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Optimization of Culture Conditions for a New Mutant *Penicillium* EZ-ZH390 to Increase Tannase Activity in Submerged Fermentation

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ABSTRACT

Back Ground and Aims: Tannins have some undesirable impact and should be broken down by tannase enzymes. One of the major problems of microbial produced tannase is the high total cost which could be reduced by using agricultural waste as a substrate.

Method: A submerged fermentation system was used to optimize production of tannase from newly isolated *Penicillium* EZ-ZH390. Dried walnut hulls were used as carbon substrate. Statistical design screening was used to find main variables. In the next step, these three factors were used to optimize tannase using response surface methodology.

Results and Discussion: According to the statistical design screening results, shaking, spore inoculum amount, and temperature had the greatest impact on tannase activity. Without optimization, the activity of tannase in walnut hull medium was 0.36 U mL^{-1}. A shaking speed of 180 rpm and an inoculum of 10^8 cfu mL^{-1} spores at 25°C were obtained as the optimal conditions using the model. It was also examined whether aeration affects tannase activity and growth kinetics of *Penicillium* EZ-ZH390 in the fermenter as well as the enzyme activity over time. The aeration 1vvm produced the greatest effect on tannase activity. The highest enzyme activity was observed between hours 60 and 75. As total sugars, reducing sugars, and tannin levels continue to decrease, Penicillium EZ-ZH390 is growing. Tannase production from *Penicillium* EZ-ZH390 in walnut hulls submerged fermentation medium is dependent on microorganism growth. The highest enzyme activity measured using this method was 3.05 U mL^{-1}.

What is "already known":	• Tannase degrades tannins; agricultural wastes can lower enzyme production costs. • Penicillium EZ-ZH390 was a stable mutant with proven high tannase activity. • Submerged fermentation is widely used, but walnut hulls hadn't been optimized with this strain.
What this article adds:	• Walnut hulls valorized for cost-effective tannase production • Penicillium EZ-ZH390 yields 3.05 U.mL^{-1} tannase in fermenter • Optimized conditions are 180 rpm, 10^8 cfu.mL^{-1}, 25°C, 1 vvm • RSM model predicts 2.38 U.mL^{-1}; validated experimentally • Tannase production is growth-associated in submerged fermentation • Aeration at 1 vvm boosts tannase activity by 22.8%

1. INTRODUCTION

Enzymes play a significant role in biochemical reactions. A principal goal in using enzymes in the industry is to decrease costs and production time [1]. Tannins are a large group of polyphenol compounds widely found in nature. Due to their ability to bind with macromolecules and minerals, these compounds perform various functions in food and the body. Also, they have been used in the leather industry for thousands of years [2-5]. Tannins have many desirable effects, including antioxidant and anticancer properties [6, 7] as well as contributing to the production of high-quality leather [8]. However, Tannins have some undesirable effects, for instance, the chelating effect on metals, protein precipitation, anti-nutritional properties, malnutrition and weight loss factor in the live stocks caused by the chelating effect on the digestive enzyme, bitter and assurgent taste, and tarnishing of fruit juices [9-12]. These compounds must therefore be closely regulated in the industry [13].

Tannins have low biodegradability and break hardly [14]. They remain in wastewaters and react with heavy metals, which cause poisoning in the environment [15]. Because of these effects, tannins should be inhibited and broken down. Tannase enzymes can break tannins into smaller compounds [16]. The product of this reaction is a primary substance for producing useful materials, such as antioxidants. Tannase is used abundantly in the food and drug industry as well as in cosmetic products [17].

The methods of fermentation and enzyme production include solid-state, submerged, and surface liquid fermentation [18, 19]. Among the three types of mediums, submerged fermentation is the most commonly used in the industry [20]. In particular, this medium offers easier control of temperature and pH on an industrial scale, usability in the current industry, easier aeration by controlling agitation rate and pump, easier sampling, shorter incubation times, use of all media, easier enzyme separation, a uniform system for better efficiency [21]. One of the major problems of submerged fermentation in the industry is the high cost of providing the substrate [22]. The production cost of enzymes using biotechnology methods can be reduced by using agricultural waste as a substrate; furthermore, separating stronger micro-organisms for increasing production and efficiency can result in a lower production cost [23]. Although it is widely known that plant wastes can be successfully used for microbial enzyme manufacture because of their low price, researching how agricultural residues can be used for tannase production still calls for optimization, both in terms of exotical strains and cultivation conditions. More concretely, it should be noted that the combined use of walnut hulls and the mutant strain Penicillium EZ-ZH390 in submerged fermentation has still gone unnoticed. Furthermore, it is necessary to investigate how principal parameters of cultivation affect the production of tannase and verify the validity of obtained values through the use of fermentative production process. Overall, the presented research was performed in order to optimize tannase production with the help of walnut hulls as a cheap substrate using strain Penicillium EZ-ZH390 and sequential statistical approach. *Penicillium* sp. EZ-ZH390 is a mutant derivative obtained through sequential induced mutagenesis of the native tannase-producing strain *Penicillium* sp. EZ-ZH190. Initially, EZ-ZH190 was subjected to heat treatment, resulting in the isolation of EZ-ZH290, and subsequent γ-irradiation of EZ-ZH290 led to the isolation of EZ-ZH390. Previous characterization demonstrated that EZ-ZH390 produced a higher level of tannase (7.66 U mL^{-1}) than its parent strain EZ-ZH290 (4.32 U mL^{-1}) and exhibited favorable enzymatic properties, including an optimum temperature of 35 °C and an optimum pH of 5.5. Moreover, the production level and pH stability of the EZ-ZH390 tannase remained consistent over five successive generations, supporting the stability of the mutant strain [24].

2. MATERIALS AND METHODS

Walnut hulls substrate was accumulated from gardens in the northwest Iran. It was dried and stored at -18°C.

2.1. Fungal Strain

The strain used in the present work was *Penicillium* EZ-ZH390 which was a mutant strain from a newly isolated microorganism *Penicillium* EZ-ZH190, which has been separated from moldy green tea in the Tarbiat Modares University [24]. The spore

suspension was obtained by growing the organism on potato dextrose agar at 25°C for 96 hours. The spores were harvested with known quantities of water.

2.2. Chemical Composition of Walnut Hulls

The dried walnut hull was crushed and screened with mesh size 120. The total sugar was measured by the Antron method [25] and the reducing sugar was assessed by the DNS method [26]. Total phenols were determined by the Makkar method [27] and nitrogen by the Kjeldahl method [28].

2.3. Medium and Culture Conditions

The spores of *Penicillium* EZ-ZH390 were inoculated in a medium that contained 7% walnut hulls in 250 mL Erlenmeyer flasks, for 96 hours at 30°C. The extract was separated with centrifuging at 10,000 g for 15 minutes at 4°C. The troublesome compounds in the extract were separated by dialysis. The supernatant obtained was stored at 4°C and used as a crud tannase enzyme.

2.4. Tannase Assay

Tannase activity was determined by spectrophotometry, according to the method of Mondal. One unit of tannase activity (U) was defined as the amount of enzyme required to release one micromole of gallic acid per minute under the defined reaction conditions. Enzyme yield was expressed in U mL^{-1} [29].

2.5. Statistical Analysis

Screening methodology (two-level fractional factorial design) and response surface methodology were used to optimize the best condition for the production of tannase from *Penicillium* EZ-ZH390. The results were analyzed by SAS and MODDE7 software.

2.5.1. Screening Methodology

For determining the effects of variables on the tannase activity in the selected medium, the screening methodology was used (**Table 1, 2**). Variables and levels (-1, +1) include time (3 and 5 days), temperature (25 and 35°C), agitation rate (80 and 150 rpm), nitrogen (0 and 3 g L^{-1}), phosphorus (0 and 1 g L^{-1}), minerals of KCl (0 and 0.5 g L^{-1}), $MgSO_4$ (0 and 0.5 g L^{-1}), $ZnSO_4$ (0 and 0.01 g L^{-1}), $FeSO_4$ (0 and 0.01 g L^{-1}) and $CuSO_4$ (0 and 0.005 g L^{-1}), as well as inoculation dose (10^5 and 10^7 cfu mL^{-1}) (**Table 1**).

2.5.2 Response Surface Methodology

To determine the optimal level of three variables selected by screening methodology (temperature, inoculation dose, and agitation rate), the central composite design with three levels for every variable and three replicates at the central point was applied using the MODDE7 statically software. The three different levels of the independent variables were 25, 32.5, and 40 °C for temperature; 80, 130, and 180 rpm for agitation rate; and 10^6, 10^7, and 10^8 cfu mL^{-1} for inoculation dose (**Table 3**).

2.6. Determination of Aeration Effect on Tannase Activity in The Fermenter

Penicillium EZ-ZH390 was cultured in the fermenter (Majer science-F1-S-3L) under the optimized condition. This examination was done at three levels, including 0.5, 1, and 1.5 vvm. The fermentation experiments were performed in a 3-L stirred-tank laboratory fermenter with a constant working volume of 2 L. Agitation was maintained at the optimized speed of 180 rpm throughout the fermentation process, while aeration was supplied through a sparger at three airflow rates (0.5, 1.0, and 1.5 vvm) to evaluate the influence of oxygen supply on tannase production. The initial pH of the culture medium was adjusted to 5.0 before sterilization according to the optimized fermentation conditions, and no automatic pH control was applied during cultivation. Dissolved oxygen concentration and the volumetric oxygen transfer coefficient (kLa) were not directly monitored during fermentation; therefore, aeration rate was used as the operational parameter for evaluating oxygen availability in the bioreactor.

TABLE 1. Screening design to evaluate impact of process variables on tannase activity from *Penicillium* EZ-ZH390

Sample	Temperature (°C)	Time (day)	Agitation rate (Rpm)	Nitrogen (g L^{-1})	Phosphour (g L^{-1})	Minerals (g L^{-1})	Inoculation (cfu mL^{-1})	Tannase activity (U mL^{-1})
1	+1	-1	+1	-1	+1	-1	-1	0.26±0.02
2	-1	-1	-1	+1	+1	+1	-1	1.48± 0.02
3	-1	+1	+1	-1	-1	+1	-1	1.76± 0.02
4	+1	+1	-1	+1	-1	-1	-1	2.78± 0.03
5	+1	+1	+1	+1	+1	+1	+1	3.64± 0.06
6	-1	-1	+1	+1	-1	-1	+1	2.92± 0.03
7	-1	+1	-1	-1	+1	-1	+1	1.98± 0.05
8	+1	-1	-1	-1	-1	+1	+1	2.86± 0.05

TABLE 2. Analyses of variance for screening method to define main effects on tannase activity from *Penicillium* EZ-ZH390

Factor	Effect	Significance (P< 0.05)	Factor	Effect	Significance (P< 0.05)
Phosphor	-0.66	0.0001	Temperature	0.4	0.0015
Minerals	-1.13	0.0001	Time	0.17	0.08
Inoculation	0.28	0.011	Agitation Rate	0.93	0.0001
Nitrogen	-0.007	0.93			

TABLE 3. Effect of variables on tannase activity from *Penicillium* EZ-ZH390 in the response surface methodology

Sample	Agitation rate	Temperature	Inoculation dose	Tannase activity	Sample	Agitation rate	Temperature	Inoculation dose	Tannase activity
1	-1	-1	-1	*0.36± 0.01	10	+1	0	0	0.92± 0.1
2	+1	-1	-1	0.35± 0.007	11	0	-1	0	0.045± 0.007
3	-1	+1	-1	1.14± 0.06	12	0	+1	0	0.35± 0.02
4	+1	+1	-1	0.3± 0.07	13	0	0	-1	0.025± 0.007
5	-1	-1	+1	0.025± 0.007	14	0	0	+1	1.4± 0.28
6	+1	-1	+1	2.37± 0.03	15	0	0	0	0.05± 0.001
7	-1	+1	+1	0.56± 0.01	16	0	0	0	0.045± 0.006
8	+1	+1	+1	2.12± 0.14	17	0	0	0	0.04± 0.001
9	-1	0	0	0.175± 0.03					

2.7. Microorganism's Growth Kinetics and Tannase Activity

For this section, fermentation was done in the fermenter under the optimized condition for 100 hours. Samples were taken from the fermenter at intervals of 5 hours. Then the total sugar, reducing sugar, tannin, and enzymatic activity of each sample were measured.

3. RESULTS AND DISCUSSION

3.1. Determination of Effective Variables on Tannase Production

The essential factors for *Penicillium* EZ-ZH390 growth were studied for the first time; therefore, all basic requirements should be considered, such as inoculation dose, phosphorus and nitrogen sources, and various minerals (zinc, sulfur, magnesium, iron, and copper), agitation rate, time and temperature. The results and their significant effects are presented in **Tables 1** and **2**.

3.2. Optimization of Effective Factors on Tannase Activity by Response Surface Methodology

The three most effective factors were selected from the screening process in this stage, and three levels of their influence were analyzed to determine optimum conditions. These factors include temperature, agitation rate, and inoculation dose (**Table 3**). Data were subjected to analysis of variance (ANOVA). The amounts of production of tannase and the experimental and predicted data are presented in **Table 4.** A quadratic regression equation described the optimization of environmental variables to produce tannase with good R^2 (0.99), explaining the validation of the model and demonstrating its quality. The interaction between the variables generated three-dimensional graphs that showed a significant increase in tannase production (2.37 U mL^{-1}) under conditions including 180 rpm agitation, 10^8 cfu mL^{-1} inoculation, and 25°C temperature. All of the factors had a significant effect, but the effect of temperature was lower than the others. In the same study presented by Aguilar et al. (2014), the effect of temperature was lower than other factors, such as pH and Mg [30]. The behavior of tannase production has been explained by the following quadratic equation of Eq. 1:

$$Y= ß_0 + ß_1A + ß_2B + ß_3C + ß_{12}AB + ß_{13}AC + ß_{23}BC + ß_{11}A^2 + ß_{22}B^2 + ß_{33}C^3$$

Eq. 1

Where Y is predicted response, $ß_0$ is intercept, $ß_1$, $ß_2$, $ß_3$ are liner coefficients, $ß_{12}$, $ß_{13}$, $ß_{23}$ are interaction coefficients, $ß_{11}$, $ß_{22}$, $ß_{33}$ are squared coefficient and A, B, C are independent variables. The response (tannase activity) can be expressed by Eq. 2 as a function of the values of agitation rate (A), temperature (B) and inoculation dose (C):

$$Y= 0.089 + 0.38A + 0.13B + 0.37C – 0.2AB + 0.59AC – 0.05BC + 0.42A^2 + 0.07B^2 + 0.32C^2$$

Eq. 2

TABLE 4. Analyses of variance for response surface methodology

Factor	Effect	Significance (P< 0.05)	Factor	Effects	Significance (P< 0.05)
Agitation rate (A)	0.763	0.0001	Inoculation dose×inoculation dose (C^2)	0.64	0.0001
Temperature (B)	0.266	0.0001	Temperature×agitation rate (AB)	-0.4	0.0001
Inoculation dose (C)	0.755	0.0001	Inoculation dose×agitation rate (AC)	1.19	0.0001
Agitation rate×agitation rate (A^2)	0.85	0.0001	Temperature×inoculation dose (BC)	-0.11	0.0011
Temperature×temperture (B^2)	0.15	0.007			

The experimental results nearly matched the predicted model, which confirms the validity of the model (**Figure 1**). **Figures 2-4** show the interaction effect of effective factors on tannase activity.

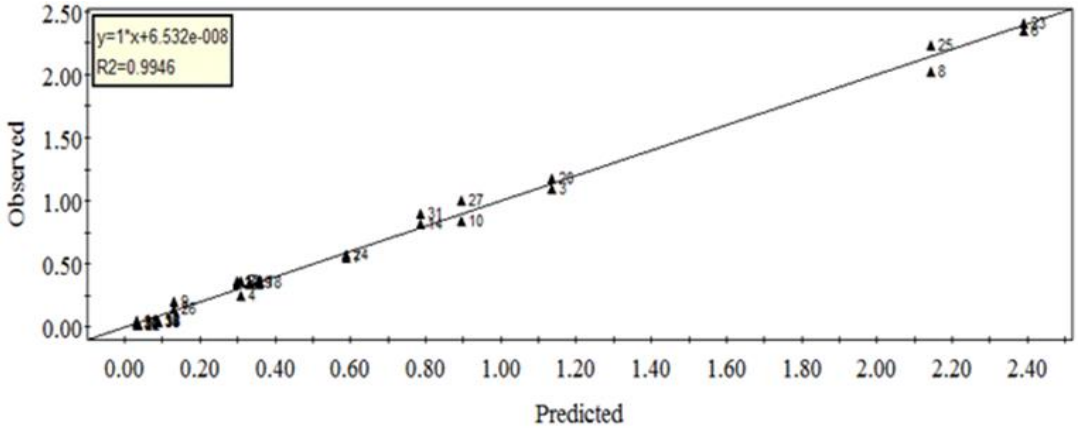
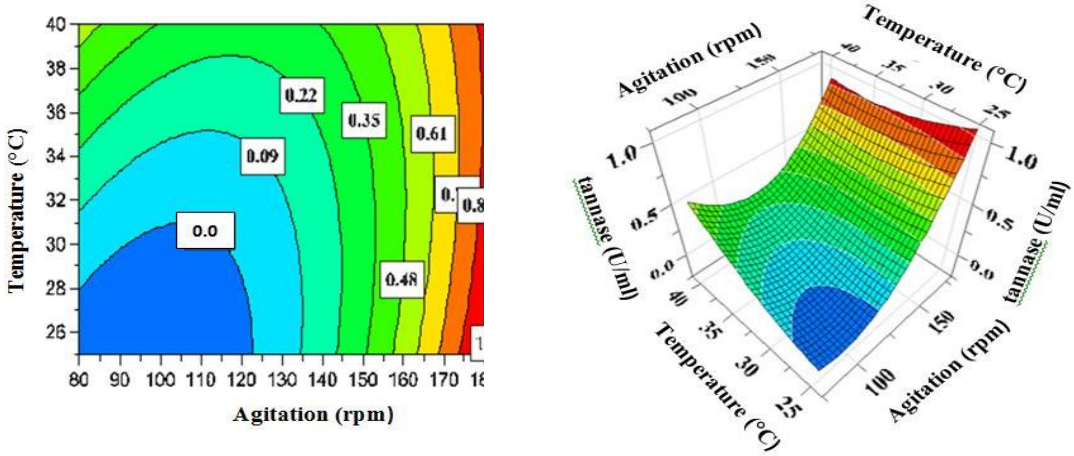


Figure 1. Comparison of response surface methodology model predictions with experimental data for tannase activity



a **b**

Figure 2. (a) Two-dimensional graph and (b) three-dimensional diagram of interaction effect of agitation rate (rpm) and temperature (°C) on tannase activity from Penicillium EZ -ZH390 in the constant inoculation dose 5 × 10^7 cfu.ml^{-1} (center point)

a **b**

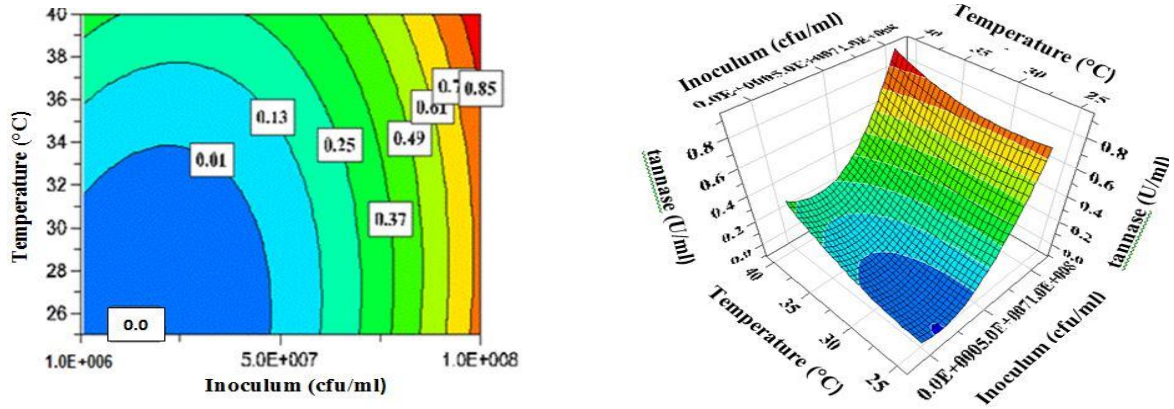
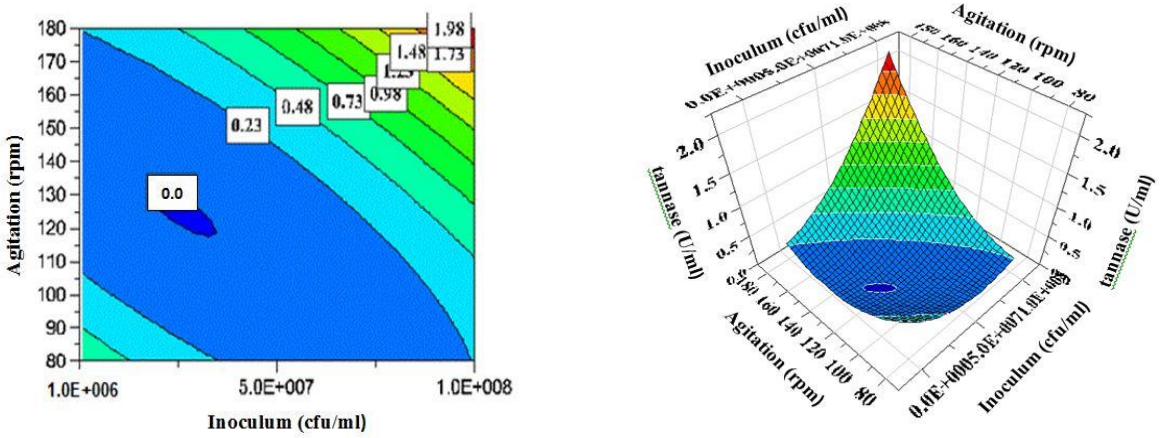


Figure 3. (a) Two-dimensional graph and (b) three-dimensional diagram of interaction effect of inoculation dose (cfu.ml^{-1}) and temperature (°C) on tannase activity (U.ml^{-1}) in the constant agitation rate 130 rpm (center point)



a b

Figure 4. (a) Two-dimensional graph and (b) three-dimensional diagram of the interaction effect of agitation rate (rpm) and inoculation dose (cfu.ml^{-1}) on tannase activity (U.ml^{-1}) at a constant temperature 32.5 ° C (center point)

3.3. Validity of The Model

In order to obtain the maximum activity of tannase, the optimization of the model was performed using response surface methodology auto-analysis software (MODDE7) by setting the maximum activity value (Y) as the goal. The resulting maximum tannase activity (Y= 2.38 U mL^{-1}) was predicted under the following optimal conditions: agitation rate 180 rpm, inoculation dose 10^8 cfu mL^{-1}, and temperature 25°C. The model was validated by repeating the experiment under optimized conditions, which resulted in the tannase production of 2.35± 0.03 U mL^{-1}, indicating the efficiency of the model for predicting the amount of tannase production under different medium conditions.

3.4. Effect of Aeration on Tannase Activity

Cultivation was carried out in the fermenter under the optimized condition with different aeration levels of 0.5, 1, and 1.5 vvm (**Table 5**). Aeration 1 vvm had maximum effect on increasing the tannase activity, while aerations 0.5 and 1.5 vvm had the same result.

TABLE 5. Effect of aeration on tannase from *Penicillium* EZ-ZH390

Aeration (vvm)	Tannase activity (U mL^{-1})
0.5	*1.65 ^{b}
1	3.05^{a}
1.5	1.72 ^{b}

*Lowercase letters indicate significant differences in the level of P>0.05

3.5. Kinetic of Penicillium EZ-ZH390 Growth and Tannase Activity

During this part of the research, growth patterns and tannase production patterns were investigated as well as the best time for using tannase for maximum effectiveness. The experiment time was 100 hours with sampling at intervals of 5 hours. 20 samples were obtained and the total phenol, total sugar, reducing sugar, and tannase activity were measured. As shown in **Figure 5**, the tannin amount in the initial growth was almost intact because tannase wasn't produced in sufficient quantities, but it decreased over the time. Tannin amount decreased more after 60 hours, which corresponds to tannase increase. During organism growth, the reducing sugar was consumed and diminished, resulting in the minimum amount of reducing sugar observed between 35 and 45 hours. At 60 to 75 hours, there was a temporary increase in reducing sugar levels and a decrease in tannin amount. The maximum tannase activity was observed at 60-75 hours.

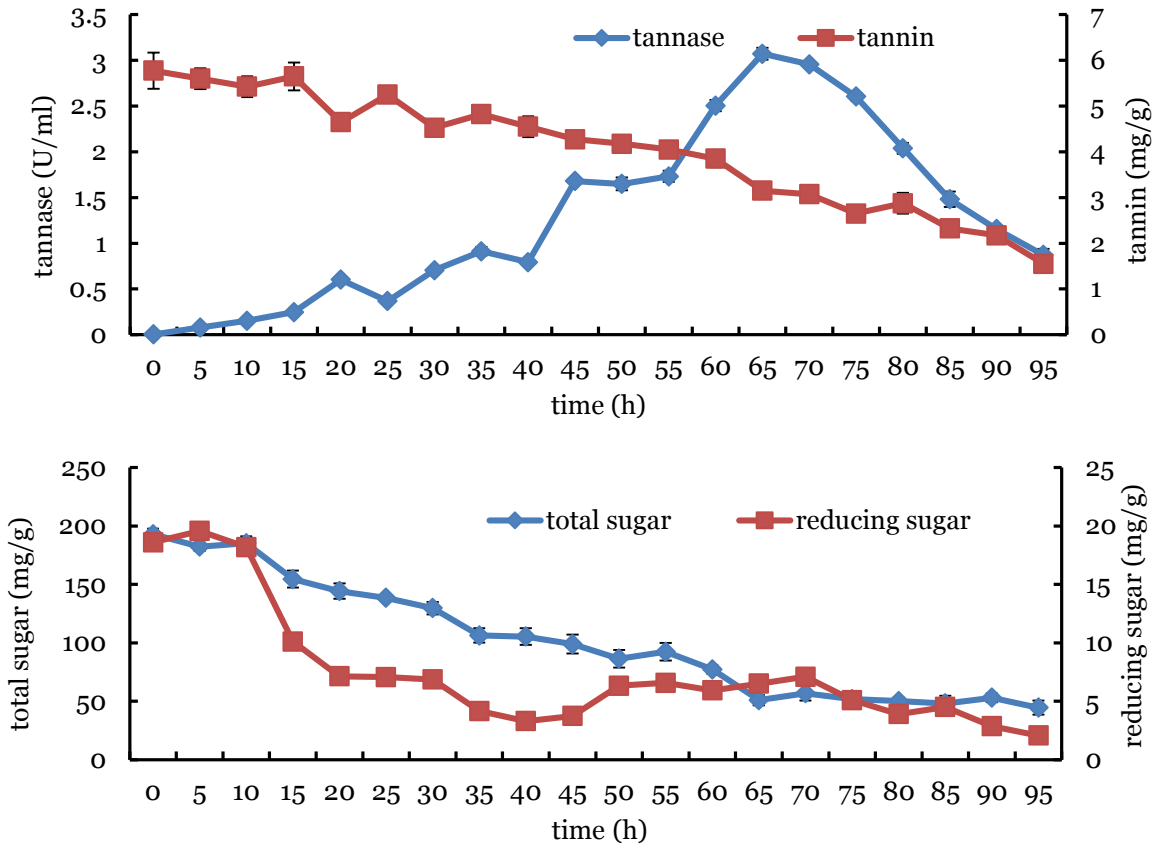


Figure 5. Consumption patterns of total sugar, reducing sugar, and tannin in the walnut hulls medium and tannase production from Penicillium EZ -ZH390.

The results show that the most effective factors were agitation rate, temperature, and inoculation dose, respectively. On the other hand, phosphorus and other mineral had a negative effect on producing tannase. Nitrogen and time had no significant effect ($p>0.05$). Adding nitrogen, phosphorus, and other minerals do not help increase enzyme activity, but may even reduce it. The use of walnut hulls has important practical and economic implications for industrial tannase production. Walnut hulls are an abundant agro-industrial by-product generated during walnut processing and are often discarded despite their high tannin content. Utilizing this residue as a fermentation substrate reduces the dependence on purified tannic acid and other relatively expensive conventional substrates, thereby lowering the overall substrate cost of the fermentation process. In addition, valorization of walnut hulls contributes to sustainable waste management by converting an agricultural residue into a value-added bioproduct, which is consistent with the principles of circular bioeconomy and sustainable bioprocessing. These characteristics make walnut hulls an attractive substrate for large-scale microbial tannase production, particularly in walnut-producing regions where this by-product is readily available [12,31]. This issue is important from an economical point because the complex media obtained from agricultural wastes are a rich resource of elements and materials required for microbial growth and enzyme production and can be provided at a low cost and don't need nutrients to be added. Nitrogen content in the walnut halls was 0.45%, which was more than the nitrogen content in the Czapek-Dox medium (0.3%); therefore, it seems that the nitrogen content was sufficient for microbial growth. Ammonium chloride 0.2% was found to be the best nitrogen source for producing tannase from *Trichoderma harzianum* [32]. Sodium nitrate 1% was introduced as the best nitrogen source for tannase production from *Aspergillus niger* [33]. Muslim et al. (2015) introduced ammonium nitrate 2% as the best nitrogen source for producing tannase from *Erwinia carotovora* [34]. In the case of phosphorus, it was shown that increasing phosphorus amount from 1/5 to 3 g L^{-1} caused a significant decrease in tannase activity [30]. Kumar et al. (2007) reported that

adding carbon and nitrogen source to complex mediums, such as Lotus leaf, Jamun leaf (*Syzygium cumini*), gooseberry leaf (*Phyllanthus emblica*), and sorghum leaf didn't increase tannase activity [35]. Agitation rate had the maximum effect on increasing the tannase activity, which can be attributed to the improvement of aeration in the viscose complex medium and consequently improving the organism's growth and tannase activity. It is worth mentioning that most fungi have aerobic life and need oxygen for growth. The strong influence of agitation rate observed in the present study can be further explained by its effect on mass transfer during submerged fungal fermentation. Appropriate agitation improves oxygen availability, homogenizes nutrient distribution throughout the fermentation broth, and reduces concentration gradients surrounding the fungal biomass, thereby promoting extracellular enzyme secretion. However, the effect of agitation is not unlimited. Excessive agitation may alter the morphology of filamentous fungi and increase mechanical stress on the mycelia, whereas insufficient agitation may limit oxygen transfer. Therefore, the optimum agitation rate of 180 rpm identified for Penicillium EZ-ZH390 appears to represent a balance between efficient oxygen transfer and favorable physiological conditions for tannase production [36]. In a study, increasing the agitation rate to 120 rpm increased *Aspergillus* tannase activity by 6 U mL^{-1} [37]. As the inoculation dose was increased, tannase activity increased as well. Dara et al. (2011) mentioned that the optimum inoculation dose for *Aspergillus niger* spores was $6*10^6$ cfu mL^{-1} [38]. The performance of *Penicillium* sp. EZ-ZH390 should also be considered in the context of its strain-development history. EZ-ZH390 was obtained through sequential induced mutagenesis of *Penicillium* sp. EZ-ZH190, a native tannase-producing isolate. The first mutagenesis step generated EZ-ZH290, while subsequent γ-irradiation of EZ-ZH290 resulted in EZ-ZH390 [24]. In the previous study, EZ-ZH390 exhibited a higher tannase production level than its parent strain EZ-ZH290, reaching 7.66 U mL^{-1} compared with 4.32 U mL^{-1} for EZ-ZH290. Furthermore, the tannase production level and pH stability of EZ-ZH390 remained consistent over five successive generations [24]. These findings support the potential of EZ-ZH390 as a stable and improved tannase-producing mutant. In the present study, we further investigated this strain under walnut-hull-based submerged fermentation and demonstrated the feasibility of optimizing its culture conditions and validating the process at fermenter scale . The temperature was the other effective factor for increasing the tannase activity. The optimal agitation rate of 150 rpm and temperature of 32°C seem to be optimal conditions for tannase activity from *Aspergillus* [39]. The optimum temperature of 25 °C obtained in the present study indicates that tannase production by Penicillium EZ-ZH390 is highly dependent on the physiological characteristics of the producing strain and the fermentation medium. Temperature influences fungal growth, membrane transport, nutrient assimilation, and the synthesis and secretion of extracellular enzymes. Therefore, the temperature supporting maximum enzyme production does not necessarily coincide with the temperature supporting the fastest fungal growth. Recent studies have reported that the optimum temperature for microbial tannase production varies considerably among fungal species, reflecting strain-specific metabolic responses and differences in fermentation substrates. These observations are consistent with the strain-dependent optimum temperature identified for Penicillium EZ-ZH390 in the present study [36].

In **Figure 2** the interaction between agitation rate and the temperature was investigated in the constant inoculation dose. The effect of the agitation rate was more significant than the temperature. Therefore, the temperature effect was negligible at high speed. The reason may be linked to the increase in aeration amount and uniform distribution of microorganisms throughout the medium during the intense agitation, as well as more access to the substrate, resulting in better feeding for microorganisms [40]. The interact-tion between agitation and temperature suggests that the influence of temperature became less pronounced when sufficient mixing was provided. Improved agitation can reduce local gradients of oxygen concentration and substrate availability within the fermentation broth, allowing fungal cells to experience a more homogeneous microenvironment. Consequently, under adequate mixing conditions, the contribution of agitation to tannase production becomes greater

than the contribution of temperature within the investigated range. Similar interactions between hydrodynamic conditions and temperature have been described for submerged fungal fermentations, where oxygen transfer efficiency influences the response of enzyme production to environmental conditions [35]. *Trichoderma* growth was induced by agitations between 150 and 200 rpm at the optimum temperature of 25-30°C [41]. Increasing the agitation rate caused an increase in enzyme activity [42]. The optimum conditions for tannase from *Lactobacillus plantarum* were a temperature of 30°C, a time of 24 hours, and an agitation rate of 120 rpm [43]. **Figure 3** showed that the inoculation dose was more effective than the temperature so that the temperature effect was negligible at the inoculation dose higher than 5×10^7 cfu mL^{-1}. Increasing the inoculation dose caused an increase in the organism's spore as well as maximizing the use of the carbon source and other sources; consequently, increasing the organism's growth and tannase activity. The optimum dose of *Aspergillus heteromorphus* for tannase production was expressed as 2.3×10^6 cfu mL^{-1} [44]. **Figure 4** showed that tannase activity was heavily dependent on agitation rate at the high inoculation dose. Due to the high agitation rate, organisms are more evenly distributed throughout the medium, thus resulting in more oxygenation. High inoculation dose increased tannase production, which can be because of the ability of walnut halls medium to feed all inoculated spores. These findings indicate that inoculum size and agitation acted synergistically under the optimized submerged fermentation conditions. A sufficient inoculum accelerated fungal establishment within the walnut hull medium, while adequate agitation improved oxygen distribution and nutrient availability throughout the culture. This combination likely promoted earlier biomass development and enhanced extracellular tannase secretion. Similar interactions between inoculum density and agitation have been reported for fungal tannase-producing systems, highlighting the importance of balancing biomass formation with oxygen transfer efficiency during submerged fermentation [35].

As a result of decreasing oxygenation of the medium, the aeration became lower, causing a decrease in tannase activity. Additionally, the higher aeration causes a decrease in tannase activity, since it causes more turbulence in the medium and breaks the mold's hyphae, so the organism's metabolites, such as tannase, are reduced. Furthermore, increasing the aeration resulted in an increase in the growth of *Pichia stipitis* and its metabolites, such as ethanol [45]. The optimal aeration amount for the growth of *Aspergillus awamori* was 0.75 vvm, and the higher aeration caused tannin oxidation [46]. Tannase activity in the fermenter with aeration 1 vvm (3.05 U mL^{-1}) was more than tannase activity in the Erlenmeyer at the optimum condition (2.37 U mL^{-1}), which shows the significant effect of aeration on the tannase activity. The higher tannase activity obtained at 1 vvm indicates that an intermediate aeration rate provided more favorable oxygen-transfer conditions for Penicillium EZ-ZH390 during submerged fermentation. Adequate aeration improves gas-liquid contact and oxygen availability, which are essential for aerobic fungal metabolism and extracellular enzyme production. However, increasing airflow beyond the optimum level does not necessarily enhance enzyme production because oxygen transfer is influenced by both aeration and mixing characteristics within the stirred-tank bioreactor. Similar observations have been reported for stirred bioreactors, where oxygen transfer efficiency depends on the interaction between agitation, aeration, and bioreactor hydrodynamics rather than airflow rate alone [47]. The higher tannase activity obtained at 1 vvm demonstrates that an intermediate aeration rate provided more favorable conditions for enzyme production than either lower or higher airflow rates. Adequate aeration is essential in submerged fungal fermentation because oxygen availability directly influences aerobic metabolism and extracellular enzyme secretion. However, increasing aeration beyond the optimum level does not necessarily improve enzyme production, as excessive airflow may alter the hydrodynamic environment of the culture and affect fungal physiology. Therefore, the results obtained for EZ-ZH390 indicate that efficient tannase production depends on maintaining a balance between oxygen supply and suitable fermentation conditions rather than simply increasing the aeration rate [35]. In research in 2014, the maximum lactic acid production from *Rhizopus microsporus* was at aeration 0.5 vvm [48]. Since dissolved oxygen concentration and the oxygen transfer coefficient

(kLa) were not measured in the present study, these mechanisms are presented as possible interpretations of the observed aeration effect rather than direct experimental observations.

By increasing tannase production, tannin was degraded in the medium into Gallic acid and glucose. These two products are used as carbon sources, stimulate organism growth, and cause more tannase production. Tannase activity decreased (probably) due to excessive accumulation of gallic acid, which reduced tannin decomposition [49]. Reducing sugar was used and decreased during organism growth, and the minimum amount of reducing sugar was reached after 35-45 hours. In this situation, it was expected that organisms would use hydrolase enzymes, such as tannase and cellulose, to break down tannins and polysaccharides and turn them into sugar for use as a carbon source. This can be considered the reason for the continuous decline in total sugar. There was a temporary increase in reducing sugar and a decrease in tannin amount at 60 to 75 hours that may be attributed to an increase in tannase production and conversion of tannins to gallic acid and glucose. During the decomposition of tannins, chelated proteins and sugars were released, which can be used by the organism. The Tannase production graph also confirms the same thing, since the maximum tannase activity was observed at 60-75 hours. Therefore, tannase activity is dependent on organism growth. The temporal relationship between tannin degradation, reducing sugar utilization, and tannase production further supports a growth-associated pattern of enzyme synthesis in Penicillium EZ-ZH390. During the active fermentation phase, hydrolysis of tannins generates gallic acid and glucose, which can subsequently be utilized as carbon sources for fungal metabolism. The maximum tannase activity observed between 60 and 75 h coincided with the period of active substrate conversion, whereas the subsequent decline in enzyme activity may be associated with depletion of readily available nutrients and accumulation of metabolic products during prolonged fermentation. Similar production kinetics have been reported for microbial tannase-producing fungi under submerged fermentation conditions [36]. In a study about the new strain of *Penicillium* EZ-ZH390, it was shown that the tannase production rate per hour was increased by 0.075 U mL^{-1} [24]. In the study of tannase from *Aspergillus niger* in the medium containing 5% tannic acid as the carbon source and incubation at 30°C for 96 hours, maximum tannase activity was observed after 50-60 hours of incubation [50]. Maximum tannase activity from *Aspergillus niger* was up to 3 days. The longer the incubation time, the less tannase activity there was because protease production can cause tannase degradation. Furthermore, the availability of medium nutrients is reduced during this period, which results in a decrease in tannase production [51]. The best time for tannase production was between 1-3 days, and increasing the time caused a decrease in the tannase activity [37]. In *Aspergillus ficcum*, 60 hours were optimum for tannase production [52]. In *Aspergillus niger*, protease activity after 20 hours of fermentation decreased tannase activity [53]. The optimum time for tannase production from *Penicillium galbrum* was 60 hours [54]. In a study by Kannan et al. (2011) on the growth kinetic and tannase production from *Lactobacillus plantarum*, it was observed that during organism growth, the reducing sugar was consumed continuously; thus, at the end of fermentation, it reached approximately zero. Tannase had the maximum production at the middle of the logarithmic phase and the beginning of the stationary phase (5.22 U mL-1). At the beginning of the stationary phase, the protease production reached the maximum amount, and then, a decrease in the tannase activity was observed. The reason can be related to tannase decomposition by protease activity [43]. The results of this particular study indicate that utilization of the previously known Penicillium EZ-ZH390 strain together with walnut shells as the agroindustrial medium, and systematic optimization of the fermentation process parameters helps to develop efficient method of tannase production that is effectively testable in the fermenter scale process.

Although the present study successfully optimized tannase production by Penicillium EZ-ZH390 under submerged fermentation and validated the process at laboratory fermenter scale, the biochemical properties of the produced enzyme were not characterized. Determination of the optimum pH, optimum temperature, pH stability, thermal stability, and kinetic parameters (such as Km and Vmax) is essential for evaluating enzyme performance and its

suitability for industrial applications. Recent studies have shown that these biochemical characteristics are critical for assessing tannase functionality in food processing, beverage clarification, bioconversion of tannins, and other industrial applications. Therefore, comprehensive biochemical characterization of tannase produced by Penicillium EZ-ZH390 will be the focus of future investigations to complement the fermentation optimization presented in this study [36]. From an industrial perspective, integrating a selected tannase-producing strain with an inexpensive lignocellulosic residue offers a practical strategy for improving the economic feasibility of enzyme production. The successful validation of the optimized process at laboratory fermenter scale suggests that walnut hulls can serve as a promising feedstock for future process development. Nevertheless, further techno-economic evaluation at pilot and industrial scales is required to quantify production costs, substrate availability, and process scalability under commercial conditions [12].

4-CONCLUSION

The most effective factors on tannase activity from *Penicillium* EZ-ZH390 in the walnut hulls medium were agitation rate, inoculation dose, temperature, and aeration. The optimum conditions for tannase production were complex medium containing 7% walnut hulls and without adding any minerals, incubation time 72 hours, agitation rate 180 rpm, temperature 25°C, inoculation dose 10^8 cfu mL^{-1}, and aeration 1 vvm. Tannase was dependent on the organism's growth. The maximum production of tannase was observed in the logarithmic phase. Finally, the maximum tannase activity in the submerged fermentation of walnut hulls medium in Erlenmeyer at the optimum condition was 2.37 U mL^{-1}. In the fermenter at the optimum condition with aeration of 1 vvm, the tannase activity was increased to 3.05 U mL^{-1}, which showed an increase of 22.8% of tannase activity. Future studies should focus on the biochemical characterization of tannase produced by Penicillium EZ-ZH390, including its catalytic properties, stability profile, and kinetic parameters, to further evaluate its potential for industrial applications [36].

5. DECLARATION

5. 1. Acknowledgments

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5.2. Author Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Nadia Oraz and Zohreh Hamidi-Esfahani. The first draft of the manuscript was written by Nadia Oraz, and Zohreh Hamidi-Esfahani commented on previous versions of the manuscript. All authors read and approved the final manuscript.

5.3. Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

5.4. Funding

No funding was received for conducting this study.

5.5. Using Artificial Intelligent chatbots

No AI chatbot has been used in this study.

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