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ARTICLE

Mucoromycota tannases: activity screening, production and enzyme purification

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ABSTRACT Tannases are industrial enzymes that catalyze the degradation of hydrolysable and complex tannins into gallic acid and glucose. While many filamentous fungi are known high-yield producers, members of Mucoromycota remain less studied regarding tannase production. This study screened Mucoromycota isolates for tannase production using plate tests with tannic acid as an enzyme production inducer. Isolates of *Rhizomucor miehei*, *Mucor corticolus*, *Mucor lusitanicus*, *Rhizopus microsporus* var. *oligosporus*, and *Rhizopus oryzae* exhibited tolerance to the inducer, with tannase production detected in most strains post-incubation. Tannic acid effectively induced tannase production under both submerged fermentation (SmF) and solid-state fermentation (SSF) conditions. In SSF, wheat bran as a substrate and Czapek-Dox solution as a moisturizing additive supported enzyme production. The highest tannase activity in SSF was observed in *R. microsporus* var. *oligosporus* and *M. corticolus*, while *R. miehei* excelled under SmF conditions. Tris buffer extraction followed by anion exchange chromatography yielded tannase-active protein fractions from *M. corticolus*. The isolated tannase exhibited optimal activity at 30 °C. These findings highlight Mucoromycota fungi as promising tannase producers for future research. **Acta Biol Szeged 68(2):77-87 (2024)**

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Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible hydrolase enzyme possessing esterase and depsidase activities, thus hydrolyzing ester bonds between galloyl groups and polyhydroxy alcohols and depside bonds between two galloyl groups. The enzyme plays a role in the biodegradation of gallotannins, ellagitannins and complex tannins (Aguilar et al. 2007; Kasieczka-Burnecka et al. 2007). The inducer molecules for enzyme production are mostly phenolic compounds such as tannic acid, gallic acid, methyl gallate or pyrogallol. Tannases are found in plants and animals, but the main producers are among the microorganisms.

Tannases are frequently applied in the leather, pharmaceutical, cosmetics and food industries. They are mainly used in the production of tea, acorn liqueur and gallic acid, and can also be used in the production of fruit juices and flavored coffee drinks (Chávez-González et al. 2012; Jana et al. 2015; Das Mohapatra et al. 2020; Ong and Annuar 2024). The use of tannase for bioremediation is also known. In environmental biotechnology, for example, the high polyphenol content of wastewater effluents from tanneries can be reduced by tannase treatment (Aissam et al. 2005). Agro-industrial waste residues can also contain significant amounts of tannins, which can lead to environmental contamination without proper tannin removal (Kumar et al. 2019).

Fungi are one of the most important producers of tannase, and many studies have been carried out on the conditions of enzyme production, the identification of new producers and the properties of the enzymes produced. Microbial tannases consist of single or more subunits, and the molecular weight of tannases obtained from fungi ranges from 45 to 310 kDa. Among filamentous fungi, *Aspergillus* and *Penicillium* species are well-known tannase producers (Aharwar and Parihar 2018). For instance, several recent studies were focused on tannases from *Aspergillus niger* (Shao et al. 2020; Papadaki and Mantzouridou 2024) and various *Penicillium* isolates (Andrade et al. 2021; Mostafa 2023). Other fungi also

Table 1. Tannase production of Mucoromycota fungi.

Fungus	Fermentation condition	Solid substrate/ broth	Fermentation temperature	Fermentation pH	Tannase activity	Reference
Rhizopus oryzae	modified solid-state	*Terminalia chebula* and *Caesalpinia digyna* residues + Czapek-Dox solution	30 °C	4.5	31.8 U/ml	Mukherjee and Banerjee 2004
Rhizopus oryzae	solid-state	Wheat bran + Czapek-Dox solution + tannic acid	40 °C	5.0	~300-1200 U	Chatterjee et al. 1996
Rhizopus oryzae	submerged	Czapek-Dox solution + tannic acid	-	5.0	6.12 U/ml	Hadi et al. 1994
Rhizopus oryzae	submerged	Czapek-Dox solution + tannic acid + *Shorea robusta* seed	30 °C	5.0	17.17 U/ml	Hota et al. 2007
Syncephalastrum racemosum	submerged	Czapek-Dox solution + tannic acid	30 °C	-	4.19 U/ml	Bradoo et al. 1996
Rhizopus oligosporus	solid-state	cranberry pomace	28 °C	-	-[1]	Vattem and Shetty 2002
Mucor circinelloides	solid-state	pomegranate rind	30 °C	5.5	2.07 IU	El-Refai et al. 2017

[1] enzyme activity was detected through HPLC determination of diphenyls.

possess tannase activity, such as *Candida*, *Cryphonectria*, *Debaryomyces*, *Fusarium*, *Hyalopus*, *Verticillium*, *Aureobasidium* and some species of the genus *Rhizopus* (Bradoo et al. 1997; Yao et al. 2014; Kumar et al. 2019; Abd-Elmotey et al. 2022). The optimum pH and temperature for most fungal tannase activities ranged between pH 4.0 and 8.0, and 40 °C and 70 °C, respectively.

Although the Mucoromycota fungi (i.e., members of the former Zygomycota) are excellent producers of different exoenzymes (Papp et al. 2016), there is little information available on the tannase production and activity of this fungal group. Some previous studies and important data on the tannase production of Mucoromycota fungi are summarized in Table 1. Various submerged fermentation (SmF) and solid-state fermentation (SSF) processes were used for production, mostly using tannic acid inducer and some plant growth substrate. Previous studies have mainly investigated the tannase production by *Rhizopus oryzae*, *Rhizopus oligosporus*, *Syncephalastrum racemosum* and *Mucor circinelloides* isolates. Most of the studies have been carried out with fungi of the genus *Rhizopus*, and the fermentation medium contained Czapek-Dox nutrient solution as an additive in many cases (Table 1). The optimum temperature of the tannases produced was generally 30 °C, while their pH optimum ranged from 4.5 to 5.5. High tannase activity (31.8 U/ml) was achieved for *R. oryzae* using plant residues as substrates (Mukherjee and Banerjee 2004) (Table 1).

For tannase production, SmF is commonly used, however, SSF systems seem to be widely applied due to their many favorable properties. Production is more advantageous in SSF systems where the fungus secretes the protein into the solid matrix providing a more efficient and easy-to-perform extraction. In addition, higher productivity and enzyme activity, increased stability of the enzyme to pH and temperature changes have been reported in SSF systems (Aguilar and Gutiérrez-Sánchez 2001; Cruz-Hernández et al. 2006). It is also important to note that the application of SSF systems can reduce the water and energy consumption, as well as the wastewater release resulting in a more ecofriendly production (Chávez-González et al. 2012; Aharwar and Parihar 2018). Substrates with high tannin content are mostly used as fermenting material in SSF systems (Jana et al. 2012), in which a mineral solution cocktail is used as moisturizing solution. Wheat bran, sugarcane bagasse, sorghum, sumac and chestnut residues are among the substrates that have been used for production (Aguilar et al. 2007). Fermentation experiments on red wine grapes were conducted in the study of Meini et al. (2021). The costs of tannase production can be reduced by using microorganisms that can produce tannase by utilizing cheap agricultural waste. In addition, optimization of tannase purification is also a key factor in reducing the production costs (Aguilar and Gutiérrez-Sánchez 2001; Aharwar and Parihar 2018).

This work describes the screening of Mucoromycota isolates for their tannase activity, including analysis and optimization of enzyme production under SmF and SSF conditions. In addition, an attempt was made on the isolation and characterization of tannase from selected producer strain.

Materials and Methods

Microorganisms

Fungal strains of *Rhizomucor miehei* SZMC 11005, *Gilbertella persicaria* SZMC 11086, *Mucor corticolus* SZMC 12031, *Rhizopus oryzae* SZMC 13611, *Rhizopus microsporus* var. *oligosporus* SZMC 13622, *Syncephalastrum racemosum* SZMC 22672 and *Mucor lusitanicus* SZMC 12028 were involved in the study. The isolates were obtained from the Szeged Microbiology Collection (SZMC, Szeged, Hungary). The fungi were maintained on potato dextrose agar (PDA, VWR International, USA) medium supplemented with 0.01 m/v% tannic acid (Sigma-Aldrich, Germany) and stored at 4 °C until use. Due to its sensitivity to heat, the tannic acid was sterilized using a syringe filter (Millex-GV, PVDF, 0.22 μm, Merck Millipore Ltd., Ireland) and added to the cooled medium.

Screening tests for tannase activity

Malt extract medium supplemented with tannic acid was used to screen fungal isolates for tannase production. The culture medium (yeast extract 0.25 m/v%, malt extract 0.5 v/v% from 20 v/v% stock solution, glucose 1 m/v%, agar 2 m/v%) was sterilized (120 °C, 30 min), and after cooling tannic acid (Sigma-Aldrich, Germany) stock solution (1 m/v%) filtered through a syringe filter (Millex-GV, PVDF, 0.22 μm, Merck Millipore Ltd., Ireland) was added at 0.1 m/v% final concentration. The culture medium prepared in a Petri dish was inoculated at one point with 50 μl of a suspension containing 10^7 spores previously washed from a culture grown on 0.01 m/v% tannic acid PDA. Subsequently, the cultures were incubated at 25 °C (*M. lusitanicus*), 28 °C (*M. corticolus* and *R. microsporus* var. *oligosporus*), or 37 °C (*R. miehei* and *R. oryzae*) until reaching of a colony diameter of 2-5 cm. After incubation, a 0.01 M iron(III) chloride solution was poured onto the colonies and the plates were allowed to stand at room temperature for 5-10 min. The iron(III) chloride forms a brown complex with the tannic acid, and enzymatic degradation of the tannic acid thus results in a clear zone around the fungal colonies. All inoculations were performed in three biological replicates.

Submerged fermentation tests

Yeast-peptone medium (peptone 0.5 m/v%, yeast extract 0.3 m/v%) without tannic acid or supplemented with 0.1 m/v% or 0.5 m/v% tannic acid (Sigma-Aldrich, Germany) was used to test the tannase production in SmF condition. Firstly, a volume of 15 ml of tannic acid free medium was transferred to 50-ml Erlenmeyer flasks. After sterilization (121 °C, 30 min), media were cooled down to room temperature, and filter sterilized (Millex-GV, PVDF, 0.22 μm, Merck Millipore Ltd., Ireland) tannic acid solution was added to each flask at appropriate concentration. Flasks were then inoculated with 10^6 sporangiospores obtained from cultures grown at inductive condition (PDA contained 0.01 m/v% tannic acid). Flasks were then incubated under continuous shaking (130 rpm) for 7 days at the optimal growth temperature of the fungi. Sampling (1 ml) was performed on days 0th, 4th and 7th in sterile Eppendorf tubes. Samples were centrifuged (14,000×*g*, 10 min) and the supernatants were stored at -20 °C until enzyme activity measurement. Three biological replicates were prepared from each cultivating system.

Solid-state fermentation tests

Wheat bran was used as a growth substrate in the SSF tests. Fermentation was carried out in 100-ml Erlenmeyer flasks testing different SSF systems for production. For the SSF1 system, 5 g of wheat bran were moisturized with 4 ml of Czapek-Dox solution ($NaNO_3$ 0.3 m/v%, KH_2PO_4 0.12 m/v%, $MgSO_4 \times 7H_2O$ 0.06 m/v%, KCl 0.06 m/v%). After sterilization (120 °C, 30 min), 1 ml of tannic acid (Sigma-Aldrich, Germany) stock solution (12.5 m/v%) filtered through a syringe filter (Millex-GV, PVDF, 0.22 μm, Merck Millipore Ltd., Ireland) was added to the moisturized wheat bran. For the tannic acid free fermentation system, 5 g wheat bran was moisturized with 5 ml of Czapek-Dox solution ($NaNO_3$ 0.25 m/v%, KH_2PO_4 0.1 m/v%, $MgSO_4 \times 7H_2O$ 0.05 m/v%, KCl 0.05 m/v%). The concentration of the components of the Czapek-Dox solution was thus the same in the two fermentation systems. Inoculation was performed with 200 μl of sporangiospore suspension (10^6 spores/ml) previously prepared from colonies adapted to tannic acid on PDA contained 0.01 m/v% tannic acid. The SSF systems were incubated for 5 days at temperatures depending on the culturing requirements of the tested fungus. Subsequently, extraction was performed with 30 ml sodium acetate buffer (50 mM, pH 6.0) and the flasks were allowed to stand for 24 h at 4 °C. The extract was then filtered on gauze and centrifuged (5040×*g*, 20 min, 4 °C).

The SSF2 system (Meini et al. 2021) consisted of 5 g wheat bran moisturized with 16 ml of Czapek-Dox solution ($NaNO_3$ 0.3 m/v%, KH_2PO_4 0.12 m/v%, $MgSO_4 \times 7H_2O$ 0.06 m/v%, KCl 0.06 m/v%). In this fermentation, a volume of 4 ml of filtered (Millex-GV, PVDF, 0.22 μm, Merck Millipore Ltd., Ireland) tannic acid (Sigma-Aldrich, Germany) stock solution (12.5 m/v%) was introduced after sterilization (120 °C, 30 min). Wheat bran moisturized with 20 ml of tannic acid free Czapek-Dox solution ($NaNO_3$ 0.25 m/v%, KH_2PO_4 0.1 m/v%, $MgSO_4 \times 7H_2O$ 0.05 m/v%, KCl 0.05 m/v%) was considered as a control medium. Flasks were inoculated with 200 μl sporangiospores (10^6 spores/ml) and incubated for three days at temperatures optimal for growth of each isolate

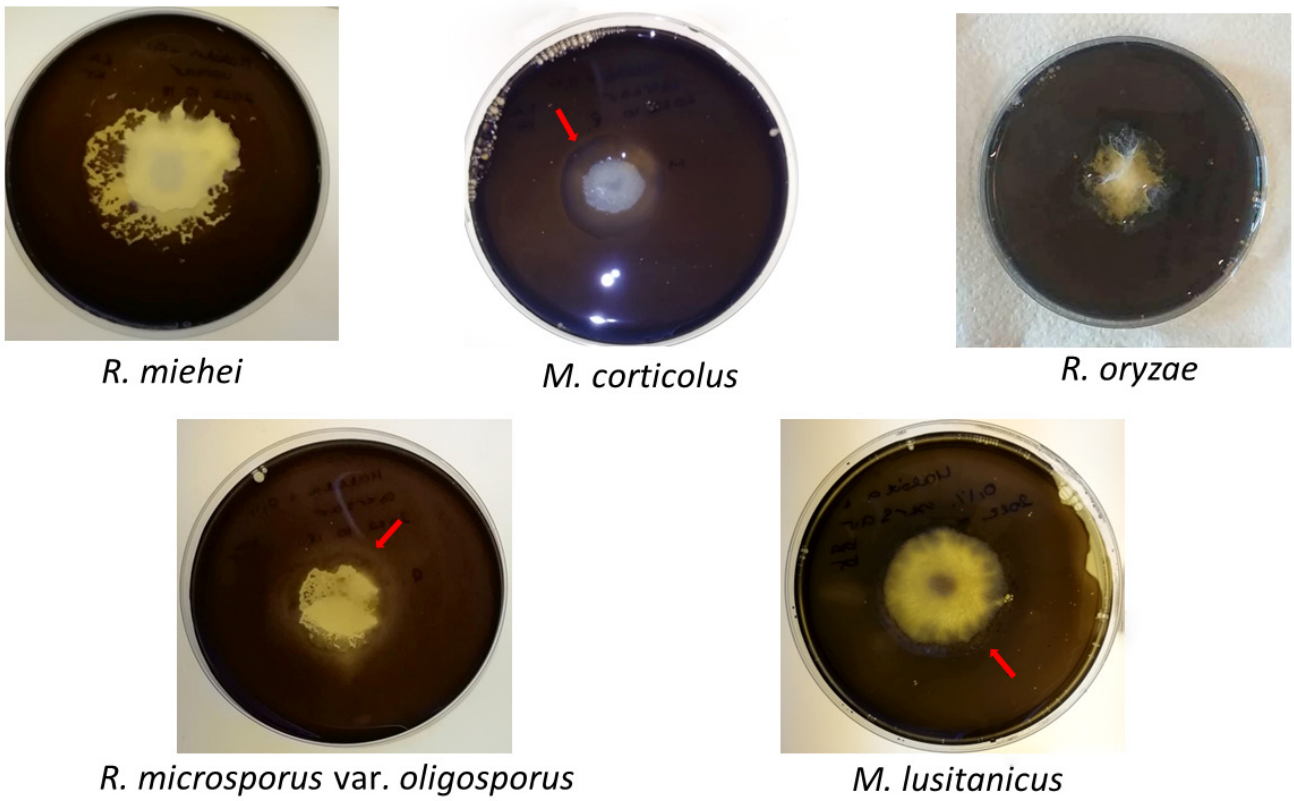

Figure 1. Study of tannase production by Mucoromycota fungi on tannic acid-supplemented medium. Cultivation was performed in malt extract agar medium (yeast extract 0.25 m/v%, malt extract 0.5 v/v% from 20 v/v% stock solution, glucose 1 m/v%, agar 2 m/v%) supplemented with 0.1 m/v% tannic acid. Plates were stained with 0.01 M iron(III) chloride solution.

tested. For SSF2, extraction was performed with 60 ml of sodium acetate buffer (50 mM, pH 6.0) following the same procedure described above for SSF1. Supernatants from both SSF1 and SSF2 systems were stored at -20 °C until use. Three biological replicates of each fermentation experiment were prepared.

Determination of tannase activity

The tannase activity was determined using the methanol-rhodanine based spectrophotometric method (Sharma et al. 2000). In this assay, the tannase releases gallic acid from the methyl gallate (Sigma-Aldrich, Germany) substrate, and the free gallic acid forms a colored complex with the rhodanine (Sigma-Aldrich, Germany) compound presenting an absorbance at 520 nm wavelength. Each step of the preparation of reaction mixtures is shown in Table 2. The reaction mixtures were set in wells of a 96-well microtiter plate (Sarstedt, Germany) and after incubation, the optical density of the mixtures was determined using a SPECTROstar Nano (BMG Labtech, Germany) microtiter plate reader. The methyl gallate (Sigma-Aldrich, Germany) stock (10 mM) and diluted (0.1 mM) solutions were prepared with sodium acetate buffer (50 mM, pH 5.0). Three replicates of the samples were placed on a plate. Calibration was performed using gallic acid solution in the concentration range of 0-100 nM. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μM of gallic acid per minute under the conditions of the assay.

Tannase purification

A mass of 25 g of wheat bran was moisturized with 80 ml of Czapek-Dox solution ($NaNO_3$ 0.3 m/v%, KH_2PO_4 0.12 m/v%, $MgSO_4 \times 7H_2O$ 0.06 m/v%, KCl 0.06 m/v%) in a 500-ml Erlenmeyer flask. After sterilization (121 °C, 30 min) and cooling, a volume of 20 ml of tannic acid (Sigma-Aldrich, Germany) stock solution (12.5 m/v%) filtered through a syringe filter (Millex-GV, PVDF, 0.22 μm, Merck Millipore Ltd., Ireland) was added to the flask. After inoculation with 1 ml of sporangiospore suspension (10^6 spores/ml), the culture was incubated for 72 h at temperature appropriate for the growth of used strain. The culture medium was extracted with 300 ml of Tris-HCl buffer (50 mM, pH 8.5) and left to stand at 4 °C for 24 h. Then, the crude enzyme extract was filtered through a gauze and centrifuged at 5040×*g* for 20 min

Table 2. Assay of tannase activity by methanol-rhodanine method, reaction mixtures and steps of measurement.

Steps	Blank sample	Test sample	Control sample
1.	12.5 µl methyl gallate (0.1 mM)	12.5 µl methyl gallate (0.1 mM)	12.5 µl methyl gallate (0.1 mM)
2.	12.5 µl buffer	12.5 µl enzyme extract	
3.	**1 h incubation at the optimum growth temperature of the fungus**		
4.	15 µl methanol-rhodanine (0.667 m/v%)	15 µl methanol-rhodanine (0.667 m/v%)	15 µl methanol-rhodanine (0.667 m/v%)
5.	**5 min incubation at the optimum growth temperature of the fungus**		
6.	10 µl KOH (0.5 M)	10 µl KOH (0.5 M)	10 µl KOH (0.5 M)
7.	-	-	12.5 µl enzyme extract
8.	**5 min incubation at the optimum growth temperature of the fungus**		
9.	200 µl distilled water	200 µl distilled water	200 µl distilled water
10.	**10 min incubation at the optimum growth temperature of the fungus**		

(4 °C). A volume of 10 ml of the supernatant was then filtered (Millex-HV, PVDF, 0.45 µm, Merck Millipore Ltd., Ireland), and applied to a Macro-Prep High Q (12.6 × 40 mm; Bio-Rad, USA) anion exchange column, which was equilibrated with 50 mM Tris-HCl buffer (pH 8.5). Elution was performed with a linear gradient of 0-1 M NaCl in Tris-HCl buffer (50 mM, pH 8.5) at a flow rate of 2 ml/min. The tannase activity of the fractions was measured by the methanol-rhodanine method.

Effect of temperature

The optimum temperature for the tannase activity was determined by applying the purified enzyme in methanol-rhodanine assay with an incubation for 1 h at temperature range from 20 °C to 60 °C.

Statistical analysis

Data are expressed as means ± standard deviation. Significance was calculated by unpaired *t*-test, or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test in the GraphPad Prism 8.00 software (GraphPad Software Inc., USA). The level of significance was determined by $p < 0.05$.

Results and Discussion

Screening for tannase activity

In these preliminary experiments, seven zygomycetes strains, namely, *R. miehei* SZMC 11005, *G. persicaria* SZMC 11086, *M. corticolus* SZMC 12031, *M. lusitanicus* SZMC 12028, *R. oryzae* SZMC 13611, *R. microsporus* var. *oligosporus* SZMC 13622 and *S. racemosum* SZMC 22672, were screened for their tannase activity using malt extract agar supplemented with tannic acid. The *R. miehei*, *G. persicaria* and *M. corticolus* strains have been shown to have excellent extracellular enzyme production capacity in previous experiments (Takó et al. 2015), thereby, these fungi are potential candidates for further studies on practical applicability. Additional isolates selected are belonging on such zygomycetes species that are capable of tannase enzyme production according to literature (Vattem and Shetty 2002; Londoño-Hernández et al. 2017; Prigione et al. 2018). In addition, the strain *R. microsporus* var. *oligosporus* SZMC 13622 included in the test was derived from tempeh like that used in the study of Vattem and Shetty (2002). In this study, the fungi were subjected to an adaptation test to tannic acid (0.01 m/v%) on PDA medium prior to starting the enzyme activity screening assays on malt extract agar. All strains except *G. persicaria* and *S. racemosum* showed growth in this inductive environment. The five strains that showed growth in the adaptation tests were then transferred to a medium specifically used for tannase production.

Since the applied fungi were not able to grow in conditions containing tannic acid as sole carbon source (data not shown), malt extract agar that can support well the fungal growth was selected as a growth environment in further experiments. The malt extract medium was supplemented with tannic acid, and after the incubation, plates were stained with iron(III) chloride solution to visualize the potential tannic acid degradation around the colonies. The colonies were allowed to grow to a diameter of about 2 to 5 cm, at which point the mycelia mass does not cover the entire surface of the medium providing the

Table 3. Diameter of fungal colonies developed on tannic acid containing malt extract agar plates.

Fungus	Colony diameter (cm)	Incubation time (h)
Rhizomucor miehei	4.67 ± 0.29	48
Mucor corticolus	2.97 ± 0.59	72
Rhizopus oryzae	3.33 ± 0.29	72
Rhizopus microsporus	3.10 ± 0.17	96
Mucor lusitanicus	4.00 ± 0.00	144

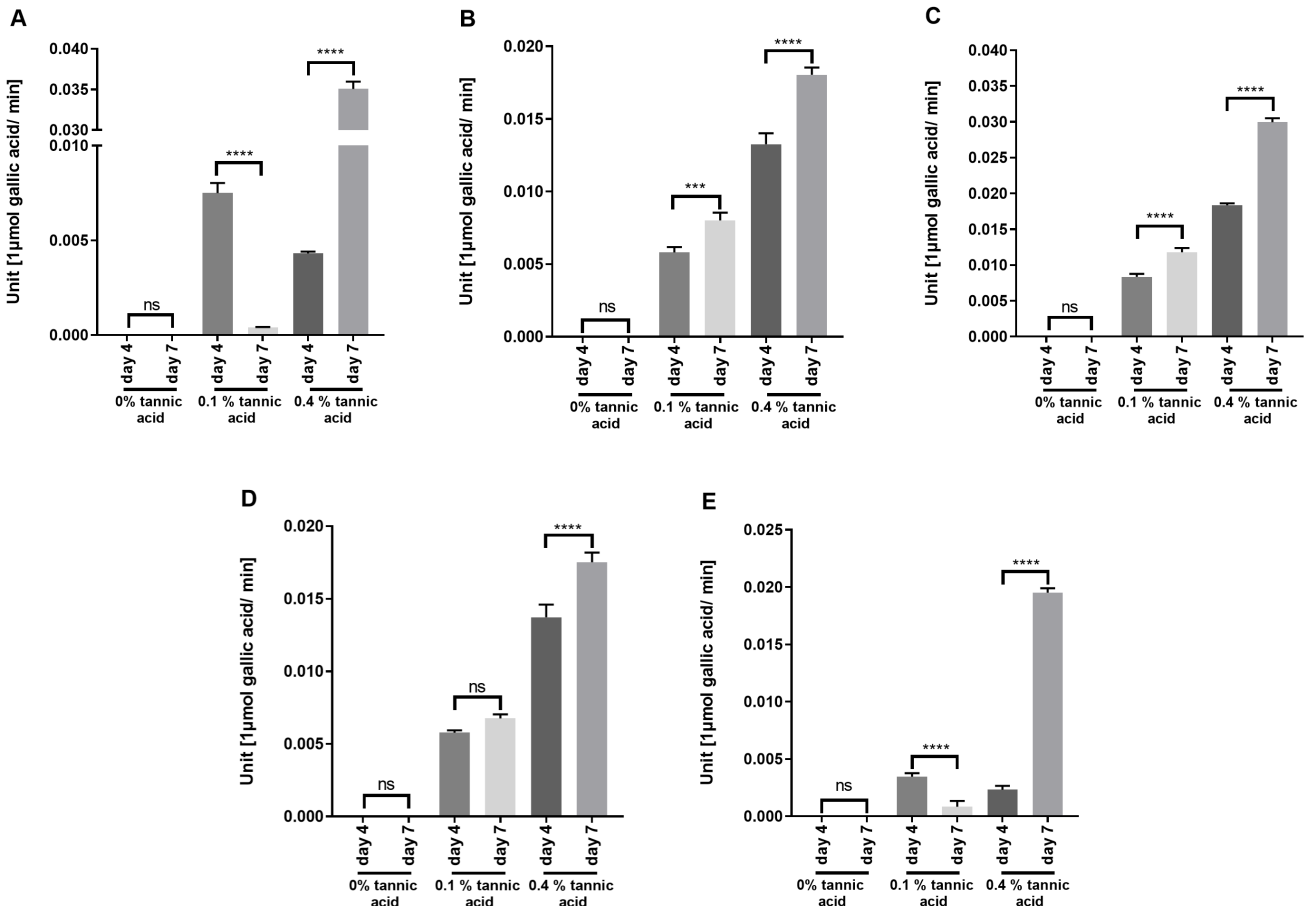

Figure 2. Tannase activity of *R. miehei* SZMC 11005 (A), *M. corticolus* SZMC 12031 (B), *R. oryzae* SZMC 13611 (C), *R. microsporus* var. *oligosporus* SZMC 13622 (D) and *M. lusitanicus* SZMC 12028 (E) strains in submerged fermentations using inductor tannic acid at different concentrations. Error bars represent the standard deviation. Significance was calculated by one-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: non-significant ($p > 0.05$).

visual detection of clear zone developed. All five isolates tested grew well on the condition applied for the tests. The diameter of the colonies together with respective incubation times are shown in Table 3. As can be seen, the *R. miehei* displayed the fastest growth, reaching an average diameter of almost 5 cm after 48 h inoculation.

After staining the plates with iron(III) chloride solution, no clear zone was observed for *R. miehei* and *R. oryzae* isolates (Fig. 1). However, a slightly less dense part around the *M. corticolus*, *R. microsporus* var. *oligosporus* and *M. lusitanicus* colonies were identified, which implies tannase activity for these fungi. In *R. miehei*, the resulting clear zone is probably not visible due to the mycelial mass formed during the intensive growth of the fungus (Table 3). Filamentous fungi with remarkable yield of production have recently been isolated from different environments such as industrial effluents (Farhaan and Patil 2019), agro-industrial byproducts (Ramli et al. 2021) and soil samples (Wakil et al. 2020; Omotayo et al. 2023) using plate assay technique for the detection.

Tannase production in SmF condition

Filamentous fungi can possess tannic acid degradation capacity in liquid culture conditions as well (Banerjee et al. 2001; Van Diepeningen et al. 2004); therefore, it was considered important to investigate the tannase production of the tested strains in SmF system. For these experiments, fungi were inoculated into yeast-peptone culture medium supplemented with 0.1 m/v% or 0.5 m/v% tannic acid as an inducer. Tannic acid free medium was used as reference for the enzyme production. Incubation was carried out for seven days with sampling performed on the 0th, 4th and 7th incubation days. The tested fungi grew well in the presence of tannic acid. However, in all isolates, the most intensive growth was observed in flasks without tannic acid, so that tannic acid inhibited the

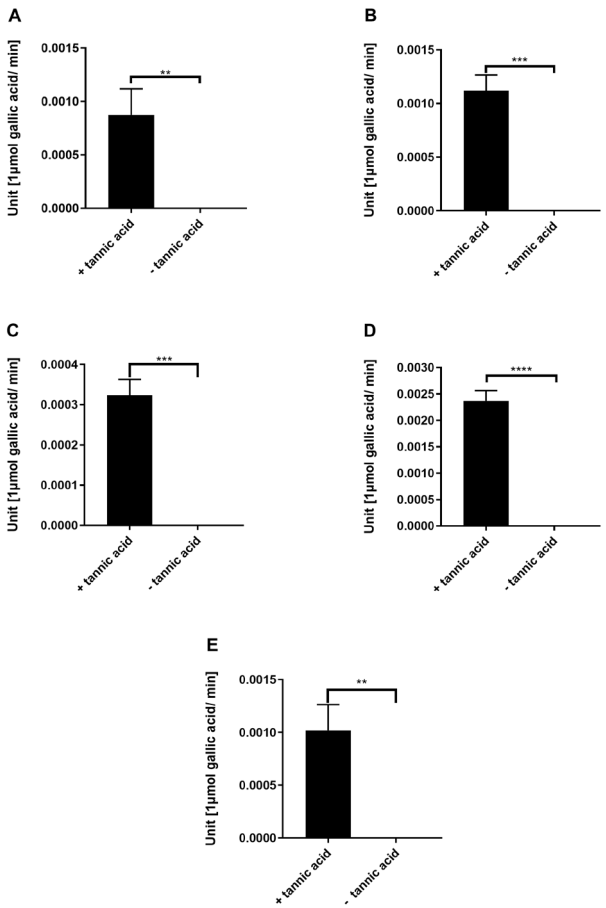

Figure 3. Tannase activity of *R. miehei* SZMC 11005 (A), *M. corticolus* SZMC 12031 (B), *R. oryzae* SZMC 13611 (C), *R. microsporus* var. *oligosporus* SZMC 13622 (D) and *M. lusitanicus* SZMC 12028 (E) strains in wheat bran-based solid-state fermentation. The SSF1 system consisted of 5 g of wheat bran moisturized with Czapek-Dox solution supplemented with tannic acid (+ tannic acid). Tannic acid-free system (- tannic acid) was considered as reference. Error bars represent the standard deviation. Significance was calculated with unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

growth of the fungi to some extent. No tannase activity was detected in the tannic acid free SmF system for all fungi tested (Fig. 2).

In *R. miehei*, the 0.1 m/v% tannic acid containing SmF resulted in maximum activity value for samples taken on the 4th day (0.0075 U) (Fig. 2A). However, it was observed that by the 7th day this value had decreased significantly ($p < 0.0001$). A possible reason for the decrease could be the protein-degrading enzymes produced by the fungus, which may lead to degradation of the enzyme protein at the used condition (Aguilar et al. 2002). However, the induction was more effective when SmF containing 0.5 m/v% tannic acid was applied, resulting in a significant ($p < 0.0001$) increase in the enzyme activity yield up to the 7th day of incubation (Fig. 2A). In *M. corticolus*, tannic acid induced the enzyme production at both concentrations used. The enzyme activity at 0.1 m/v% tannic acid concentration was 0.0058 U on the 4th day of incubation, which enhanced in parallel to the incubation time to 0.0080 U by the 7th day ($p < 0.001$). An improvement in tannase yield was obtained by increasing the tannic acid concentration to 0.5 m/v% (Fig. 2B). The *R. oryzae* demonstrated 0.0084 U activity at 0.1 m/v% tannic acid concentration after 4 days of incubation that was increased by 1.4-times up to the 7th day ($p < 0.0001$). Elevated tannic acid amount (0.5 m/v%) in medium resulted in improved enzyme yield for this fungus as well (Fig. 2C). Activities of 0.0058 U and 0.0067 U were measured for the *R. microsporus* var. *oligosporus* tannase on the 4th and 7th day, respectively, in the presence of 0.1 v/v% tannic acid. The highest enzyme yield for this fungus in SmF system was 0.018 U reached in the presence of 0.5 m/v% tannic acid after 7 days incubation (Fig. 2D). In *M. lusitanicus*, a decrease was shown in tannase yield from the 4th to 7th day in the presence of 0.1 m/v% tannic acid (Fig. 2E). This may be due to the effect of protein-degrading enzymes produced by the fungus. In addition, it is known that the tannic acid can denature proteins which may also explain the decrease in activity (Aguilar et al. 2007). However, tannases from fungi are glycosylated mostly with neutral sugars, thus protected from being precipitated by tannic acid as other proteins (Chávez-González et al. 2012). The tannase glycosylation, however, can be different even in the same microorganism that is dependent on the culture system used for fermentation (Renovato et al. 2011). The higher the tannic acid content of SmF the higher the enzyme activity obtained for *M. lusitanicus* as well (Fig. 2E).

Tannase production in SSF condition

SSF is a well applicable method for tannase enzyme production. Under solid conditions, the extracellular enzyme production is stimulated to a greater extent and more extractable enzyme proteins are present in the culture medium. In addition, the enzyme produced in SSF showed stability over a wider range of environmental parameters (Aguilar et al. 2007). Shorter incubation time is generally required to achieve maximum enzyme yield in SSF than in SmF (Chávez-González et al. 2012). In SSF systems, wheat bran was found to be a suitable substrate for fungal tannase production (Yao et al. 2014). For instance, tannic acid supplemented wheat bran-based SSF systems resulted in high tannase production yields in *Aspergillus aculeatus* (Banerjee et al. 2007) and *R. oryzae* fungi (Table 1; Chatterjee et al. 1996). Based on these previous experiments, both tannic acid-induced and uninduced wheat bran-based SSF systems were set up in our experiments.

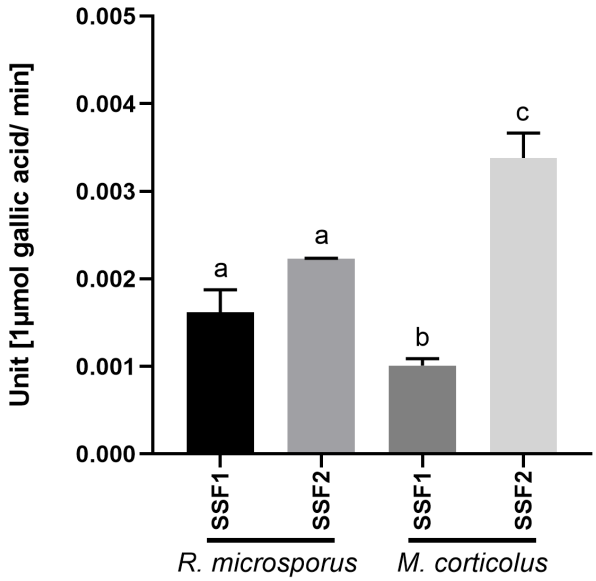

Figure 4. Comparative evaluation of tannase yields of *R. microsporus* var. *oligosporus* and *M. corticolus* achieved in wheat bran-based SSF1 and SSF2 systems. In SSF2 condition, the wheat bran was moisturized with four times the amount of water to that used in SSF1. After 120 h (SSF1) or 72 h (SSF2) incubation, extracts were made using 30 ml (SSF1) or 60 ml (SSF2) sodium acetate buffer. Error bars represent the standard deviation. Different letters above the columns indicate significant differences according to one-way ANOVA ($p < 0.05$).

The fermentation systems were moisturized with Chapek-Dox solution to provide minerals for proper growth and reproduction. Two fermentation approaches, i.e. SSF1 and SSF2, were set up to test the optimal condition for enzyme production. All fungi tested grew well in both fermentation conditions, indicating that the tannic acid did not inhibit their growth at the concentration applied.

After incubation and extraction, the following highest tannase activity values were obtained in extracts from SSF1 system: *R. miehei*, 0.000875 U (Fig. 3A); *M. corticolus*, 0.001119 U (Fig. 3B); *R. oryzae*, 0.000324 U (Fig. 3C); *R. microsporus*, 0.002365 U (Fig. 3D) and *M. lusitanicus*, 0.001018 U (Fig. 3E). There were significant differences in the enzyme activities measured in extracts of induced and tannic acid-free uninduced fermentations, indicating that tannic acid can stimulate the tannase production of the fungi tested. In the wheat bran system, *R. microsporus* var. *oligosporus* (Fig. 3D) showed the highest tannase activity, while the lowest activity was obtained for *R. oryzae* (Fig. 3C). For comparison, lower tannase yields were obtained in SSF than in SmF condition. A potential explanation for the phenomenon can be that some tannase enzymes may also have β-glucosidase activity (Aguilar et al. 2007; Ramírez-Coronel et al. 2003). For instance, if tannic acid and cellobiose were present simultaneously as substrates, only the glucosidase activity of the purified enzyme was detectable (Aguilar et al. 2007). Since crude extracts of SSF systems were used for the activity assay, the reaction mixture could contain significant amounts of cellobiose or other oligosaccharides that could be potential substrates for β-glucosidase activity. It is important to note, however, that the β-glucosidase activity of the extracts was not determined in this experimental system.

A subsequent testing procedure was also performed to optimize the enzyme production in a modified SSF system (SSF2). Here, the wheat bran substrate was moisturized with an elevated amount of nutrient solution, as well as a shorter incubation time and double volume of extraction buffer were applied (Meini et al. 2021). The *M. corticolus* and the *R. microsporus* var. *oligosporus* were selected for these tests due to their growth characteristics and tannase production capacity in SSF1 system. For comparison, cultivation in SSF1 system was performed again, where no significant differences were identified in the two separate fermentations (see Fig. 3B and D and Fig. 4). Application of the SSF2 condition did not result in a significant change in enzyme production of *R. microsporus* var. *oligosporus* compared to SSF1 (Fig. 4) ($p > 0.05$). However, the enzyme activity obtained in SSF2 system for *M. corticolus* was three times higher than that measured in SSF1 condition (Fig. 4) ($p < 0.05$). It was observed that the tannase activity of *R. microsporus* var. *oligosporus* in SSF1 was higher compared to that of *M. corticolus*. In contrast, *M. corticolus* demonstrated more tannase yield in SSF2 than *R. microsporus* var. *oligosporus* (Fig. 4) ($p < 0.05$), indicating that the enzyme production capacity of each fungus may differ under different culture conditions.

Purification of tannase enzyme

Purification of the *M. corticolus* tannase was carried out using chromatography approaches. The *M. corticolus* could produce the enzyme in high amounts at SSF2 condition, therefore, a larger scale version of this cultivation system was used to achieve a high crude enzyme yield.

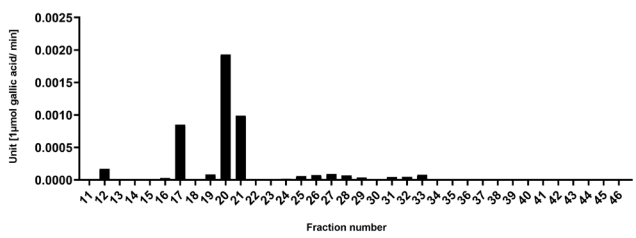

Figure 5. Tannase activity in fractions collected during *M. corticolus* SZMC 12031 tannase protein purification by anion exchange chromatography.

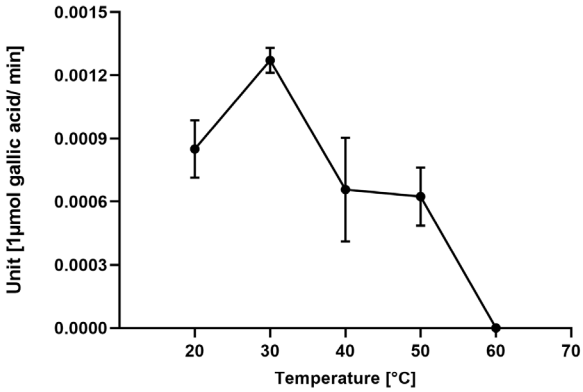

Figure 6. Effect of temperature on the activity of the tannase purified from *M. corticolus* SZMC 12031. Error bars represent the standard deviation.

The purification procedure was started by extracting the enzyme with Tris buffer from the wheat bran-fungal mycelia environment. The concentrated extract was then filtered through a syringe filter. After filtration, the extract was further purified on an anion exchange column. The tannase activity measured in the unfiltered extract was 0.004729 U, while in the filtered extract it was 0.005504 U. The higher activity is probably due to the fact that filtration could have removed substances (e.g., saccharides, proteins from wheat bran) that could interfere with the activity of the hydrolytic enzymes (Sun et al. 2008). The enzyme activity of fractions 11-46 collected by anion-exchange chromatography is shown in Fig. 5. Highest tannase activity was measured in fraction 20, but fractions 17 and 21 also showed remarkable activity. The enzyme activity obtained in fractions 25-29 (Fig. 5) was probably due to the presence of another tannase enzyme. Anyway, the extraction with Tris buffer followed by syringe filtration and anion exchange chromatography could also be an efficient procedure for tannase purification. Ion exchange chromatography is a preferred method for purification of tannase enzyme from different fermentations (Aharwar and Parihar 2018). Anion exchange resin was also used for the purification of *Penicillium herquei* tannase produced by SmF (Qiu et al. 2011) and for *A. niger* enzyme produced by SSF (Ramírez-Coronel et al. 2003).

Temperature optimum of the purified tannase

Activity of the purified *M. corticolus* tannase was monitored at incubation temperatures between 20 °C and 60 °C. Results showed that the temperature optimum for maximal tannase activity was 30 °C (Fig. 6). The purified tannase exhibited about 48% of its original activity at 50 °C. The hydrolysis of methyl gallate by the enzyme decreased markedly above 50 °C, while at 60 °C the enzyme completely lost its activity (Fig. 6). In general, the tannases have their optimum at temperatures between 30 °C and 70 °C (Aharwar and Parihar 2018). Fungal-derived tannase enzymes with an optimal temperature around 30 °C have been described in previous studies (Chávez-González et al. 2012; Kumar et al. 2019). For instance, tannases from *P. herquei* (Qiu et al. 2011), and *Saccharomyces cerevisiae* (Lopes et al. 2018) have been shown to have maximal activity at 30 °C.

Conclusion

In this study, the tannase production of Mucoromycota isolates was investigated in a chromogenic plate assay system and under various fermentation conditions containing tannic acid as an inductor compound. The tannic acid-supplemented malt extract agar was found to be a suitable environment for tannase activity detection in the Mucoromycota strains. The tannic acid environment was tolerated by five isolates among the seven fungi tested, from which the *M. corticolus*, *R. microsporus* var. *oligosporus* and *M. lusitanicus* exhibited the highest tannase production after staining with iron(III) chloride. Tannic acid has been shown to be an excellent inducer of tannase production both in SmF and SSF systems as well. In addition, it was found that the Czapek-Dox solution effectively supports the enzyme production in wheat bran-based SSF at higher moisture content. The highest enzyme yields were obtained in *R. miehei* for SmF and in *M. corticolus* for SSF. Anion exchange chromatography was a suitable approach for the purification of tannase enzyme protein from *M. corticolus*, that showed maximal activity at 30 °C in temperature tolerance tests. The active fungi identified can be reliable sources of tannase among zygomycetes, however, further studies are needed to comprehensively screen the tannase production of this fungal group.

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References

Abd-Elmotey S, Nasar FR, Nasar SA, Abou-Taleb KA (2022) 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. Egypt J Chem 65:21-35.
Aguilar CN, Favela-Torres E, Viniegra-González G, Augur C (2002) Culture conditions dictate protease and tannase production in submerged and solid-state cultures of *Aspergillus niger* Aa-20. Appl Biochem Biotechnol 102-103:407-414.
Aguilar CN, Gutiérrez-Sánchez G (2001) Review: Sources, properties, applications and potential uses of tannin acyl hydrolase. Food Sci Tech Int 7:373-382.
Aguilar CN, Rodríguez R, Gutiérrez-Sánchez G, Augur C, Favela-Torres E, Prado-Barragán LA, Ramírez-Coronel A, Contreras-Esquivel JC (2007) Microbial tannases: advances and perspectives. Appl Microbiol Biotechnol 76:47-59.
Aharwar A, Parihar DK (2018) Tannases: production, properties, applications. Biocatal Agric Biotechnol 15:322-334.
Aissam H, Errachidi F, Penninckx MJ, Merzouki M, Benlemlih M (2005) Production of tannase by *Aspergillus niger* HA37 growing on tannic acid and olive mill waste waters. World J Microbiol Biotechnol 21:609-614.
Andrade PML, Baptista L, Bezerra CO, Peralta RM, Góes-Neto A, Uetanabaro APT, Costa AMD (2021) Immobilization and characterization of tannase from *Penicillium rolfsii* CCMB 714 and its efficiency in apple juice clarification. J Food Meas Charact 15:1005-1013.
Banerjee D, Mondal KC, Pati BR (2001) Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. J Basic Microbiol 41:313-318.
Banerjee D, Mondal KC, Pati BR (2007) Tannase production by *Aspergillus aculeatus* DBF9 through solid-state fermentation. Acta Microbiol Immunol Hung 54:159-166.
Bradoo S, Gupta R, Saxena RK (1996) Screening of extracellular tannase-producing fungi: development of a rapid and simple plate assay. J Gen Appl Microbiol 42:325-329.
Bradoo S, Gupta R, Saxena RK (1997) Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus*. Process Biochem 32:135-139.
Chatterjee R, Dutta A, Banerjee R, Bhattacharyya BC (1996) Production of tannase by solid-state fermentation. Bioprocess Eng 14:159-162.
Chávez-González M, Rodríguez-Durán LV, Balagurusamy N, Prado-Barragán A, Rodríguez R, Contreras JC, Aguilar CN (2012) Biotechnological advances and challenges of tannase: an overview. Food Bioprocess Technol 5:445-459.
Cruz-Hernández M, Augur C, Rodríguez R, Contreras-Esquivel JC, Aguilar CN (2006) Evaluation of culture conditions for tannase production by *Aspergillus niger* GH1. Food Technol Biotechnol 44:541-544.
Das Mohapatra PK, Biswas I, Mondal KC, Pati BR (2020) Concomitant yield optimization of tannase and gallic acid by *Bacillus licheniformis* KBR6 through submerged fermentation: An industrial approach. Acta Biol Szeged 64:151-158.
El-Refai HA, Abdel-Naby MA, Mostafa H, Amin MA, Salem HAA (2017) Statistical optimization for tannase production by *Mucor circinelloides* isolate F6-3-12 under submerged and solid state fermentation. Curr Trends Biotechnol Pharm 11:167-180.
Farhaan MM, Patil RC (2019) Screening and characterisation of tannase producing fungi from soil near tannery industry. European J Biotechnol Biosci 7:68-70.
Hadi TA, Banerjee R, Bhattacharyya BC (1994) Optimization of tannase biosynthesis by a newly isolated *Rhizopus oryzae*. Bioprocess Eng 11:239-243.
Hota SK, Dutta JR, Banerjee R (2007) Immobilization of tannase from *Rhizopus oryzae* and its efficiency to produce gallic acid from tannin rich agro-residues. Indian J Biotechnol 6:200-204.
Jana A, Halder SK, Ghosh K, Paul T, Vágvölgyi C, Mondal KC, Das Mohapatra PK (2015) Tannase immobilization by chitin-alginate based adsorption-entrapment technique and its exploitation in fruit juice clarification. Food Bioprocess Technol 8:2319-2329.
Jana A, Maity C, Halder SK, Mondal KC, Pati BR, Das Mohapatra PK (2012) Tannase production by *Penicillium purpurogenum* PAF6 in solid state fermentation of tannin-rich plant residues following OVAT and RSM. Appl. Biochem Biotechnol 167:1254-1269.
Kasieczka-Burnecka M, Kuc K, Kalinowska H, Knap M, Turkiewicz M (2007) Purification and characterization of two cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain *Verticillium* sp. P9. Appl Microbiol Biotechnol 77:77-89.
Kumar SS, Sreekumar R, Sabu A (2019) Tannase and its applications in food processing. In Parameswaran B, Varjani S, Raveendran S, eds., Green Bio-processes. Enzymes in Industrial Food Processing. Springer, Singapore, 357-381.
Londoño-Hernández L, Ramírez-Toro C, Ruiz HA, Ascacio-Valdés JA, Aguilar-Gonzalez MA, Rodríguez-Herrera R, Aguilar CN (2017) *Rhizopus oryzae* – Ancient microbial resource with importance in modern food industry. Int J Food Microbiol 257:110-127.
Lopes LMDM, Batista LHC, Gouveia MJ, Leite TCC, de Mello MRF, de Assis SA, de Sena AR (2018) Kinetic and thermodynamic parameters, and partial characterization of the crude extract of tannase produced by *Saccharomyces cerevisiae* CCMB 520. Nat Prod Res 32:1068-1075.
Meini M-R, Cabezudo I, Galetto CS, Romanini D (2021) Production of grape pomace extracts with enhanced antioxidant and prebiotic activities through solid-state fermentation by *Aspergillus niger* and *Aspergillus oryzae*. Food Biosci 42:101168.
Mostafa HS (2023) Potato peels for tannase production from

Penicillium commune HS2, a high tannin-tolerant strain, and its optimization using response surface methodology. Biomass Conv Bioref 13:16765-16778.

Mukherjee G, Banerjee R (2004) Biosynthesis of tannase and gallic acid from tannin rich substrates by *Rhizopus oryzae* and *Aspergillus foetidus*. J Basic Microbiol 44:42-48.

Omotayo MA, Olaleye ON, Odetunde SK, Avungbeto MO (2023) Isolation and molecular identification of tannase producing fungi from soil. Afr J Biotechnol 22:322-328.

Ong CB, Annuar MSM (2024) Potentialities of tannase-treated green tea extract in nutraceutical and therapeutic applications. Appl Biochem Biotechnol 196:7534-7553.

Papadaki E, Mantzouridou FT (2024) Application of *Aspergillus niger* for extracellular tannase and gallic acid production in non-sterile table olive processing wastewaters. Waste Biomass Valor 15:1199-1212.

Papp T, Nyilasi I, Csernetics Á, Nagy G, Takó M, Vágvölgyi C (2016) Improvement of industrially relevant biological activities in Mucoromycotina fungi. In Schmoll M, Dattenböck C, eds., Gene Expression Systems in Fungi: Advancements and Applications. Fungal Biology. Springer, Cham, 97-118.

Prigione V, Spina F, Tigini V, Giovando S, Varese GC (2018) Biotransformation of industrial tannins by filamentous fungi. Appl Microbiol Biotechnol 102:10361-10375.

Qiu Y, Niu H, Huang W, He Y, Wu XH (2011) Properties and secondary structure of tannase from *Penicillium herquei*. Biotechnol Bioprocess Eng 16:858-866.

Ramírez-Coronel MA, González GV, Darvill A, Augur C (2003) A novel tannase from *Aspergillus niger* with β-glucosidase activity. Microbiology 149:2941-2946.

Ramli MS, Siva R, Rashid NYA, Sharifuddin SA, Samat N, Hasibuan S, Lani MN, Mansor A (2021) Screening of potential tannase-producing fungi from local agri-industrial by-products using a plate assay and submerged fermentation. Int J Adv Sci Eng Inf Techno 11:1209-1213.

Renovato J, Gutiérrez-Sánchez G, Rodríguez-Durán LV, Bergman C, Rodríguez R, Aguilar CN (2011) Differential properties of *Aspergillus niger* tannase produced under solid-state and submerged fermentations. Appl Biochem Biotechnol 165:382-395.

Shao Y, Zhang YH, Zhang F, Yang QM, Weng HF, Xiao Q, Xiao AF (2020) Thermostable tannase from *Aspergillus niger* and its application in the enzymatic extraction of green tea. Molecules 25:952.

Sharma S, Bhat TK, Dawra RK (2000) A spectrophotometric method for assay of tannase using rhodanine. Anal Biochem 279:85-89.

Takó M, Kotogán A, Krisch J, Vágvölgyi C, Mondal KC, Papp T (2015) Enhanced production of industrial enzymes in Mucoromycotina fungi during solid-state fermentation of agricultural wastes/by-products. Acta Biol Hung 66:348-360.

Van Diepeningen AD, Debets AJM, Varga J, Van Der Gaag M, Swart K, Hoekstra RF (2004) Efficient degradation of tannic acid by black *Aspergillus species*. Mycol Res 108:919-925.

Vattem DA, Shetty K (2002) Solid-state production of phenolic antioxidants from cranberry pomace by *Rhizopus oligosporus*. Food Biotechnol 16:189-210.

Wakil SM, Ajayi OD, Fasiku SA (2020) Production of tannase by fungi isolated from different soils. J Basic Appl Sci 16:1-8.

Yao J, Guo GS, Ren GH, Liu YH (2014) Production, characterization and applications of tannase. J Mol Catal B Enzym 101:137-147.