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Statistical Optimization for Tannase Production by *Mucor circinelloides* Isolate F6-3-12 under Submerged and Solid State Fermentation

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Abstract

Mucor circinelloides isolate F6-3-12 was selected out of three fungal isolates that can utilize tannic acid as substrate. The fungus was identified by phenotypic analysis and 18S rRNA gene sequencing. Extracellular tannase was produced by solid-state fermentation (SSF) and submerged fermentation (SmF) from plants known for their high tannin content to be used as sole carbon source and supporting solid substrate namely pomegranate rind, sumac leaves and green tea leaves powder , pomegranate rind was selected for maximum tannase production under SSF. Optimization using sequential statistical strategy for some vital factors; substrate concentration, temperature, substrate: moisture ratio, incubation period, inoculum age and KH_2PO_4 were adjusted by the sequential two design of response surface methodology. *Mucor circinelloides* isolate F6-3-12 produced the highest tannase level (2.07 IU) during the first 72 h of fermentaton using pomegranate rind concentration of 1 g/flask in SSF then decline after 96 h. Tannase production was enhanced from 2.07 IU to 5.83 U/gds using Plackett-Burman design and improvement up to 12.24 U/gds upon applying the Box–Behken design that considered 2.82 and 2.1 fold enhancement.

Key Words: *Mucor circinelloides,* tannase, 18S rRNA gene sequencing, sequential statistical strategy, solid-state fermentation.

Introduction

Tannase referred as tannin acyl hydrolase (E.C. 3.1.1.20), is hydrolytic enzyme that is an inducible enzyme which hydrolyze ester bonds present in ellagitannins, gallotannins, complex tannins (1). They are significant enzymes distributed throughout the animal, plant and microbial kingdoms of wide application in food, pharmaceutical and chemical industries. Tannins are natural water-soluble polyphenols found in plants as secondary metabolites (2, 3). Industrially it is produced by microbial means using SmF, where the activity is expressed intracellular that imply additional costs in its production (4). Commercially, tannase were used for preparing instant tea, manufacture of acorn liquor also in gallic acid production, which is used as precursor in the synthesis of antibacterial agent trimethoprim, and also propylgallate as food preservative. Gallic acid also exhibits biological activities as antioxidant (5 ,6) and clarifying agent in juices and coffee-flavored drinks (1, 7) and printing inks. SSF has advantages over SmF techniques for tannase production as using available raw materials as substrates, low energy used, less expensive downstream processing, non-aseptic conditions, less water usage, little fermentation space, higher concentration of the products and high reproducibility (1, 8, 9, 10). Fungi are the most adapted microorganisms for SSF as they are known to tolerate low water and high osmotic pressure conditions and can utilize

agro wastes (11). Changing one variable of the fermentation conditions at a time keeping the others at fixed levels is time-consuming and never guarantees determination of optimal conditions. Designing suitable fermentation conditions is of significant importance because it reduces time, expense and increase product yield and productivity (12).

Materials and Methods

Chemicals: Tannic acid, Quinine HCL, Potato dextrose agar was obtained from Sisco India. Commercial green tea, pomegranate rind and sumac leaves were obtained from local market. Other chemicals were of analytical grade.

Microorganism and inoculums: Fresh fungal spores inoculated on potato dextrose agar were used as inoculum, 2 ml spore suspension (contain-ing around 10^6 spores/ml) was added to 100 ml of the production medium in 250 ml Erlenmeyer flasks and incubated at 30°C for 7days on a reciprocal shaker (200 rpm). The fungal strain was routinely grown on potato-dextrose agar (PDA) medium at 30°C and preserved at - 80ºC in 50%(v/v) glycerol.

Phenotypic and genotypic Characterization: Genetic identification using molecular taxonomy was performed to identify and study the phylogeny. Molecular identification of the selected fungal isolate was performed based on its internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. Mycelia were collected by centrifugation (at 4 °C and 5000 rpm for 20min) and DNA was extracted by using protocol of Gene Jet Plant genomic DNA purification Kit (Thermo#K0791). PCR was performed by using Maxima Hot Start PCR Master Mix (Thermo# K0221), and PCR clean up to the PCR product made by using GeneJET™ PCR Purification Kit (Thermo#K0701) in Sigma Company of Scientific Services, Egypt (www.sigma-co-eg.com). Finally, sequencing of the PCR product was performed a t GATC Company (German) by using ABI3730xlDNA sequencer, using forward and reverse primers, and by combining the traditional Sanger technology with the new 454 technology. Purified DNA was subjected to PCR amplification using a pair of ITS1(52 -TCCGTA GGTGAA CCTGCGG-32) and ITS4(52 -TCCTCCGCT TATTGATAT GC-32) primers for ITS-rDNA amplification (13). Sequence data was analyzed in the Gene Bank data base by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The unknown sequence was compared to all of the sequences in the database to assess the DNA similarities (14). Multiple sequence alignment and molecular phylogeny were performed using BioEditsoftware. The phylogenetic tree was displayed using the TREEVIEW program (15, 16).

Culture conditions: Two culture systems; submerged fermentation (SmF) and solid state fermentation (SSF) were used for tannase production. Composition of production medium (Modified Dox medium) was (g/L): $NaNO_3$; 6, KCL; 0.52, $MgSO_4.7H_2O$;0.52, KH_2PO_4; 0.52, $ZnSO_4$; 0.01, $FeSO_4$;0.01 (17) and 3gm green tea leaves powder per flask (In case of SSF 1gm of green tea leaves per flask) at 30ºC, initial pH 5.5 and varying incubation time (200 rpm agitation for SmF). Two ml spore suspension were used as inoculum of the production medium, the growth kinetics of the fungal culture in SmF and SSF were periodically monitored. Crude enzyme extracts from SmF were obtained by filtering, while the extracts from SSF were obtained by centrifugation at 5000 rpm, all experiments were conducted in triplicate.

Three solid substrates which are pomegranate rind, sumac leaves and green tea leaves powder were examined for enzyme production. Each substrate was dried at 70 ºC and finely powdered in a grinder mixer and used in SSF. These substrates were used as solid media for tannase production without any pretreatment. One gram powder of each substrate was added to 250 ml Erlenmeyer conical flasks, moistened with 3 ml of modified Dox medium of pH 5.5. The contents were autoclaved at 121.5ºC for 20 min, cooled to room temperature and inoculated with 2ml of fungal spore inoculum. The

contents were mixed thoroughly and incubated at 30°C for different incubation periods.

Extraction of tannase enzyme: For the submerged fermentation, after incubation for the desired period, the fungal mycelia were removed by filtration through Whatman No.1 filter paper and the supernatant was treated as crude enzyme for assaying tannase activity. For solid state fermentation, after incubation, the fermented substrates were mixed properly by adding 50 ml of 0.2M acetate buffer (pH 5.5) to the fermented medium. Then, the flasks were kept on the rotary shaker for 1hr and centrifuged at 5000 rpm for 10 min to remove all fungal debris and residue of substrate (18) the clarified extract that represented the crude enzymes was used for assaying tannase activity.

Tannase assay: Tannase assay was estimated by the modified colorimetric method (19). Based on violet colour produced when $FeSO_4$ in presence of Rochelle salt (color reagent) that react with gallic acid. The reaction mixture contained 0.5ml of tannic acid (2.0 % w/v in 0.2M sodium acetate buffer, pH 5.5) was added to 0.5ml of crude enzyme. This reaction mixture was incubated at 40°C for 30 min. The enzymatic reaction was terminated by adding 3.0 ml of precipitant solution (100 ml Quinine HCL (1.0%) mixed with 50 ml of 10.0 % NaCl). A control was prepared side by side using heat denatured enzyme. Then 1ml of the mixture was transferred in open Dwarf tube and centrifuged at 9000 rpm in ultra micro-centrifuge for 15 min. 300µl of the supernatant was transferred to a dry clean test tube and mixed well with 0.5ml of the color reagent. The volume was completed to 5 ml by adding distilled water. The developed color (reddish violet) was measured at 555 nm against boiled enzyme as control, using Spectro UV-VIS labomed.Inc.USA. One unit of enzyme (international units) was defined as the amount of enzyme able to release 1 ìmol gallic acid per minute of culture filtered under the standard assay conditions.

Determination of hydrolysable tannins: One gram of each of green tea or sumac leaves or pomegranate rind was mixed with 10 ml water, autoclaved, then substrates were mixed properly by adding 50 ml of 0.2M acetate buffer (pH 5.5) to each substrate. Then, the flasks were kept on the rotary shaker for 1hr and centrifuged at 5000 rpm for 10 min to remove all residue of substrate; one ml of the clarified extract was transferred to a clean test tube. The tannin compounds are precipitated by adding 2 ml of precipitant solution. One milliliter of the mixture is transferred to Open Dwarf tube and centrifuged at 9000 rpm for10 min. Five hundred micro liter of the supernatant was transferred to a clean and dry test tube and mixed with 0.5 ml of the color reagent. The volume was completed to 5 ml by adding distilled water. The developed color was measured at 555nm. The reading of OD ×6.0 is equivalent to hydrolysable tannins (19).

Protein content: The protein content was determined according to Lowry method (20).

Culture conditions

Effect of incubation time: The fungal culture was inoculated in the autoclaved SSF flasks then incubated at 30°C for different time periods ranging from 72h to 216 h. The enzyme was extracted as discussed above and the crude enzyme was preserved at 4°C for further analysis.

Effect of moistening agents: Different types of moistening agents; modified Dox medium, tap water and distilled water were examined for their role in enzyme production. The solid substrates were moistened by one of these moistening agents and mixed properly, autoclaved, inoculated and incubated at 30°C for the proper time. Then, the enzyme was extracted as above and assayed for tannase activity.

Effect of moisture level: The solid substrate was moistened using modified Dox medium with different ratios (w/v) ranging from 1:4, 1:5and 1: 6 to determine the best ratio of substrate: moistening agent for enzyme production under SSF. The enzyme was extracted and assayed for tannase activity.

Statistical analysis

Plackett-Burman design: The Plackett-Burman experimental design is a fractional factorial design

recommended when more than five factors are under investigation (21,22). This design is practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or near optimal responses (23). In this study, the design was used to reflect the relative importance and concentrations of some medium components and fermentation conditions on tannase activity. Seven independent variables were screened in nine combinations, organized according to the Plackett Burman design matrix described in the results section. For each variable, a high level (+) and low level (-) was tested. All trials were performed in duplicates and the averages of products percentage were treated as the responses. The main effect of each variable was determined by the following equation:

$Exi = (©Mi^{+} - ©Mi^{-}) / N$

Where Exi is the variable main effect, Mi^{+} and Mi^{-} are tannase activity in trials where the independent variable (xi) was present in high and in low levels, respectively, and N is the number of trials divided by 2.

Box-Behnken Design:In the second phase of medium formulation for optimum tannase activity, the Box-Behnken experimental design was applied (24). In this model, the most significant independent variables, named (X_1), (X_2) and (X_3) were included and each factor was examined at three different levels, low (-), high (+) and central or basal (0). These factors included incubation time (X_1), concentration of $NaNO_3$ (X_2) and moisture content (X_3), all were treated as independent variables. Thirteen combinations and their observations (shown in the results section) were fitted to the following second order polynomial mode:

$$Y=b_o+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$$

Where, Y is the dependent variable (tannase activity); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient at center point; b_1 , b_2 and b_3 are linear coefficients; b_{12} , b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP software. The quality of the fit of the polynomial model equation was expressed by R2 (regression coefficient). If the proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA), contour plots can be usefully employed to study the response surface and locate the optimum operational conditions (25). The 3D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level for maximal production of tannase. The F-test was calculated to determine factors having a significant effect.

Results and Discussion

Biosynthesis of tannase enzyme under submerged fermentation: The present part of the study aims to the examination of three fungi for the biosynthesis of tannase by submerged culture using green tea as substrate. Fermentation was undertaken by shaking culture technique at 30°C using production medium contain 3% green tea in Erlenmeyer flask (250 ml) contain 50 ml modified Dox medium. Fermentation lasted for 2, 3, and 4 and 5 days at 30°C, the fermentation broth was centrifuged and assayed for tannase activity, final pH and protein content.

The results given in table-1 indicated that no relationship between the final pH and the synthesis of tannase, in all cases, the pH values of the culture filtrate of the investigated cultures varied from 4.26-7.83. The results also indicated that the synthesis of tannase depends on the fungus and on the time of incubation. Isolate 1 exhibited the highest tannase activity (1.78 U/ml after 3 days). The other two fungal species showed lower tannase activities; 0.22U/ml after 48 h for isolate 2 and 0 .15 U/ml for isolate 3 after 48 h. Of all the three investigated fungi,isolate 1 was the most promising tannase producer, therefore it was selected for further investigation.

Identification of the fungus: Sequence analysis of 18S rDNA of the selected fungus (isolate 1)

with available NCBI Gen Bank database showed that the tested fungus revealed high identity (99%) to *Mucor circinelloides* (Gene Bank accession number KX349462). The phylogenetic tree was displayed using the TREEVIEW Program that showed the sequences of close relatives obtained from Gen Bank to resolve the phylogenetic relations with ancestor (Fig. 1). *Mucor* is commonly used in industry to produce important organic acids and other industrial enzymes.

Biosynthesis of tannase enzyme by Mucor circinelloides isolate F6-3-12 ***using solid state fermentation (SSF):*** In this experiment three agriculture residues namely pomegranate rind, sumac leaves and green tea powder were used as substrates. This was carried out by inoculating the selected fungus ***Mucor circinelloides*** isolate F6-3-12 in 250 ml Erlenmeyer flasks each containing 3gm of each substrate and 10 ml modified dox medium. At the end of the fermentation period, the fermented substrates were treated as mentioned above. Data recorded in table (2) showed that by using pomegranate rind powder the tannase activity reached 3.82 U/gds (U/g substrate) with high protein content (354 mg/g substrate) this may be due to the highest tannin content of pomegranate rind powder among the other considered substrates (hydrolysable tannin 8.9%).

It's clear that pomegranate rind powder was the best inducer for tannase biosynthesis (3.82 U/gds) after 3 days with specific activity 0.01 and decrease to 2.62U/gds after 4 days, the enzyme production decreased due to the depletion of the substrates. Therefore pomegranate rind powder was used as a sole solid substrate a in the next experiments instead of green tea. Utilization of agricultural wastes into valuable products helped in solving waste disposal problems.

Results obtained from this comparative study of tannase production using both SmF and SSF systems showed that ***Mucor circinelloides*** isolate F6-3-12 had a higher tannase activity using SSF (more than 1.85 times) as compared to SmF, and this observation is similar to the results previously reported (7). It is important to note that for tannase production, the used model (substrate- support) resulted in enhanced enzyme induction and also indicates that ***Mucor circinelloides*** isolate F6-3-12 strain can be adapted for SSF system and utilizes the nutrients in a better form than when it is grown in other culture systems, like SmF. The other two substrates namely sumac leaves and green tea

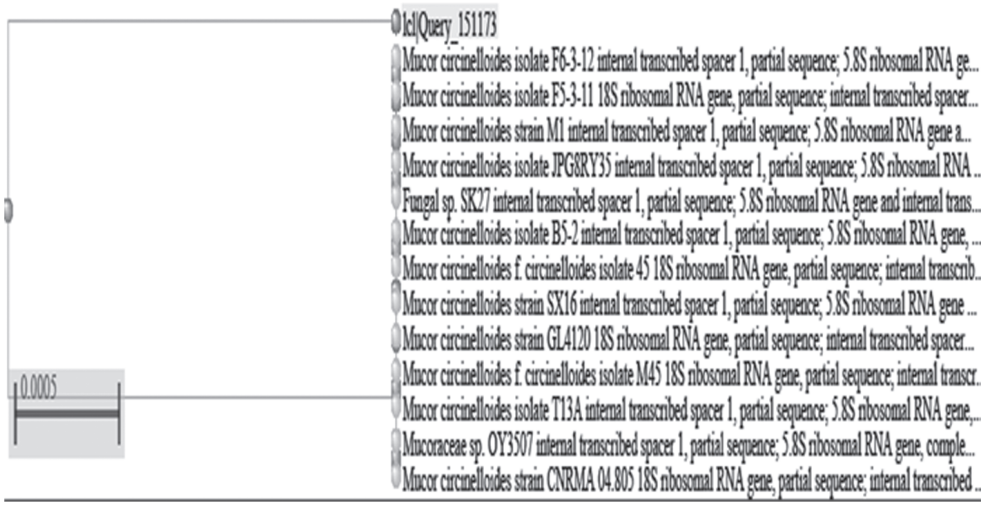

Fig. 1. The phylogenetic tree of *Mucor circinelloides* isolate F6-3-12

leaves produce 2.07 U tannase /gds respectively at 72h. After 120 h, there was a reduction in the amount of tannase due to the depletion of the substrates and the accumulation of gallic acid as reported by Kar *et a*l., (26). Also Bradoo *et al.* (27); and Aguilar and Gutiérrez-Sánchez, ((1) mentioned that tannase is produced during the phase of growth and thereafter declines, also gallic acid showed end product repression (26,27,28). Maximum tannase production was obtained using pomegranate rind as substrate, so it was selected for our further studies on tannase enzyme production.

Influence of incubation time on tannase production: The effect of the incubation period (3, 5, 7 and 9 days) on the tannase productivity was investigated at 30^{o}C. The results (table 3) indicated that an incubation period of 3 days was the most appropriate for the production of tannase (3.82U/gds) with specific activity 0.01U/mg protein, after 5 days the activity decrease to 2.62U/gds with specific activity 0.007U/mg protein and at 7 days decrease to 0.76 U and no activity was detected after 9 days. From the mentioned data, incubation periods of 3 and 5 days were chosen for extra investigation.

Effect of moisturizing agents on tannase biosynthesis:In this experiment the effect of different types of moisturizing agent (dox medium, tap water and distilled water) was examined for tannase biosynthesis. The pomegranate rind was moistened with one of these moistening agents, mixed, autoclaved and incubated at 30 ^{o}C for 3 and 5 days. The results in table (4) indicated that dox medium was the most favorable moisturizing agent with activity 3.82 U/gds and specific activity 0.011U/mg protein after 3 days then decreased to 2.62 after 5 days, followed by tap water and distilled water gave 3.17 U/gds after 3 days, and after 5 days the activity decreased to 2.61 and 2.13 respectively. . Our results agree with that reported by Beniwal *et al*. (29). They found that Czapek Dox medium was the best moisturizing agent for enzyme (2.3U/g) production followed by tap water and distilled water. Majit *et al*. and others (30,31,32) found that tap water was the best moisturizing agent for enzyme production. Ahmed *et al*. (33) also reported distilled water-mediated extraction from the fermented masses.

Extraction of the enzyme from solid substrate at the end of the fermentation process is critical step as it determines the cost of enzyme production due to bounding of the enzyme with the fungal mycelia by means of different noncovalent bonds.

The modified Dox medium was used in different ratios (w/v): 1:4, 1:5 and 1:6 to moist the substrate, a ratio of 1:6 was found to be the best for tannase yield due to the low oxygen supply as the moisture level increases hence a reduction in both enzyme production and biomass. Our results agree with Jana *et al*. (34) using 1:6 solid to solvent ratio.

Optimization of Mucor circinelloides isolate F6-3-12 tannase biosynthesis by multi-factorial design: Fermentation conditions required for maximum rate of tannase biosynthesis were then explored by carrying out a two- phase multi-factorial optimization approach. The first approach involved the application of an incomplete factorial experiment to screen for the optimum culture conditions and medium composition that affect the tannase biosynthesis. The second part was to optimize the most significant factors using a response surface methodology.

Elucidation of fermentation factors affecting tannase biosynthesis: The Plackett- Burman design (21) was used to determine the different culture and medium conditions involved on tannase biosynthesis process. Seven variables were used at two levels, high concentration (+) and low concentration (-) as showed in table (5). The factors tested were shown in Table (6); incubation time, sodium nitrate, KHP_2O_4, moisture level, substrate concentration, temperature and inoculums age. Each row represents a trial run and each column represents an independent variable. All experiments were performed in triplicates and the results (average of the observations) of tannase activity and protein content were calculated. The difference between

Table 1. Survey of some fungal strains for the biosynthesis of tannase in submerged culture using green tea as carbon source

Strain	Final pH Activity U/ml	Tannase mg/ml	Total proteins	Specific activity (U/mg protein)
isolate 1				
48 hrs	4.26	1.08	29.5	0.037
72 hrs	4.80	**1.78**	29.5	0.06
96 hrs	5.27	0.86	43.0	0.02
120 hrs	5.02	-	45.6	-
Isolate				
248 hrs	7.08	0.22	23.3	0.009
72 hrs	6.55	0.073	25.0	0.003
96 hrs	6.52	0.073	30.1	0.002
120 hrs	6.84	0.044	33.0	0.001
Isolate 3				
48 hrs	7.83	0.15	19.5	0.007
72 hrs	7.7	0.073	21.0	0.003
96 hrs	7.39	-	23.0	-
120 hrs	7.22	-	23.0	-

Table 2. Effect of different substrates (containing tannins) as carbon source for biosynthesis of *Mucorcircinelloides*isolate F6-3-12 tannase enzyme by using solid state fermentation (SSF) technique

Substrate	Tannase (U/g ds)	Total proteins (mg/ml)	Specific activity (U/mg protein)
Pomegranate rind72 hrs120 hrs	3.822.62	354354	0.010.007
Sumac leaves72 hrs120 hrs	2.07-	768503.9	0.003
Green tea leave72 hrs120 hrs	2.07-	336372	0.006-

Amount of initial gallic acid in natural substrates (mg/g) :

Pomegranate rinds: 89.520; Sumac Leaves : 57.524; Grean tea leaves : 70.952

both averages of measurements made at high level (+) and the low level (-) of the factor was estimated as the main effect of each variable upon tannase biosynthesis (Table-7).

Table-6 showed a wide variation tannase activity from 0.12 to 5.83 U/gds, this reveal that medium optimization is necessary for high enzyme production. The main effects of the examined factors on the enzyme activity were calculated and ranked by analyzing the Plackett-Burman design data. The tannase biosynthesis is positively affected by incubation time, moisture level and $NaNO_3$ concentration, and temperature in the medium. On the other hand other variables had the negative effect.

A formula composed of (g/ l) is predicted to be near optimum: pomegranate rind, 7g; K_2HPO_4, 2.0; sodium nitrate, 7.0; inoculum age, 9 days

Table 3. Effect of incubation period on *Mucorcircinelloides*isolate F6-3-12tannase biosynthesis under Solid State Fermentation

Time	Tannase (U/g ds)	Total proteins (mg)	Specific activity (U/mg protein)
3 days	3.82	354	0.011
5 days	2.62	354	0.007
7 days	0.76	516	0.001
9 days	-	546	-

Table 4. Effect of moisturizing agents on *Mucorcircinelloides*isolate F6-3-12tannase biosynthesis

Moisturizing agents	Tannase (U/g ds)	Protein (mg/g ds)	Specific activity (U/mg protein)
Modified Dox media			
72 hrs	3.82	258	0.015
120 hrs	2.62	192	0.014
Tape water			
72 hrs	3.17	300	0.011
120 hrs	2.61	270	0.010
Distilled water			
72 hrs	3.17	192	0.016
120 hrs	2.13	108	0.019

Table 5. Factors examined as independent variables affecting Tannase biosynthesis by *Mucorcircinelloides*isolate F6-3-12 and their levels in the Plackett-Burman experiment.

Factor	Symbol	-	0	+
Time	T	2	3	4
Sodium nitrate% $NaN0_3$	NN	5	6	7
KH_2P0_4%	K	1	1.5	2
Moisture level	M	1:4	1:5	1:6
Substrate concentration	S	3	5	7
Temperature	Temp	27	30	33
Inoculum age	in	5	7	9

Table 6. Optimization of media fermentation for the biosynthesis of Mucorcircinelloidesisolate F6-3-12 tannase with Plackett-Burmandesign

Trial No.	T	NN	K	M	S	Temp	IN	Tannase (U/g ds)	Protein (mg/g ds)	Specific activity (U/mg protein)
1	-	-	-	+	+	+	-	1.62	215.6	0.007
2	+	-	-	-	-	+	+	0.42	213.9	0.001
3	-	+	-	-	+	-	+	0.63	217.1	0.002
4	+	+	-	+	-	-	-	3.79	269.1	0.014
5	-	-	+	+	-	-	+	0.12	120.7	0.001
6	+	-	+	-	+	-	-	0.52	274.8	0.001
7	-	+	+	-	-	+	-	0.31	182.4	0.001
8	+	+	+	+	+	+	+	5.83	316.1	0.018
9	0	0	0	0	0	0	0	3.82	223.8	0.017

Table (7): Main effects of independent variables on tannase biosynthesis produced by *Mucorcircinelloides*isolate F6-3-12according tothe results of Plackett-Burman design

Independent variables	Main effect
Time	2.06
$Na NO_3$	1.88
$KH_2 PO_4$	0.03
Moisture	2.7
Substrate	0.8
Temperature	0.97
Inoculum age	0.24

Table 8. Examined concentration and the results of the Box-Behnkendesign experiment

Trial	Independent variable			Tannase activity (U/g ds)	Total protein (mg/g ds)	Specific activity (U/mg protein)
	X1 Incubation period	X2 Moisture level	X3 $NaNO_3$			
1	-	-	0	0.42	316.7	0.001
2	+	-	0	3.49	428.7	0.008
3	-	+	0	3.14	214.3	0.01
4	+	+	0	0.4 4	287.4	0.001
5	-	0	-	6.22	347.1	0.017
6	+	0	-	0.87	187.2	0.004
7	-	0	+	12.24	136	0.09
8	+	0	+	11.99	318.3	0.037
9	0	-	-	0.32	315.1	0.001
10	0	+	-	0.41	333.4	0.001
11	0	-	+	1.11	284.7	0.003
12	0	+	+	9.89	496.9	0.02
13	0	0	0	8.14	365.4	0.022

Table 9. Results of ANOVA analysis for optimization of *Mucorcircinelloides*isolate F6-3-12Tannase enzyme production by the Box-Behnken experiment.

Term	Coefficient estimate	*df*	SE	Sum of Squares	t-value	*F*-value	*P*-Value
Corrected Model	-	9	-	262.58678	-	6.9408	0.0230*
Intercept	8.14	1	1.183722	-	6.88	-	0.0010*
X_1	-0.66625	1	0.724879	3.55111	-0.92	0.8448	0.4002
X_2	1.055	1	0.724879	8.90420	1.46	2.1182	0.2053
X_3	3.42625	1	0.724879	93.91351	4.73	22.3412	0.0052*
$X_1 * X_2$	-1.5675	1	1.025134	9.82823	-1.53	2.3381	0.1868
$X_1 * X_3$	1.275	1	1.025134	6.50250	1.24	1.5469	0.2687
$X_2 * X_3$	2.1725	1	1.025134	18.87903	2.12	4.4912	0.0876
X_1^2	-0.7475	1	1.066993	0.52650	-0.70	0.1253	0.5148
X_2^2	-5.645	1	1.066993	119.77498	-5.29	28.4935	0.0032*
X_3^2	0.4375	1	1.066993	0.70673	0.41	0.1681	0.6988

R^2 =R Squared = 0.92589(Adjusted R Squared = 0.792492)

with initial pH 7.0 at 33 °C for 4 days incubation. The remaining variables with less significant main effect were used in all next trials at their zero level; this optimized medium was used for further investigations.

A verification experiment was carried out in triplicate for determining the accuracy of the Plackett- Burman design, the applied near optimum condition resulted in 5.83 U/gds tannase biosynthesis. These results represent about 1.53 fold increases in tannase biosynthesis in comparison to the results showed using the basal condition (3.82U/g).

Culture conditions optimization by Box – Behnken design: In order to search for the optimum concentration of the most significant variables (incubation time, sodium nitrate and moisture level), experiments were performed according to Box - Behnken experimental design. The coded and levels of the three independent variables investigated at three different levels (-1, 0, +1) were listed in Table (8) with 13 trials.

The regression equation coefficients were calculated, and the data were fitted to a second-order polynomial equation.

$$Y_{activity} = 8.14 - 0.66625X_1 + 1.055X_2 + 3.42625X_3 - 1.5675X_1X_2 + 1.275X_1X_3 + 2.1725X_2X_3 - 0.7475X_1^2 - 5.645X_2^2 - 0.4375X_3^2$$

Y activity is the response (tannase yield) and X_1, X_2 and X_3 are the coded values of the test (incubation period, moisture level and sodium nitrate), respectively. The two dimensional contour plots as the graphical representations of the regression equation (Fig.2) determine the interactions of the three factors and showed the optimum levels of each and their interactions using SAS JMP 8 NULL program tools.

Regression results from the Box- Behnken designed were shown Table (9). The more significant is the corresponding coefficient reflected the high magnitude of the *t*- value and low magnitude of the *p*- value. The variable with the largest effect was the moisture level- the linear effect- and the squared term of sodium nitrate, the results obtained in this study are obviously lower than those reported by Aguilar and Gutiérrez-Sánchez(1), Aguilar *et al.* (28), Kar and Banerjee (35) and Kar *et al*. (36). Liu *et al*., (37) reported that maximum production of tannase by *Aspergillus spp*.UCP1284 (SSF) using a factorial design (2^3) was 12.26U/g dry substrate with initial moisture content of 40% and using 2.0% of tannic acid.

(R^2) was calculated as 0.92 for tannase activity which indicates that the statistical model can explain 92.1 % of variability in the response (a value of R2 > 0.75 indicated the correctness of the model). The aptness of the model can be tested by the value of coefficient (R^2) and correlation coefficient (R), the closer the value of R to 1, the better the correlation between the measured and the predicted values.

Verification of the optimization methods: A verification experiment was done using the predicted optimal conditions for tannase biosynthesis while the basal medium was used as control. Under the optimization condition 12.24 U/gds tannase biosynthesis was reached. The result indicated that the optimization conditions accelerated about 5.91 fold times more tannase yield (12.24/g) of the basal medium (2.07 U/g). The two steps of optimization resulted in a formula of the following composition (g/l): pomegranate rind, 7g/flask; KH_2PO_4, 2.0; $MgSO_4.7H_2O$,0.52; KCl, 0.52; $ZnSO_4$ $8H_2O$,0.01 and $FeSO_4$ $8H_2O$,0.01 at initial pH 5.5 after 72 h incubation with moisture content of 1:7 under solid state fermentation.

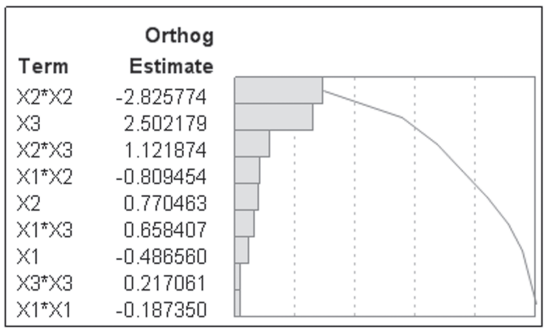

Fig. 2. Pareto Plot of Transformed Estimates

Conclusions

The present investigation suggests that agro residues such pomegranate rind are one of the best and most cost effective alternatives for the costly pure tannic acid for industrial production of microbial tannase. The culture conditions for the production of tannase enzyme from *Mucor circinelloides* isolate F6-3-12 were evaluated and standardized. These conditions were: solid-state fermentation, incubation temperature of 30 °C, fermentation time of 72 h. The first step response surface methodology resulted in a 2.8 fold increase in tannase production and 2 fold after the second step. As the range of application of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse field of applications. By using cheaper and easily available substrates and use of dox medium as moistening agent without need of any mineral salt, we have tried to lower down the input cost for enzyme production which is one of the reasons that limit the use of tannase at the industrial level and thus it can be possible to use this enzyme in large scale in beer, food, fruit and leather industries.

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