.55 and 1.34 d^{-1}**.** The growth parameters (doubling time (t_d) and multiplication rate (MR)) of *A. niger* ok 626231 (0.45 d^{-1} and 2.24) and *P. griseoroseum* ok 626651 (0.52 d^{-1} and 1.93) were calculated at the log phase of the growth curves from μG **(Fig. 3A-iii)**

Whereas the maximum amount of enzyme activity of (147.0 and 148.7 U/ml) *A. niger* ok 626231 and *P. griseoroseum* ok 626651 were recorded on the 4th day and 5th day, respectively **(Fig. 3B-i)**. Data in **Fig. (3B-ii)** showed a high determination coefficient ranging between 0.95 to 0.99 between the enzyme production by *A. niger* ok 626231 and *P. griseoroseum* ok 626651, and fermentation period at log phase. Also, the exponential equation ($y= 7.0465e^{0.79x}$ and $y= 5.9599e^{0.66x}$) expressed the specific enzyme production rate (μE) of both *A. niger* ok 626231 and *P. griseoroseum* ok 626651 was 0.79 and 0.66 d^{-1}**.**

As well the maximum concentration of gallic acid produced by both strains of *A. niger* ok 626231 and *P. griseoroseum* ok 626651 were recorded on the 4th day and 5th day, respectively **(Fig. 3C-i)**. The specific gallic acid production rates (μA) of *A. niger* ok 626231 and *P. griseoroseum* ok 626651 were 0.21 and 0.24 d^{-1} which were achieved from the exponential equation $105.05e^{0.21x}$ and $75.274e^{0.24x}$ with high R^2 of 0.96 to 0.99, respectively **(Fig. 3C-ii)**. These results are in agreement with those of **[32]** who found that the best incubation period was 4 days for tannase production from *A. niger* FETL FT3 using a shake-flask technique.

3.5 Tannase and gallic acid production using tannin-rich raw materials under submerged and solid-state fermentation by the selected strains

The tannin content was determined in eleven tannin-rich raw materials used in this study were tea (0.033 g/L), clove (0.031g/L), pomegranate peel (0.029 g/L), thyme (0.025 g/L), mulberry leaves (0.011 g/L), guava leaves (0.010 g/L), chard leaves (0.010 g/L), banana peel (0.008 g/L), sesame (0.006 g/L), camphor (0.005 g/L), and wheat bran (0.004 g/L).

The selected strains *A. niger* SWP33 and *P. griseoroseum* T11 were cultivated on these materials as a sole carbon source under solid-state fermentation (SSF) and submerged fermentation (SmF).

In the case of cultivation by SmF technique, results are tabulated in **Table (3)** showed that the ability of the tested strains *A. niger* SWP33 and *P. griseoroseum* T11 to grow in medium contained tannin-rich agricultural wastes extract using SmF and give biomass yield ranging from 2.075 to 4.040 g/L, and from 2.855 to 3.872 g/L, tannase activity ranging from 12.5 to 155.8 U/ml, and from 26.5 to 151.3 U/ml, and gallic acid content from 39.2 to 258.8 mg/ml, and from 57.6 to 245.1 mg/ml with tannin degradation ranging from 6.4 to 57.2, and 15.5 to 55.4 %, respectively. Pomegranate peel extract (151.3

U/ml, and 245.1 mg/ml) was found to be significantly the best agricultural waste followed by guava leaves (149.5 U/ml, and 241.6 mg/ml), and banana peel (143.5 U/ml, and 235.8 mg/ml) for tannase, and gallic acid production by *A. niger* SWP33 with tannin degradation percentage of 55.4, 52.5, and 44.1 %, and the cell dry weight reached to 3.872, 3.843, and 3.830 g/L, respectively.

Whereas guava leaves extract (155.8 U/ml, and 258.8 mg/ml) was found to be significantly the best agricultural waste followed by banana peel extract (151.4 U/ml, and 255.0 mg/ml), and pomegranate peel extract (148.3 U/ml, and 247.4 mg/ml) for tannase, and gallic acid production by *P. griseoroseum* T11 with tannin degradation percentage of 57.2, 53.6, and 49.2% and the cell dry weigh reached to 4.040, 3.935, and 3.910 g/L, respectively.

The lowest percentage of tannin degradation of 15.5, and 6.4 % by *A. niger* SWP33 and *P. griseoroseum* T11 was exhibited in the presence of camphor, and clove extracts with cell dry weight of 2.855, and 2.075 g/L, enzyme activity of 26.5, and 12.5 U/ml, and gallic acid content of 57.6, and 39.2 mg/ml, respectively.

In the case of cultivation by SSF technique, results in **Table (3)** showed that the capability of *A. niger* SWP33 and *P. griseoroseum* T11 to grow on agricultural wastes of pomegranate peel, guava leaves, and banana peel, and degrade with percentage reached to range from 68.1 to 72.4 %, and from 65.8 to 77.8 %. Likewise, both strains *A. niger* SWP33 and *P. griseoroseum* T11 gave more significant amounts of cell dry weight (ranging from 2.12 to 2.155 g/L, and from 1.93 to 2.37 g/L), tannase activity (ranging from 177.2 to 185.7 U/ml, and from 161.7 to 179.5 U/ml), and gallic acid content (ranging from 259.7 to 268.0 mg/ml, and from 267.2 to 273.5 mg/ml) when cultivated on pomegranate peel, guava leaves, and banana peel, respectively.

Whereas the ability of *A. niger* SWP33 and *P. griseoroseum* T11 to cultivate on camphor, and clove wastes were low with cell dry weight of 1.93, and 1.32 g/L, the degradation percentage reached up to 38, and 9.2 %, and then led to a decrease in the production of the enzyme (72.5, and 21.4 U/ml), and gallic acid (111.7, and 86.8 mg/ml), respectively.

From the above results, it could be observed that the SSF was more preferred technique for cultivating *A. niger* SWP33 and *P. griseoroseum* T11 and producing enzyme on the best wastes of pomegranate peel, guava leaves, and banana peel than SmF which increased approximately ranging from 24.4 to 54.4-fold of tannin degradation. Furthermore, the SSF technique was selected for further study.

Moreover, the above results indicated that the maximum amounts of enzyme and gallic acid were observed when *A. niger* SWP33 and *P. griseoroseum* T11 were cultivated on pomegranate peel, and guava leaves and the enzyme activity was increased about from 9 to 23.4-fold, and gallic acid from 5.6 to 10.1-fold as compared to other wastes under SSF**,** respectively.

This confirms the results recorded by **[33]** that the production of tannase can be done by using agro-industrial wastes as substrates either by SmF, or SSF from bacteria, yeasts, and fungi. In addition, **[7]** reported that SSF of pomegranate peel led to the maximum production of tannase activity, and gallic acid was 2.8 and 4.4-fold, and 1.1-fold over increase by *A. niger* A8 compared to the liquid surface fermentation (LSF), and SmF, respectively.

So, the wastes of pomegranate peel, banana peel, and guava leaves were selected for the next study.

3.6 Effect of different inoculum sizes of single and mixed cultures on tannase and gallic acid production.

In the case of inoculation of pomegranate peels, guava leaves, and banana peels with *A. niger* SWP33 or *P. griseoroseum* T11 as a monoculture at different concentrations ranging from 3-10% of inoculum size under solid-state fermentation.

Results in **Fig. (4 A and B)** revealed that the inoculum size of *A. niger* SWP33 and *P. griseoroseum* T11 at 6, and 7 % (v/v) was the best ones for the enzyme and gallic acid production, respectively.

Also, results in **Fig. (4 A and B)** exhibited that the enzyme and gallic acid production were significantly increased by increasing the inoculum size of *A. niger* SWP33 or *P. griseoroseum* T11 up to 6, and 7% (v/v) when inoculated the tested agricultural wastes, respectively. The highest pomegranate peel, guava leaves, and banana peel tannin degradation were reached to 79.2, 75.4, and 68.6 when their inoculated 6% (v/v) of *A. niger* SWP33 and reached 66.80, 75.80, and 69.25% when

their inoculated 7% (v/v) of *P. griseoroseum*, respectively.

Results also indicated that the highest enzyme and gallic acid production appeared in presence of pomegranate peel, and guava leaves inoculated with a monoculture of *A. niger* SWP33 or *P. griseoroseum* T11, respectively.

Results in **Fig. (4C)** show that the inoculation of tested wastes with co-cultures of *A. niger* SWP33 and *P. griseoroseum* T11 at a ratio of inoculum sizes (1.5:1.5, 2.0:2.0, 2.5:2.5, 3.0:3.0, 3.5:3.5, 4.0:4.0, 4.5:4.5, and 5.0:5.0 v/v) was increased the enzyme, and gallic acid production more than inoculated with a monoculture. The significantly maximum production of the enzyme (ranging from 197.4 to 232.3 U/ml), gallic acid (ranging from 286.7 to 329.1 mg/ml), and percentage of tannin degradation (ranging from 83.5 to 89.4%) were recorded when pomegranate peel, guava leaves, and banana peel were inoculated with co-cultures at inoculum size ratio of 3.5:3.5% (v/v). The banana peel was more preferred for the enzyme and gallic acid production after inoculation with co-cultures.

Tannase produced by co-cultures of *A. niger* SWP33 and *P. griseoroseum* T11 was applied for decolorizing reactive blue 19 (RB19) and red 24 (RR24) dyes at different concentrations (0.5 – 1.2 %) using the agar well diffusion method. Results in Fig. (5) and Table (4) indicated that the ability of the fungal enzymes to degrade textile dyes of RB19 and RR24 with various concentrations gave a clear inhibition zone with a diameter ranging from 10 to 25, and 10 to 30, respectively. The significantly largest zone diameter was recorded at 0.8 mg/L concentration of RB19, and 0.4, and 0.5 mg/L concentrations of RR24 after 6 h of the incubation period.

Furthermore, results also indicated that fungal tannase activity was more efficient for decolorizing of RR24 textile dye than RB19 textile dye.

These results coincided with **[34]** who said that laccase and tannase from endophytic fungi were the most eco-friendly, and cost-effective dye removal techniques.

3.7 Application of co-cultures of tannase as dyes decolorization

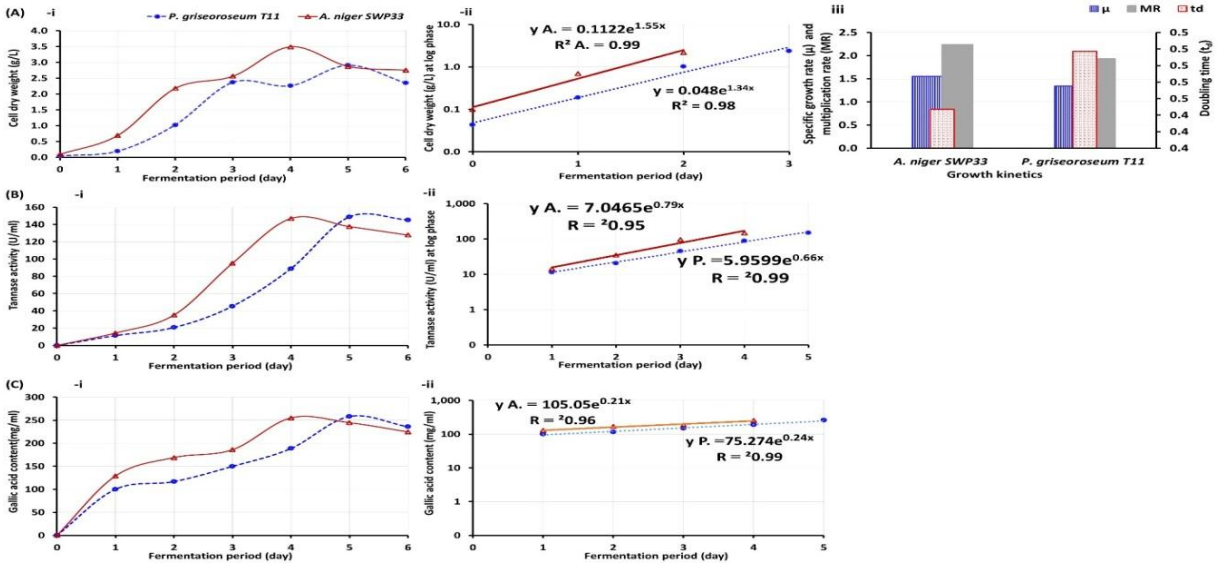

Fig. 3. Fungal growth (A), tannase production (B) and gallic acid content (C) profiles of *Penicillium griseoroseum* T11 and *Aspergillus niger* SWP33 cultivated on tannic acid broth medium at 28°C during 6 days of fermentation period using submerged culture.

i- Growth, tannase production, and gallic acid content curves.
ii- Determination coefficient between fermentation period and each of cell dry weight, enzyme production, or gallic acid content at log phase for both strains.
iii- Growth kinetics of both strains.

Table (3). Biomass, tannase, and gallic acid production by *Penicillium griseoroseum* T11 and *Aspergillus niger* SWP33 using agricultural wastes as a carbon source under submerged and solid-state fermentation.

Cultivation method	Agricultural waste extracts	*Penicillium griseoroseum* T11				*Aspergillus niger* SWP33			
		CDW (g/L)	TA (U/ml)	GAC (mg/ml)	TD (%)	CDW (g/L)	TA (U/ml)	GAC (mg/ml)	TD (%)
Submerged fermentation	**Guava leaves**	4.040^{a}	155.8^{a}	258.8^{a}	57.2^{a}	3.843^{b}	149.5^{b}	241.6^{b}	52.5^{b}
	Banana peel	3.935^{a}	151.4^{b}	255^{b}	53.6^{b}	3.830^{b}	143.5^{c}	235.8^{c}	44.1^{c}
	Pomegranate peel	3.910^{a}	148.3^{b}	247.4^{c}	49.2^{c}	3.872^{a}	151.3^{a}	245.1^{a}	55.4^{a}
	Tea dregs	3.700^{b}	121.6^{c}	231.6^{d}	42.4^{d}	3.780^{c}	130.5^{d}	227.7^{d}	38.3^{d}
	Thyme	3.510^{c}	98.3^{d}	188.7^{e}	34.3^{e}	3.763^{c}	117.2^{e}	209.8^{e}	36.4^{e}
	Mulberry leaves	3.370^{d}	51.4^{e}	109.7^{f}	30.6^{f}	3.470^{e}	54.4^{g}	98.8^{g}	28^{g}
	Chard leaves	3.320^{d}	51.2^{e}	102.3^{g}	29.8^{g}	3.520^{d}	58.8^{f}	103.9^{f}	32.4^{f}
	Camphor	3.150^{e}	45.3^{f}	102.3^{g}	22.5^{h}	2.855^{i}	26.5^{k}	57.6^{j}	15.5^{j}
	Sesame	$3.085^{e,f}$	42.9^{f}	88.2^{h}	20.4^{i}	3.130^{g}	44.5^{i}	88.8^{h}	24.2^{h}
	Wheat Bran	2.970^{f}	42.1^{f}	80.8^{i}	20.1^{i}	3.240^{f}	52.2^{h}	92.5^{h}	26.7^{g}
	Clove	2.075^{g}	12.5^{g}	39.2^{j}	6.4^{j}	3.070^{h}	34.5^{j}	64.6^{i}	18.6^{i}
Solid-state fermentation	**Guava leaves**	2.370^{a}	179.5^{a}	273.5^{a}	77.8^{a}	2.140^{b}	182.4^{b}	261.8^{b}	$70.6^{a,b}$
	Banana peel	2.035^{b}	174.2^{b}	$270.7^{a,b}$	66.7^{b}	2.120^{c}	177.2^{c}	259.7^{b}	$68.1^{b,c}$
	Pomegranate peel	1.930^{b}	161.7^{c}	267.2^{b}	65.8^{b}	2.155^{a}	185.7^{a}	268^{a}	72.4^{a}
	Tea dregs	1.650^{c}	159.2^{d}	246.8^{c}	63.6^{c}	2.115^{c}	172.3^{d}	261.2^{b}	66.2^{c}
	Thyme	1.600^{c}	141.9^{e}	222.4^{d}	56.9^{d}	2.030^{d}	160.5^{e}	230.6^{c}	61^{d}
	Mulberry leaves	$1.550^{c,d}$	77.2^{f}	183.5^{e}	55.1^{e}	1.970^{f}	83.4^{g}	203.2^{e}	50.7^{e}
	Chard leaves	$1.445^{d,e}$	71.5^{g}	174.9^{f}	52.6^{f}	2.010^{e}	85.6^{f}	218.5^{d}	50.8^{e}
	Camphor	$1.420^{e,f}$	62.5^{h}	134.7^{g}	39.4^{g}	1.925^{h}	72.5^{k}	111.7^{i}	38^{h}
	Sesame	$1.410^{e,f}$	56.2^{i}	122.3^{h}	25.9^{h}	1.950^{g}	77.6^{i}	160.4^{h}	45.6^{f}
	Wheat Bran	$1.370^{e,f}$	49.7^{j}	101.7^{i}	22.6^{i}	1.950^{g}	79.1^{h}	175.9^{f}	49.5^{e}
	Clove	1.320^{f}	21.4^{k}	86.8^{j}	9.2^{j}	1.935^{h}	75.9^{j}	166.7^{g}	40.9^{g}

CDW= Cell dry weight, TA= Tannase activity, GAC= Gallic acid content, TD= Tannin degradation. ^{a,b}-Means followed by different letters are significantly different at $P< 0.05$ level.

Table (4). Dye decolorization by *A. niger* SWP33 and *P. griseoroseum* T11 co-cultures tannase at 37˚C for 6 hrs of incubation period.

Textile dye concentrations (%)	Inhibition zone diameter (mm)	
	Reactive blue 19	Reactive red 24
0.05	10^{i}	18^{f}
0.10	12^{h}	20^{e}
0.20	15^{g}	20^{e}
0.30	17^{f}	23^{d}
0.40	17^{f}	30^{a}
0.50	18^{e}	30^{a}
0.60	18^{e}	28^{b}
0.70	20^{c}	27^{c}
0.80	25^{a}	15^{g}

0.90	22[b]	12[h]
1.00	22[b]	12[h]
1.10	19[d]	10[i]

The well diameter was 5 mm, [a,b]-Means followed by different letters in the same column are significantly different at *P*< 0.05 level.

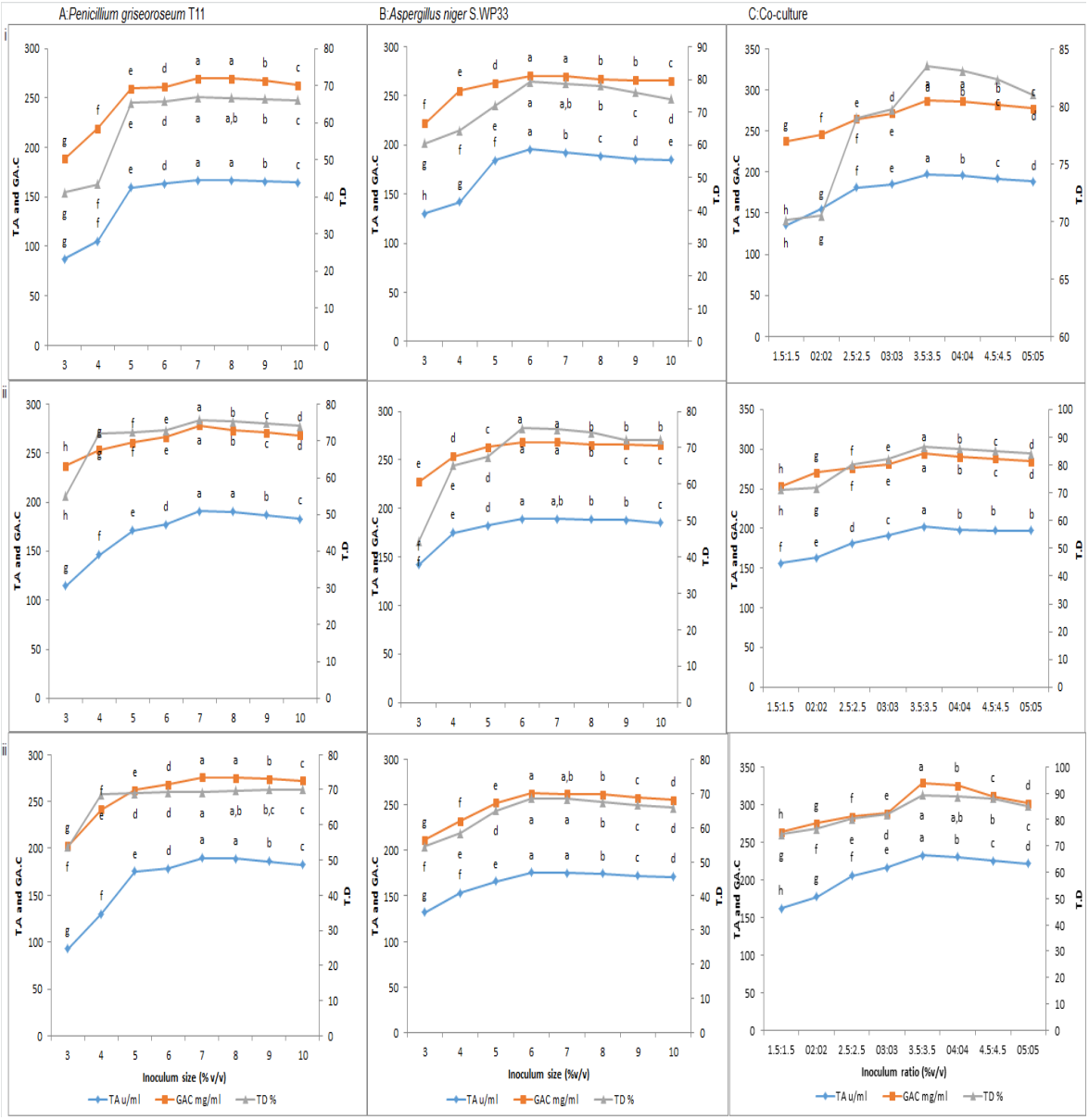

Fig. 4. Effect of inoculum size of mono-cultures and co-cultures on tannase and gallic acid production under solid-state fermentation.

i. pomegranate peel
ii. guava leaves
iii. banana peel

TA= Tannase activity, GAC= Gallic acid content, TD= Tannin degradation.

[a,b] Values with small letters above column having different superscripts are significantly different (at p≤0.05).

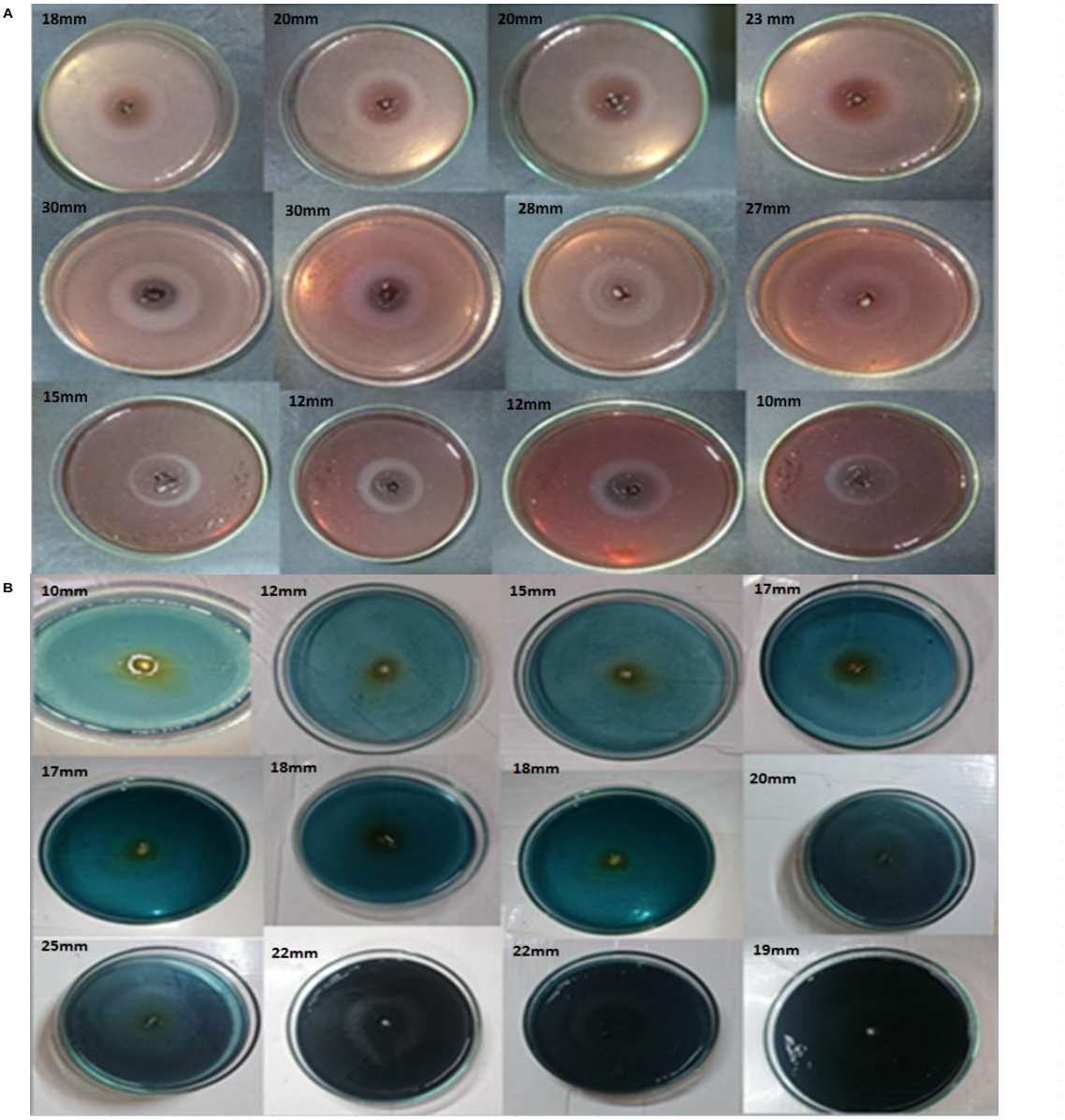

Fig. 5. *A. niger* SWP33 and *P. griseoroseum* T11 tannase activity against (A) reactive red 24 and (B) reactive blue 19 textile dyes at different concentrations (0.5 – 1.1 %) using agar well diffusion method.

4 Conclusions

Based on the results of this study, it could be concluded that the both *A. niger* SWP33 and *P. griseoroseum* T11 strains have the potential to degrade tannin-rich agricultural waste through synthesizing tannase and gallic acid using submerged and solid-state fermentation approaches. The solid-state fermentation approach was preferred by tested strains for the synthesis of tannase and gallic acid over the submerged fermentation technique using wastes of pomegranate peel, guava leaves, and banana peel. During a 6-hours incubation time, fungal tannase has the potential to decolorize textile dyes. As a result, this enzyme is thought to be eco-friendly, and it may be used in the future in the environmental remediation of dye effluents to remove pollutants.

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الملخص العربي

إنتاج التانيز وحمض الجاليك بواسطة *Penicillium griseoroseum* T11 و *Aspergillus niger* SWP33 باستخدام النفايات الزراعية تحت التخمير المغمور والصلب وتطبيقاته

في هذا العمل ، يمكن استخدام المصادر الميكروبية في التخليق التكنولوجي الحيوي للتانيز. يُفضل التانيزات الميكروبية لأنها أكثر ثباتًا وانتاجها بعوائد اعلي من تلك التي تم الحصول عليها من المصادر الأخرى ، بالاضافة الي ذلك ، يمكن ان يتعرضوا للتلاعب الجيني بسهولة أكبر من النباتات والحيوانات. ويستخدم التانيز في العديد من الصناعات ، بما في ذلك الاغذية والتكنولوجيا الحيوية البيئية. من بين ١٦١ عزلة فطرية تم اختيار اثنتين (، تم 18S rRNA). بناءً على السمات المظهرية والجينية (التسلسل الجيني GA) لإنتاج المزيد من التانيز وحمض الجاليك (T11 و SWP33(. وقد تم تحقيق أقصى إنتاج من التاناز T11 *Penicillium griseoroseum* و SWP33 *Aspergillus niger*تعريف هذه العزلات على أنها في وجود T11 *P. griseoroseum* و SWP33 *A. niger*(١٤٧.٠ و ١٤٨.٧ وحدة / مل) وحمض الجاليك (٢٥٥ و ٢٥٨ مجم / مل) بواسطة حمض التانيك في اليومين الرابع والخامس من التخمر ، مع تحديد معدلات الإنزيم و حمض الجاليك ٠.٧٩ و ٠.٦٦ د$^{-1}$ و ٠.٢١ و ٠.٢٤ د$^{-1}$ على التوالي. عندما تزرع على نفايات غنية بالتانين كوسيط منخفض التكلفة لإنتاج التانيز و حمض الجاليك ، فضلت السلالات المختبرة تخمير الحالة الصلبة أكثر من التخمير المغمور. وخلال ٦ ساعات من الحضانة ، أظهر التانيز الفطري نشاطًا في إزالة اللون الأزرق التفاعلي ١٩ والأحمر ٢٤ من أصباغ النسيج.