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Original article

Pomegranate peel is a low-cost substrate for the production of tannase by *Bacillus velezensis* TA3 under solid state fermentation

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ABSTRACT

Tannase production from *Bacillus velezensis* TA3 was optimized by sequential statistical approach using pomegranate as a solid substrate. Easily available and low-cost substrates such as cow dung, coffee husk, apple peel and pomegranate peel were initially screened. Among these substrates, pomegranate peel was found to enhance tannase activity. Further, pomegranate peel was used as the substrate to optimize the factors by traditional methods. In one-variable-at-a-time approach optimum process conditions were, 48 h incubation (1.92 U/g), 0.3% tannic acid (1.33 U/g), 0.08% minerals (2.3 U/g) with 55% initial moisture content (2.7 U/g). To screen the impact of variables such as fermentation period, moisture content, sodium chloride level, tannic acid concentration and required minerals on tannase production under solid state fermentation, a two-level full factorial design was used. Among these factors, incubation time, moisture and tannic acid concentrations were found to have significant influence on tannase production ($p < 0.001$). The optimum concentration of these three significant factors was further evaluated by central composite design (CCD) and response surface methodology (RSM). The optimum concentration of these variables was determined as 57 h incubation, 72.5% moisture and 0.68% tannic acid. Under optimized conditions, the experimental result of 32 U/g substrate was very close with predicted value (33.1 U/g substrate) which validated the experimental design. RSM mediated statistical approach showed 9-fold enzyme activity than unoptimized medium.

1. Introduction

Tannase is an extracellular or intracellular enzyme, adaptive, that comes under the super family of esterase (Aguilar et al., 2007). It is mainly involved in the catalysis of tannins by hydrolyzing their ester and depside bonds giving glucose, gallic acid as well as various galloyl esters of glucose (Haslam and Stangroom, 1996). It has varied industrial applications in feed, food, brewing, chemical and pharmaceutical firms extending from the making of acorn wine, instant tea, gallic acid, and flavored cool drinks (Madeira et al., 2011). Tannase is synthesized by various microorganisms such as fungi, yeast and bacteria. Of these sources, most of the research work has been done on fungal tannase. But the application of fungal isolates for the large-scale production of tannase is limited due to its genetic complexity and because of its slow growth rate. Bacterial strains which can resist high pH and/or temperature may be considered as a novel source for the production of industrially useful tannase (Beniwal et al., 2015). Production of tannase by bacteria such as *Lactobacillus plantarum, Serratia ficaria* and *Bacillus massiliensis* has been published (Ayed and Hamdi, 2002; Belur and Mugeraya, 2011). Industrial production and purification of tannases can be done by submerged or solid-state fermentation techniques. The important benefits of solid-state fermentation (SSF) include lower production cost, low wastewater generation, simplicity and high enzyme yield. On other hand, submerged fermentation (SmF) has advantages in sterilization, process control, incubation time, utilization of whole substrates and simple recovery technique of extracellular enzymes. In both these

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processes, substrates with rich tannin are used in order to achieve high enzyme yield.

The crude tannin obtained from various natural resources is used for the tannase production to minimize the making cost. Amla leaves, jamum leaves, rice bran, peanut oil cake, wheat bran, baggase, powdered tamarind seeds, tea and coffee leaf residues have been already reported as substrates for tannase production. Yield of tannase was found to be more in SSF when compared to SmF (Aguilar et al., 2007). Application of castor bean residues (Madeira et al., 2011) and rose wood dust (Beniwal et al., 2013) as substrates for the making of tannase using SSF had been reported. Since, various factors are involved in fermentation process, various statistical methods can be used to improve enzyme production by optimizing the factors (Vijayaraghavan and Vincent, 2015). Two level full factorial experimental design is one of the important statistical tools to screen the important variables for optimization of various fermentation factors. Even though there are various kinds of optimization designs available, central composite design (CCD) is frequently used.

The present study aims to screen and optimize various process variables for tannase production from *B. velezensis* TA3 isolated from a saltpan using low cost medium under SSF by a two-level full factorial design and response surface methodology.

2. Materials and methods

2.1. Isolation of halotolerant bacteria for tannase production

B. velezensis TA3 used in this study was isolated from a hypersaline environment at Kanyakumari, Tamil Nadu, India. Soil samples were collected from saltpan and bacterial isolation was performed by standard method. Briefly, 1 gm of soil sample was suspended in 100 ml distilled water and different dilutions were plated on minimal medium and enriched medium with 0.5% (w/v) tannic acid. The morphologically different bacterial isolate was picked and pure culture was made. Tannase producing bacterial isolates were determined based on zone of hydrolysis. (Jana et al., 2012). The strains were then inoculated into nutrient broth medium and kept for 18 h incubation at 37 °C with an agitation rate of 175 rpm.

2.2. Substrates

The solid substrates such as Orange peel, Wheat bran, Cow dung, Coffee husk, Apple peel and Pomegranate peel were used in this study. The substrate such as orange, apple and pomegranate peels were obtained from the local market. Substrates were sun dried for 3 days, after which oven dried at 60 °C and finally finely ground using a mixer grinder. The powdered substrates were then sieved using a standard sieve to remove impurities and were stored in a sterilized container for further use.

2.3. Tannase production by solid state fermentation

Five grams of each substrate was taken in 250 ml Erlenmeyer flask. These act as an inert material and also provide nutrients to the organism. The particle size was ranged between 2.0 mm and 3 mm. The moisture content of the culture medium was kept as 60% (v/w) using mineral salt solution ($MgSO_4$ (0.1%), NH_4NO_3 (0.5%), KCl (0.1%), Na_2HPO_4 (0.1%) with pH 7.0. The flasks were sterilized at 121 °C for 30 min and cooled. 5% inoculum which was prepared earlier in nutrient broth was then inoculated and incubated for 2 days. 50 ml phosphate buffer was added to the fermented substrate after two days. The crude enzyme source was filtered followed by centrifugation (10,000 rpm for 10 min) and assayed.

2.4. Tannase assay

It was performed following the protocol of Sharma et al. (2007) using spectrophotometric method of analysis with little modifications using tannic acid as the substrate.

2.5. Optimization of tannase production by one-variable-at-a-time approach

Optimum nutritional factors and physical parameters required to enhance tannase production by *B. velezensis* TA3 was screened under SSF using pomegranate as the substrate. The effect of fermentation period (24 h–72 h), moisture content of the culture medium (50–75%), supplementation of tannic acid (0.1–0.5%), carbon sources (glucose, xylose, maltose, starch and sucrose, 0.5%) and mineral salts such as, $MgSO_4$, NaH_2PO_4, $CaCl_2{\cdot}2H_2O$ and KCl) at 0.02%–0.1% were added.

2.6. Screening of variables for the production of tannase by statistical approach

A two-level full factorial design having a set of 32 experimental runs with two replications was made to evaluate the significance of the selected five factors on tannase production by *B. velezensis* TA3 with pomegranate peel as substrate. The variables selected were fermentation period (24 and 48 h), moisture content of the culture medium (50–70%), sodium chloride (3.0–6.0%), supplementation of tannic acid (0.05–0.5%) and mineral salts (0.01% – 0.1%). In this experimental design, the highest (+) and lowest (−) level was described in Table 1a and the response was tabulated in Table 1b. Factors that showed more than 95% confidence levels were considered to have significant influence on tannase production (Table 1c). After elucidating the effect of the selected variables, the process parameters that had influenced tannase production were determined. Then, three factors – moisture content of the medium, incubation time as well as tannic acid concentration were further evaluated using CCD to optimize their concentration to acquire maximum tannase activity. The software Design Expert (version 10) was used for the design and interpretation of results. The mean value of (−1 and +1) was used to assess the response of the designed model, which was calculated by following first-order polynomial equation.

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm} \alpha_{ijklm} x_i x_j x_k x_l x_m$$

where Y is the response, α_{ij} = ijth, α_{ijk} = ijkth, α_{ijkl} = $ijkl$th, and α_{ijklm} = $ijklm$th interaction coefficients, α_0 was an intercept, and α_i was the ith linear coefficient.

2.7. Central composite rotary design and response surface methodology

Using CCD at five levels in 20 different runs, incubation time, moisture content and tannic acid were further optimized (Table 2a). The selected variables and the concentrations of the variables were initially fixed based on the analysis of statistical screening. The designed matrix and results were described in Table 2b. The model for calculating optimum response is in accordance with the second-order polynomial equation as follows.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{ij=1}^{3} \beta_{ij} X_{ij},$$

Table 1a
Independent variables and levels of a two-level full factorial design for the production of tannase.

Factor	Name	Units	−1	+1
A	Fermentation period	h	24.00	48.00
B	Moisture	%	50.00	70.00
C	Tannic acid	%	0.0500	0.5000
D	Sodium chloride	%	3.00	6.00
E	Minerals	%	0.0100	0.1000

Table 1b
Design matrix and response for the selected five variables in two-level full factorial design.

Run	Fermentation period h	Moisture %	Tannic acid %	Sodium chloride %	Minerals %	Enzyme activity (U/g)
1	48	70	0.05	3	0.1	3.2
2	48	70	0.5	6	0.01	5.4
3	24	50	0.05	6	0.01	2.9
4	48	50	0.5	6	0.01	6
5	24	50	0.5	6	0.01	3.47
6	24	50	0.05	6	0.1	7.58
7	48	70	0.05	6	0.01	7.26
8	48	50	0.05	6	0.01	7.4
9	24	50	0.5	6	0.1	4.5
10	24	70	0.05	6	0.1	4.8
11	24	70	0.05	3	0.01	5.5
12	48	50	0.05	6	0.1	1.6
13	48	70	0.05	3	0.01	6.8
14	48	50	0.5	3	0.01	3.9
15	48	50	0.5	3	0.1	9.8
16	48	70	0.5	3	0.01	5.8
17	48	50	0.05	3	0.01	11.9
18	48	50	0.5	6	0.1	9.3
19	24	50	0.5	3	0.1	4.6
20	24	70	0.5	6	0.1	5.19
21	48	70	0.05	6	0.1	9.9
22	24	50	0.05	3	0.1	1.9
23	24	70	0.05	6	0.01	4.82
24	24	50	0.05	3	0.01	3.29
25	24	70	0.5	6	0.01	2.9
26	48	70	0.5	3	0.1	6.9
27	48	70	0.5	6	0.1	12.2
28	24	70	0.5	3	0.01	8.27
29	24	70	0.5	3	0.1	5.8
30	48	50	0.05	3	0.1	7.5
31	24	70	0.05	3	0.1	9.2
32	24	50	0.5	3	0.01	13.2

in which, Y corresponds to the response (tannase activity), $\beta_0$is the offset term, β_i is the coefficient of linear terms, β_{ii} is the coefficient of square terms whereas β_{ij} is the coefficient of interactive terms.

3. Results

3.1. Screening of low-cost substrates for tannase production

The selected bacterium, *B. velezensis* TA3 utilized all selected agro-residues such as, orange peel, wheat bran, cow dung, coffee husk, apple peel and pomegranate peel for tannase production. Among the selected substrates, pomegranate peel has significant influence on enzyme production followed by orange peel and coffee husk (Fig. 1). For further evaluation pomegranate peel was selected.

3.2. Optimization of tannase production by B. Velezensis TA3 using traditional method

Traditional method was used for tannase production in SSF using pomegranate peel as a selected solid medium. In SSF, moisture content is an important factor influence on enzyme production. In this study 55% moisture content was found to be optimum (Fig. 2a). The effect of fermentation period (24 h–72 h) on tannase production was studied. The maximum production of tannase was found after 48 h of incubation at 37 °C (Fig. 2b). Since tannic acid is an inducible enzyme, supplementation of tannic acid with the medium induces tannase production. In our study, addition of 0.4% tannic acid to the medium was found to have high impact on tannase production (Fig. 2c). Even though, various carbon sources (glucose, xylose, maltose, starch and sucrose) were also tested to analyze their effect on tannase production, they did not show any influence (Fig. 2d). Enzyme activity was inhibited by these sources. The influence of minerals on enzyme production is also screened and the selected mineral medium supported tannase production (Fig. 2e).

3.3. Screening of variables for the production of tannase by a two-level full factorial design

In order to assess the impact of significant factors on tannase yield, a 32 experimental run was made. The production of tannase varied widely, which showed the significance of the optimization process parameters to gain maximum yield of enzyme (Table 1b).

Table 1c
Analysis of variance for the production of tannase in a two-level full factorial design.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	276.86	28	9.89	569.90	0.0001
A-Fermentation period	22.68	1	22.68	1307.21	<0.0001
B-Moisture	0.8128	1	0.8128	46.85	0.0064
C-Tannic acid	4.26	1	4.26	245.72	0.0006
D-Sodium chloride	4.76	1	4.76	274.27	0.0005
E-Minerals	0.8320	1	0.8320	47.96	0.0062
AB	0.7750	1	0.7750	44.67	0.0068
AC	0.5512	1	0.5512	31.77	0.0110
AD	11.12	1	11.12	640.67	0.0001
AE	1.41	1	1.41	81.34	0.0029
BC	2.95	1	2.95	170.17	0.0010
BD	6.43	1	6.43	370.38	0.0003
BE	7.72	1	7.72	445.10	0.0002
CD	1.23	1	1.23	71.03	0.0035
CE	5.73	1	5.73	330.21	0.0004
DE	19.03	1	19.03	1097.09	< 0.0001
ABC	6.84	1	6.84	394.52	0.0003
ABD	23.43	1	23.43	1350.26	< 0.0001
ACD	20.74	1	20.74	1195.20	< 0.0001
ACE	57.73	1	57.73	3327.23	< 0.0001
ADE	2.42	1	2.42	139.48	0.0013
BCE	0.3916	1	0.3916	22.57	0.0177
CDE	3.32	1	3.32	191.08	0.0008
ABCD	25.35	1	25.35	1460.93	< 0.0001
ABCE	9.61	1	9.61	554.13	0.0002
ABDE	29.26	1	29.26	1686.53	< 0.0001
ACDE	5.93	1	5.93	342.02	0.0003
BCDE	0.9730	1	0.9730	56.08	0.0049
ABCDE	0.5671	1	0.5671	32.69	0.0106
Residual	0.0521	3	0.0174		
Cor Total	276.91	31			

Table 2a
Independent factors and levels for the production of tannase in central composite design and response surface methodology.

Factors	Symbol	Coded values				
		-α	−1	0	1	α
Fermentation	A	7.63	24	48	72	88.3
Moisture	B	41.4	50	62.5	75	83.5
Tannic acid	C	0.004	0.2	0.5	0.8	1.004

Table 2b
Design matrix and interactive response for central composite design.

Std	A:Fermentation	B:Moisture	C:Tannic acid	Enzyme activity
	h	%	%	U/g
20	48	62.5	0.5	29.21
8	72	75	0.8	28.2
10	88.363	62.5	0.5	19.2
12	48	83.5224	0.5	21.1
4	72	75	0.2	23
13	48	62.5	−0.00453785	0.2
9	7.63697	62.5	0.5	0
16	48	62.5	0.5	30.2
19	48	62.5	0.5	28.4
18	48	62.5	0.5	30.1
6	72	50	0.8	10.5
1	24	50	0.2	2.1
17	48	62.5	0.5	29.4
11	48	41.4776	0.5	1.3
5	24	50	0.8	12.5
3	24	75	0.2	10.5
2	72	50	0.2	4.25
14	48	62.5	1.00454	25.2
15	48	62.5	0.5	29.4
7	24	75	0.8	15.3

In this model design, moisture content of the medium, incubation time, sodium chloride, tannic acid concentration and minerals exhibited positive influence on tannase production. The capability of the designed model was examined and the significant factors were further analyzed using analysis of variance which confirmed that the designed model is statistically significant ($p < 0.05$).

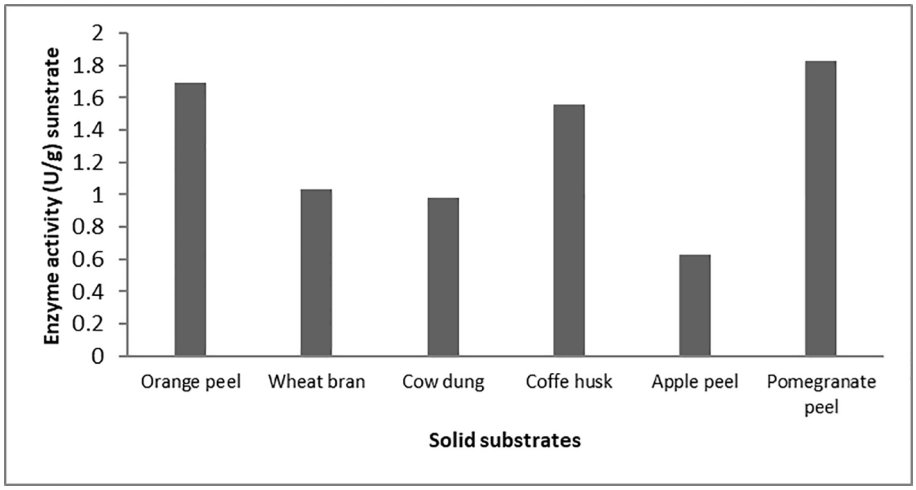

Fig. 1. Effect of various agro-industrial wastes on tannase production in solid state fermentation.

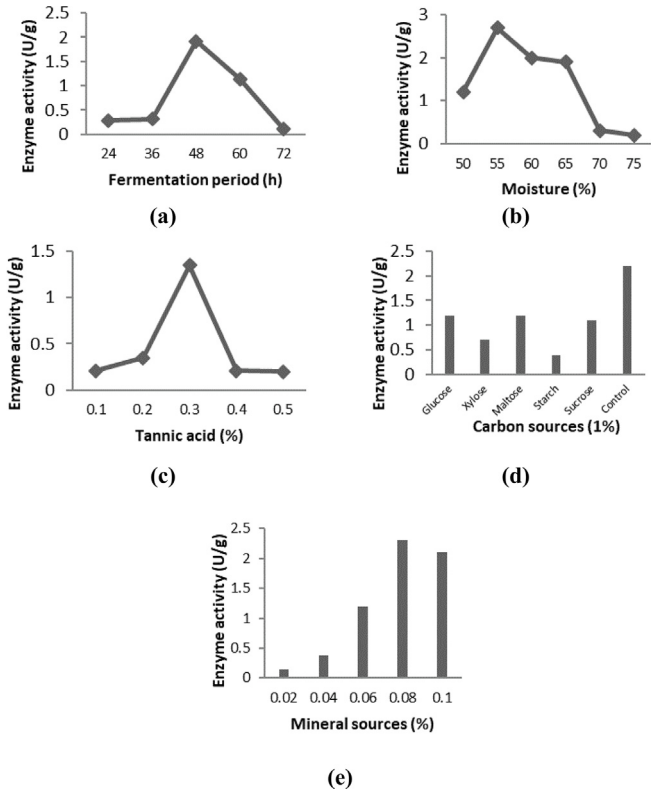

Fig. 2. Optimization of enzyme production by one-variable-at-a-time approach.

The F-value of the model is 1301.21 and the p value is < 0.0001. Incubation time, moisture content and tannic acid also show strong influence on tannase production. But variables such as sodium chloride and mineral salts do not have any positive impact on tannase yield. So these two factors were ignored and the rest of the significant variables were selected for further optimization study with CCD and RSM.

3.4. Optimization of significant variables by response surface methodology

In this CCD model, 20 experiments were conducted in duplicates (Table 2b) and found that tannase activity got varied from 0.2 to 30.1 U/g substrate. The ANOVA results of the designed CCD model showed its statistically significance with an F value of 22.95 and p value < 0.01 (Table 2c). The value < 0.05 was found to be significant. In this model incubation time of the culture medium, moisture content and tannic acid concentration were significant (<0.001). Also, in this case, AB (0.02) was significant model term. The R^2 value of the designed model was found to be 0.9538, which showed that 95.38% of the variation noted in tannase yield could be described by the new designed model. It was also observed that the lack-of-fit F-value was 0.151 which was not significant ($p > 0.05$). The predicted R^2 value was 0.9722 and the adjusted R^2 value was 0.961. In this model adequate precision value was more than 4 which indicated adequate signals. To visualize variable interactions, response surface plots and contour plots were used (Fig. 3). In our study, the obtained response plot was found to be convex in shape and this shows that the optimum process condition was well defined. Fig. 3a–f shows the interactions between selected variables. Tannase yield got increased at higher incubation time and decreased after 57 h of incubation at 37 °C. Likewise, moisture content of the medium had positive influence up to an optimum level and further decreased at higher concentrations. Also, tannase production increased positively up to an optimum value while high concentration of tannic acid inhibited enzyme production.

4. Discussion

A halotolerant tannase producing bacterium was isolated from the hypersaline environment and the potent bacterial strain was identified as *B. velezensis* TA3. The production of tannase enzyme by the selected bacterial strain was enhanced by optimizing various process parameters by response surface methodology. Many studies on tannase from bacterial isolates suggest the application of enriched culture medium and further by solid culture medium for isolating and screening enzyme producing organisms. Therefore, we used enriched medium as well as minimal medium followed by a solid medium for the isolation and screening of bacterial strains. 0.5% tannic acid was solely used as the carbon source and the ability to hydrolyze tannic acid was determined by well diffusion method. Out of 50 tannase positive isolates, TA3 identified as *B. velezensis* showed maximum tannase activity (>15 mm zone) than other isolates. The selected bacterial isolate (TA3) showed various morphological and biochemical characters. It was a rod shaped, gram-positive and motile bacterium. The

Table 2c
Results of the regression analysis of the central composite design.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	2372.73	9	263.64	22.95	<0.0001
A-Fermentation	244.97	1	244.97	21.32	0.0010
B-Moisture	479.82	1	479.82	41.76	<0.0001
C-Tannic acid	345.54	1	345.54	30.08	0.0003
AB	79.70	1	79.70	6.94	0.0250
AC	1.76	1	1.76	0.1530	0.7039
BC	5.53	1	5.53	0.4811	0.5037
A^2	578.48	1	578.48	50.35	<0.0001
B^2	479.80	1	479.80	41.76	<0.0001
C^2	395.65	1	395.65	34.44	0.0002
Residual	114.89	10	11.49		
Lack of Fit	8.75	5	1.75	0.151	0.702
Pure Error	2.15	5	0.4300		
Cor Total	2487.63	19			

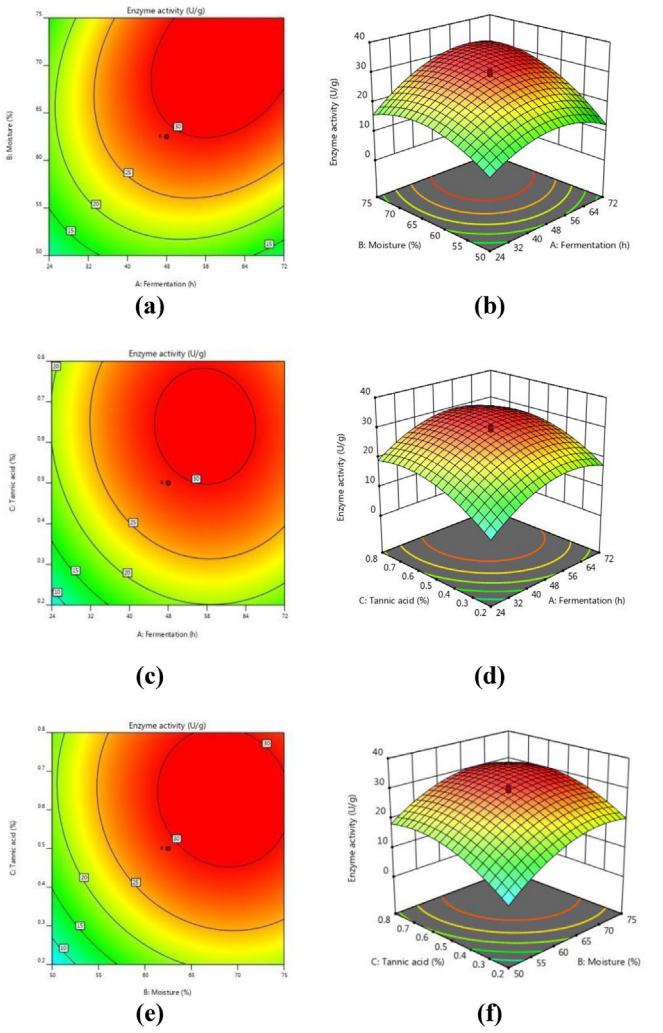

Fig. 3. Contour and response surface plot for the production of tannase in solid state fermentation by *Bacillus velezensis* TA3. Interactive effect between fermentation period and moisture content (a & b), fermentation period and tannic acid (c & d) and between moisture and tannic acid (e & f).

16S rDNA sequence having 1329 bp revealed more than 99% sequence similarity with *B. velezensis*.

The selected bacterial strain was cultured in SSF for tannase production and pomegranate peel was found to be suitable for the production of tannase. In bacteria, tannase is regarded as an inducible enzyme whose production depends on the availability of tannic acid (Mondal and Pati, 2000). The availability of tannins in the fruit peel induces the production of tannase and hence they can be used as cheap substrates for enzyme production. The pomegranate peels are considered as rich source of natural tannins (Saad et al., 2012). Tannic acid is commonly used as a source of carbon to induce the yield of tannase enzyme. However, an inhibitory effect of pure carbon sources in tannase synthesis was reported by Aguilar et al. (2001).

In this study, tannase producing organism was screened by minimal and enriched medium. Previous studies also suggested the use of these methods for the isolation of tannase producing bacteria. In a study, Raghuwanshi et al. (2011) isolated and screened tannic acid degrading bacteria using minimal medium containing 1% tannic acid. Many authors have reported the advantage of using enriched medium with tannin for the isolation of bacteria from tannin rich environment for tannase production (Chhokar et al., 2010). In *Lactobacillus plantarum*, tannic acid concentration in culture medium has significantly influenced the production of tannase (Ayed and Hamdi, 2002). Because of various industrial applications of tannase from bacterial sources, it is important to optimize the process parameters for enhancing the production of tannase. The impact of process variables such as moisture content, sodium chloride, carbon source, incubation time and minerals on tannase activity in solid state fermentation using *B. velezensis* TA3 was investigated by one-variable-at-a-time (OVAT) method. Later, five factors were selected and screened statistically by a two-level full factorial design followed by central composite design (CCD) and response surface methodology (RSM).

Optimization of tannase production using statistical methods has various advantages than traditional methods. Tannase production varied based on microorganisms, type of fermentation, culture conditions and experimental process. In our study, tannase production was found to be high after statistical approach. Optimum conditions for enhanced tannase production were 57 h fermentation, 72.5% moisture content with 0.68% tannic acid. Fig. 2a-c shows that a rise in incubation time lead to increased tannase yield up to 57 h after which tannase activity dropped gently. Moisture content of the medium is one of the prime variables that affect tannase yield. At lower as well as higher moisture levels, the tannase activity was found to be very less. The maximum enzyme activity was obtained at 72.5% moisture content. Also, up to 0.68% tannic acid in the culture medium enzyme activity enhanced and depleted at higher concentrations. The experimental tannase activity of 32 U/g substrate was very close with the predicted value of 33.1 U/g substrate validated the designed model. Under optimized process conditions nine times increase in tannase activity was attained when compared with unoptimized conditions. In another study, Sharma et al. (2007) used a statistical approach for tannase synthesis by *Aspergillus niger* and 5% tannic acid showed high rate of production. Mohan et al. (2014) reported 35.5 °C and 97 h of

incubation time as optimum for enhanced tannase production from *Aspergillus foetidus* MTCC 3557 using statistical approach. Although a number of fungal species have been reported to produce tannase, studies on bacterial strains capable of tannase production were limited (Mondal and Pati, 2000). The obtained enzyme yield was higher than that of various bacterial species. For instance, Prasanna et al. (2012) optimized tannase production using response surface methodology from *Bacillus massiliensis* and the yield was found to be less than that of present study. However, Raghuwanshi et al. (2011) used RSM for optimized production of tannase using *Bacillus sphaericus* and over nine-fold increase on enzyme production was achieved. In general, the yield of enzyme differs based on strain, substrate, inducer, enzyme assay method and types of fermentation.

5. Conclusion

Enhanced production of tannase from *B. velezensis* TA3 with pomegranate peel as a solid substrate was optimized using different methods. Among the selected variables, tannic acid concentration, incubation time and initial moisture content significantly affected tannase activity. The optimum process conditions for increased tannase production were found to be 57 h incubation period, 72.5% moisture content and 0.68% tannic acid. The optimized medium increased 9-fold tannase yield than unoptimized medium.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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