Optimization of tannase production by a novel *Klebsiella pneumoniae* KP715242 using central composite design☆

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ABSTRACT

A novel tannase producing bacterial strain was isolated from rhizospheric soil of *Acacia* species and identified as *Klebsiella pneumoniae* KP715242. A 3.25-fold increase in tannase production was achieved upon optimization with central composite design using response surface methodology. Four variables namely pH, temperature, incubation period, and agitation speed were used to optimize significant correlation between the effects of these variables on tannase production. A second-order polynomial was fitted to data and validated by ANOVA. The results showed a complex relationship between variables and response given that all factors were significant and could explain 99.6% of the total variation. The maximum production was obtained at 5.2 pH, 34.97 °C temperature, 103.34 rpm agitation speed and 91.34 h of incubation time. The experimental values were in good agreement with the predicted ones and the models were highly significant with a correlation coefficient (R^2) of 0.99 and a highly significant F-value of 319.37.

1. Introduction

Tannase (tannin acyl hydrolase EC 3.1.1.20) is an inducible and largely extracellular enzyme that causes the cleavage/hydrolysis of ester and depside bonds of hydrolysable tannins yielding glucose and gallic acid [1,2]. Tannase has wide applications ranging from preparation of instant tea, beer, wine, coffee flavored soft drinks to the production of gallic acid. It is also used as a clarifying agent to reduce the haze and bitterness in case of beer and fruit juices, as well as in the treatment of wastewater contaminated with polyphenolic compounds [1,3]. Gallic acid, the major hydrolytic product of tannic acid, is used in food, cosmetics, adhesives and in the synthesis of potent antioxidant, propyl gallate [4]. It is also used in the production of trimethoprim which have immense role to play in food and pharmaceutical industries [3,5].

Although tannase has been reported in plants, animals, and microorganisms, it is mainly produced by the latter [6,7]. Among tannin degrading microbes, majority of the research work has been carried out using the filamentous fungi of *Aspergillus* and *Penicillium* genus but many bacterial species including *Lactobacillus plantarum*, *L. paraplantarum*, and *L. pentosus* are also known to produce tannase [8,9]. The fungi exhibit better activity for the degradation of hydrolysable tannins. However, a major problem in usage of fungal strain at industrial level is its relatively slow growth rate and genetic complexity. On the other hand, the growth rate of bacteria is very high and they can be easily manipulated at genetic level. The bacteria are also capable of living under extreme temperature conditions, therefore they may prove potential source of thermostable tannase [10,11]. Beside this, bacterial tannase can also degrade and hydrolyze natural tannins and tannic acid very efficiently [12,13]. However, studies on tannase production by bacteria are very scanty.

Tannase production by microorganisms can be carried out by various methods such as liquid surface, submerged, solid state cultures and modified solid-state cultures [14]. The main advantages of solid state cultures include simplicity, lower production costs, high enzyme yield and low wastewater. However, submerged cultures have advantages in process control and recovery easiness of extracellular enzymes. Keeping this in view, an effort has been made to optimize the submerged culture conditions for the maximum tannase production.

Traditional methods for the optimization of culture conditions through changing one variable at one time and keeping the other

variables constant is a laborious and costly process. In addition to this, these methods also fail to give any information about the interaction between the different variables. These limitations have been overcome by using response surface methodology [15,16]. Now a days, response surface methodology (RSM) is extensively used to optimize the various culture conditions as well as to assess the interactions between different physiological and nutritional variables [17,18]. Therefore, in the present study, we report the optimization of the production process of tannase by *Klebsiella pneumoniae* using response surface methodology (RSM) under submerged fermentation.

2. Materials and methods

2.1. Chemicals

All the chemicals used in the present investigation were of analytical grade and were procured from Himedia Biosciences.

2.2. Enrichment and isolation of tannic acid utilizing bacteria

A total of 30 samples including compost, soil under high tannin containing plants, farm yard manure, fruit and vegetable garbage and local farms soil were collected. All the samples were collected aseptically in sample bags and were stored at 4 °C until isolation was performed. One gram of each soil sample was suspended in 50 ml minimal medium [19] containing K_2HPO_4: 0.5 g/l, KH_2PO_4: 0.5 g/l, $MgSO_4$: 2.0 g/l, $CaCl_2$: 1.0 g/l and NH_4Cl: 3.0 g/l supplemented with 1% tannic acid (pH 5.5). The flasks were incubated at 37 °C on rotary shaker at 150 rpm for 24 h. A portion of each culture was transferred to fresh medium and cultured again under same conditions. The above incubation and transfer operations were repeated twice. Finally cultures were serially diluted with sterile saline and plated on nutrient agar medium. These plates were incubated at 37 °C for 24 h. Repeated streaking of single colony was performed on nutrient agar medium until pure cultures were obtained. The strains were maintained on nutrient agar slants in refrigerator at 4 °C by regular transfer.

2.3. Screening of tannic acid utilizing bacteria

All the isolates were screened for tannic acid utilization by visual reading method. The isolates were harvested with sterile cotton buds and suspended aseptically in 5 ml substrate medium (pH 5.5) containing sodium dihydrogen phosphate (33 mmol/l) and methyl gallate (2 mmol/l) to prepare a dense suspension (at least equivalent to a number 3 Mc Farland standard). The substrate medium was then incubated aerobically at 37 °C for 24 h. At 30 min, 2, 4 and 24 h of incubation, 1 ml of suspension was aseptically removed, centrifuged at $3000 \times g$ for 1 min and filter sterilized. The aliquot thus prepared was used for the detection of gallic acid, the presence of which was interpreted as positive for tannase. The samples were alkanized with equal amounts of saturated sodium bicarbonate solution (pH 8.6) and then left at room temperature for an hour. This alkalization facilitated nonenzymatic oxidation of gallic acid to form polymerized compounds of *o*-quinine derivatives resulting in green colouration of medium. Green to brown coloration of the medium was judged as positive result for tannase [20]. In the second step, the isolates were screened for extracellular tannase production in tannic acid containing nutrient agar (NA) plates. Filter sterilized tannic acid solution 1% (w/v) was overlaid on nutrient agar plates. After 30 min of incubation, the plates become opaque due to the formation of protein tannic acid insoluble complex. The remaining overlaid tannic acid solution was removed. Pure colonies of the isolates were picked (point inoculation) and were spotted at the centre of the above plates

and incubated at 37 °C for 2–3 days. Isolates showing clear zone around the colonies were interpreted as positive for extracellular tannase production.

2.4. Identification of potential tannase producing isolates

The strain that possessed highest tannase activity was selected for subsequent studies and was identified on the basis of its morphological, physiological biochemical charactristics and 16S rRNA gene sequence. Genomic DNA was isolated using Easy-DNA Kit (Invitrogen) and quality was evaluated on 1% Agarose Gel. Fragment of 16S rDNA gene was amplified. The PCR amplicon was purified and further processed for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 27F and 1492R primers using BDT v3.1Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1416 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

2.5. Production of tannase

The selected isolate was used for the production of extra cellular tannase in submerged conditions. Fermentation was carried out in 250 ml flask containing 50 ml of minimal medium supplemented with 1% tannic acid. Two percent of overnight grown culture was used as an inoculum. The biomass was separated by centrifugation and supernatant was used for tannase assay.

2.6. Tannase assay

Enzyme solution (0.2 ml) was incubated with 0.3 ml of 1.0% (w/v) tannic acid, in 0.2 M acetate buffer (pH 5.5) at 40 °C for 40 min and then the reaction was terminated by the addition of 3 ml BSA (1 mg/mL), which precipitates the remaining tannic acid. A control reaction was also processed with heat denatured enzyme. The tubes were then centrifuged (10,000 rpm, 10 min) and the precipitates were dissolved in 3 ml of SDS–triethanolamine (1% w/v, triethanolamine) solution. 1 ml of $FeCl_3$ reagent (0.13 M) was added and kept for 15 min for stabilization of the colour. The absorbance of both the test and control tubes were measured at 530 nm against the blank (without tannic acid). One unit of the tannase was defined as the amount of enzyme, which is able to hydrolyse 1 mM of substrate tannic acid in 1 min under assay conditions [7].

2.7. Optimization of culture conditions using RSM

Optimization of physical parameters for maximum tannase production was carried out by using response surface methodology, a statistical technique that is widely used as a tool for checking the efficiency of several processes. Central composite factorial design was used in the optimization of culture conditions for tannase production. Four-factors and five-level face-centered cube design requiring a total of 30 experiments were used in this study. The independent variables studied were pH (X1), incubation temperature (X2, °C), incubation time (X3, h) and agitation speed (X4, rpm). The response (dependent variable) was tannase activity (U/ml). Each independent variable was studied at five coded levels ($-\alpha$, -1, 0, $+1$, $+\alpha$). The minimum and maximum levels of each independent variable and the experimental design with respect to their coded and uncoded levels are presented in Table 1. The

Table 1
Experimental range and levels of physical parameters (independent variables).

independent variables	Range and levels				
	$-\alpha$	-1	0	$+1$	$+\alpha$
pH (X1)	2.5	4.0	5.5	7.0	8.5
Incubation temperature (X2) (°C)	12.5	25	37.5	50	62.5
Incubation time (X3) (h)	12	24	48	84	120
Agitation speed (X4) (rpm)	0	50	100	150	200

Table 2
Central composite design of the variables with tannase activity as response.

Run	pH	Incubation (h)	Temp (°C)	RPM	Actual value	Predicted value
1	0	2	0	0	0.022	0.021
2	0	0	0	0	0.013	0.012
3	−1	1	1	1	0.039	0.040
4	1	1	1	1	0.030	0.028
5	1	−1	1	−1	0.019	0.018
6	0	0	0	2	0.011	0.011
7	0	0	0	0	0.019	0.020
8	−1	1	−1	1	0.009	0.009
9	0	−2	0	0	0.021	0.021
10	−1	1	−1	−1	0.019	0.018
11	1	1	−1	1	0.039	0.039
12	−2	0	0	0	0.030	0.031
13	−1	−1	1	−1	0.018	0.020
14	−1	−1	1	1	0.019	0.018
15	1	−1	−1	1	0.019	0.020
16	2	0	0	0	0.012	0.013
17	−1	−1	−1	−1	0.031	0.030
18	0	0	0	0	0.013	0.015
19	0	0	0	−2	0.005	0.006
20	1	1	1	−1	0.021	0.021
21	0	0	0	0	0.017	0.019
22	0	0	−2	0	0.001	0.001
23	0	0	0	0	0.036	0.038
24	0	0	2	0	0.045	0.043
25	1	1	−1	−1	0.065	0.064
26	1	−1	−1	−1	0.063	0.064
27	−1	1	1	−1	0.064	0.064
28	−1	−1	−1	1	0.064	0.064
29	0	0	0	0	0.063	0.064
30	1	−1	1	1	0.065	0.064

minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form is listed in Table 2. The relation between the coded values and actual values were described as in the following Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad I = 1, 2, 3, \ldots, k \qquad (1)$$

where x_i is the coded value, X_i is the actual value of an independent variable; X_0 is the real value of X_i at center point, ΔX_i is the step change of the variable. The second-order model used to fit the response to the independent variables is shown in Eq. (2):

$$Y = \beta_0 + \sum_{i=1}^{k} b_{ij} X_i + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_i<1}^{k} \sum_{j}^{k} b_{ij} + X_i X_j + e \qquad (2)$$

where, i, j are linear, quadratic coefficients, respectively, while 'b' is regression coefficient, Y is the tannase activity (U/ml), k the number of factors studied and optimized in the experiment, 'e' is random error and β_0 is the intercept. A second-order regression analysis of the data was carried out to get empirical model that defines response in terms of the independent variables. Analysis of Variance (ANOVA) was performed in coded level of variables to study the effects of independent variables. 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.

3. Results

3.1. Isolation and screening of bacteria

Enrichment medium containing 0.2% tannic acid as sole carbon source, when inoculated with soil suspension, showed notable increase in turbidity because of bacterial growth, which was accompanied by proportionate decrease in brown- black coloration of the medium. Appropriately diluted aliquots from these enrichment cultures produced pure isolated colonies. When pure cultures were tested for their ability to utilize tannic acid as sole carbon source, it was observed that 13 isolates out of total 20 tested were able to show significant growth. These 13 isolates were further screened for their ability to degrade tannic acid by visual reading method and gel diffusion method (zone of hydrolysis). Out of these 13 isolates, 6 (TAH 3, TAH 4, TAH 7, TAH 8, TAH 9 and TAH 10) were found to be positive for visual detection method as indicated by color change of the medium from green to brown using methyl gallate as the substrate. However, a distinct zone of hydrolysis on the agar plates could not be obtained by these bacteria except TAH 10. Out of these six potential isolates, isolate no. TAH 10 (0.02 U/ml) exhibited maximum tannase activity and was selected for further study.

3.2. Identification of bacterial isolate

Morphological characterization suggests that the bacterial strain TAH 10 is a gram negative, non motile, rod shaped bacterium. According to Bergey's manual of Determinative Bacteriology, certain physiological and biochemical tests were performed as shown in Table 3. Amplification of 16S rDNA fragment of genomic DNA produced a single PCR amplicon of 1416 bp. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W (Table. 4) Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA (Fig. 1). The 16S rDNA sequence of the strain TAH 10 has been deposited in the NCBI GeneBank (Accession number KP715242).

### 3.3. Optimization of culture conditions using RSM

In the present study, response surface methodology (RSM) has been used to optimize the culture conditions (pH, incubation

Table 3
Results of biochemical tests Biochemical tests for the isolate *TAH10*.

Characterization	TAH 10
Colony morphology	
Configuration	Round
Elevation	Raised
Surface	Smooth
Cell Shape	Rods
Arrangement	Occurring singly
Spore(s)	−
Motility	−
Biochemical tests	
Indole test	+
Lactose	+
Citrate	+
Oxidase	−
Catalase	+

Table 4
Sequence producing significant alignments (BLAST).

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB513734.1	*Klebsiella pneumoniae*, strain: 319	2608	2608	100%	0.0	99%
DQ520801.1	*Enterobacteriaceae bacterium* NR58	2608	2608	100%	0.0	99%
AY540111.1	*Klebsiella* sp. HE1	2603	2603	100%	0.0	99%
AB641122.1	*K. pneumoniae*, strain: SW	2597	2597	100%	0.0	99%
HF543828.1	*K. pneumoniae* subsp. pneumoniae strain DSM:30104T, clone K5	2597	2597	100%	0.0	99%
AB680063.1	*K. pneumoniae*, strain: NBRC 3321	2597	2597	100%	0.0	99%
HQ407264.1	*K. pneumoniae* strain T89	2597	2597	100%	0.0	99%
AB548822.1	*Klebsiella* sp. NCCP-142	2597	2597	100%	0.0	99%
EU661374.1	*K. pneumoniae* strain K8	2597	2597	100%	0.0	99%
EU661377.1	*K. pneumoniae* strain K30	2597	2597	100%	0.0	99%
AF228919.1	*K. pneumoniae* subsp. ozaenae	2593	2593	100%	0.0	99%
KJ210572.1	*Klebsiella* sp. BAB-3527	2591	2591	100%	0.0	99%
KC434996.1	*K.pneumoniae* strain U5	2591	2591	100%	0.0	99%
JX966429.1	*K. pneumoniae* strain MBR11	2591	2591	100%	0.0	99%
AB647144.1	*K.pneumoniae*, isolate: TR17	2591	2591	100%	0.0	99%
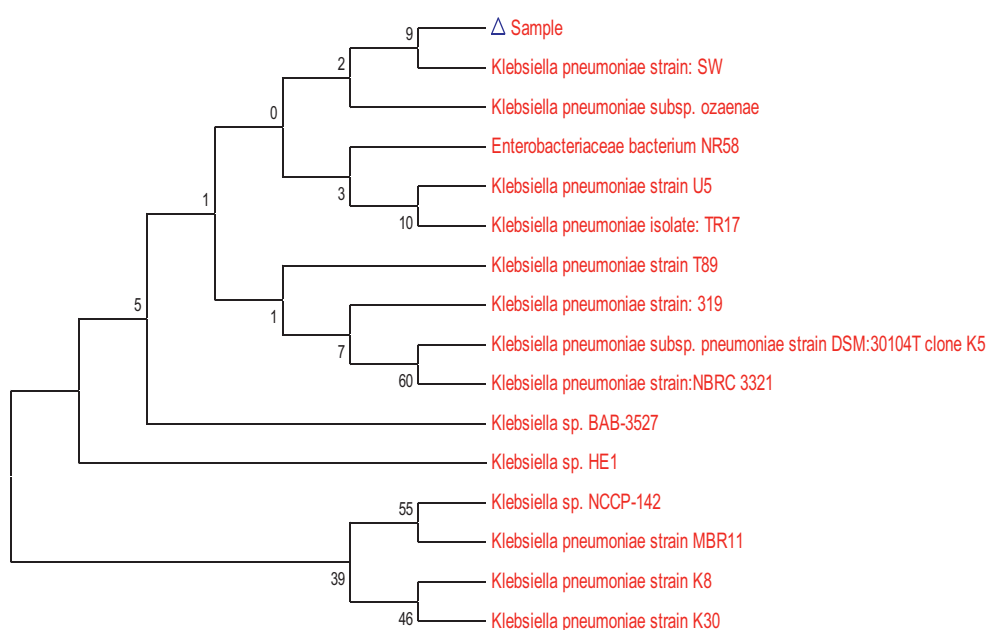
Fig. 1. Neighbour-joining tree showing the position of isolate *Klebsiella pneumoniae* (GenBank Accession Number KP715242) shown as sample.

Table 5
ANOVA (analysis of variance) for response surface quadratic model for optimization of tannase production of *Klebsiella pneumoniae* (GenBank Accession number KP715242).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	
Model	0.011707	14	0.000836	319.3724	<0.0001	Significant
A—pH	0.000339	1	0.000339	129.3078	<0.0001	
B—Incubation Time	0.000338	1	0.000338	128.9218	<0.0001	
C—Temperature	0.000589	1	0.000589	224.9014	<0.0001	
D—Agitation	4.26E-05	1	4.26E-05	16.27803	0.0011	
AB	1.91E-05	1	1.91E-05	7.30829	0.0163	
AC	1.23E-06	1	1.23E-06	0.470142	0.5034	
AD	2.12E-05	1	2.12E-05	8.080501	0.0123	
BC	0.000314	1	0.000314	119.9768	<0.0001	
BD	4.22E-06	1	4.22E-06	1.612841	0.2234	
CD	3.22E-06	1	3.22E-06	1.228438	0.2852	
A^2	0.003012	1	0.003012	1150.393	<0.0001	
B^2	0.004463	1	0.004463	1704.576	<0.0001	
C^2	0.005234	1	0.005234	1999.026	<0.0001	
D^2	0.000948	1	0.000948	361.9857	<0.0001	
Residual	3.93E-05	15	2.62E-06			
Lack of fit	3.51E-05	10	3.51E-06	4.161693	0.0645	Not significant
Pure error	4.21E-06	5	8.43E-07			
Cor total	0.011746	29				

temperature, incubation time and agitation speed) for the maximum tannase production. In order to examine the cumulative effect of four different culture conditions (independent variables) on tannase production, a Central Composite Design having 5 centre points leading to a total of 30 experiments were performed. Equation 3 represents the mathematical model relating the production of tannase with the independent process variables, X_i and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the design expert. The experimental and predicted values of yields of tannase are given in Table 2. It was observed that the predicted values for tannase production were in good agreement with observed values.

3.4. Model validation

The competence of the model and fitness were evaluated by using ANOVA (analysis of variance) and regression coefficients for the experimental design used (Tables 5 and 6). The ANOVA for the quadratic model was highly significant with an F value of 319.372 as shown by Fisher's F-test, along with a very low probability value (P model > F = 0.0001), which was significant at 95% confidence interval. At the same time, relatively lower value of coefficient of variation (CV = 5.43%) indicated a better precision and reliability of the experiments carried out. The goodness fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2 = 0.996$) indicates that 99.6 of the total variation in the tannase yield is attributed to the independent variables. The predicted R^2 of 0.982 for tannase production is in reasonable agreement with the adjusted R^2 of 0.993. A comparison between range of predicted values at the design points and the average prediction error shows adequate precision. "Adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case ratio is 56.95 for tannase production which indicates an adequate signal. The insignificant lack of fit value also indicated model can be used to navigate the design space. The model was found to be significant for production within the range of variables employed. The final predictive equation was as follows:

Tannase activity $(Y) = -7.81835 + 1.70037*A + 0.20623*B + 0.030253*C + 0.011065*D - 1.37500E - 003*A*B + 1.25000*A*C + 1.66667*A*D - 2.08333E-004*B*C - 9.33333*B*D - 1.66667*C*D - 0.16508*A^2 - 2.56583*B^2 - 2.36481*C^2 - 2.73926*D^2$ (3)

where, Y represents the tannase produced as a function of the coded levels of pH. (A) incubation, (B) temperature, (C) incubation period and agitation rate (D).

3.5. Three dimensional response surface plots

The three-dimensional (3-D) response surfaces plots (Fig. 2a–f) were designed on the basis of the model equation to explore the interaction among different variables and also the optimum level

Table 6
Statistical analysis for tannase production.

Model terms	Values
Std. Dev.	0.001618
Mean	0.029773
C.V.%	5.434793
PRESS	0.000208
R-squared	0.996656
Adj R-squared	0.993536
Pred R-squared	0.98229
Adeq precision	56.95118

of each factor for maximum tannase production by K. pneumoniae. In the response surface plots, two variables were kept constant at their optimum level, while the other two are present within their investigational range. The three-dimensional plots (Fig. 2) reveal that as the pH and incubation temperature increases, it causes an increase in the tannase production to optimum values of 5.2 and 34.97 °C, respectively. A further increase leads to the decrease of enzyme production. The maximum tannase production was obtained after 91.34 h with an agitation speed of 103.34 rpm. Increase in the agitation speed and incubation period beyond the optimum level caused a decrease in the enzyme production.

4. Discussion

In view of the economic importance of the enzyme tannin acyl hydrolase at the industrial level, the present study was carried out for the isolation and identification of potential tannse producing bacterial strain. The enzyme production by the strain was maximized by optimizing the various parameters through response surface methodology (RSM). Most of the literature on bacterial tannase suggests the use of enrichment liquid medium followed by solid medium for the isolation and identification purpose. In this study also, we used enrichment medium containing 0.2% tannic acid as sole carbon source. The selected pure cultures were examined for their ability to degrade tannic acid by visual appraisal and gel diffusion method (zone of hydrolysis). Out of the potentials isolates, isolate no. TAH 10 identified as K. pneumoniae (GenBank Accession number KP715242) exhibited maximum enzyme activity. Raghuwanshi et al. [21] isolated potential bacterial strains in minimal medium supplemented with 1% tannic acid. These isolates failed to exhibit distinct zone of hydrolysis but were positive for visual detection method. A number of other authors have also used enrichment medium having condensed tannin as the sole carbon source. [22–24].

The isolate (TAH 10) exhibited various morphological characteristics. It was a gram negative, non motile, rod shaped bacterium when grown in broth. A single band of 1416 bp was obtained on amplification of the 16S rDNA sequence. The isolate was found to be having 99% sequence similarity with K. pneumoniae, strain SW (GenBank Accession number AB641122.1). The phylogenetic tree constructed by the neighbor joining method [25] suggests that the isolate TAH 10 is a novel strain (K. pneumoniae GenBank Accession number KP715242) in the K. pneumoniae cluster.

Because of the immense applications of bacterial tannase it is necessary to optimize the culture conditions for maximum enzyme production. The effect of different process parameters namely pH, temperature, agitation speed and incubation time on tannase enzyme activity in submerged fermentation using K. pneumoniae, Accession number KP715242 (TAH 10) was studied and optimized with central composite design of RSM. Optimum conditions for maximum enzyme production were 5.2 pH, 34.97 °C temperature, 103.34 rpm agitation speed and 91.34 h of incubation time. Under these optimum conditions, 3.25 fold increase in enzyme activity was obtained as compared to the initial un-optimized conditions.

Fig. 2(a, d, e), reveals that an increase in incubation period caused an increase in tannase activity up to 92 h after that the tannase activity declines which may be attributed due to depletion of nutrients in the medium. Fig. 2(b, d, f) shows the effect of temperature on tannase activity. At lower and higher temperatures, the tannase activity was found to be low. The maximum tannase activity was observed at a temperature of 34.97 °C. Similar results have been reported by Beniwal et al. [10]. Similarly the enzyme activity was found to be low at lower and higher pH (Fig. 2a–c). Acidic pH favored the maximum activity whereas it
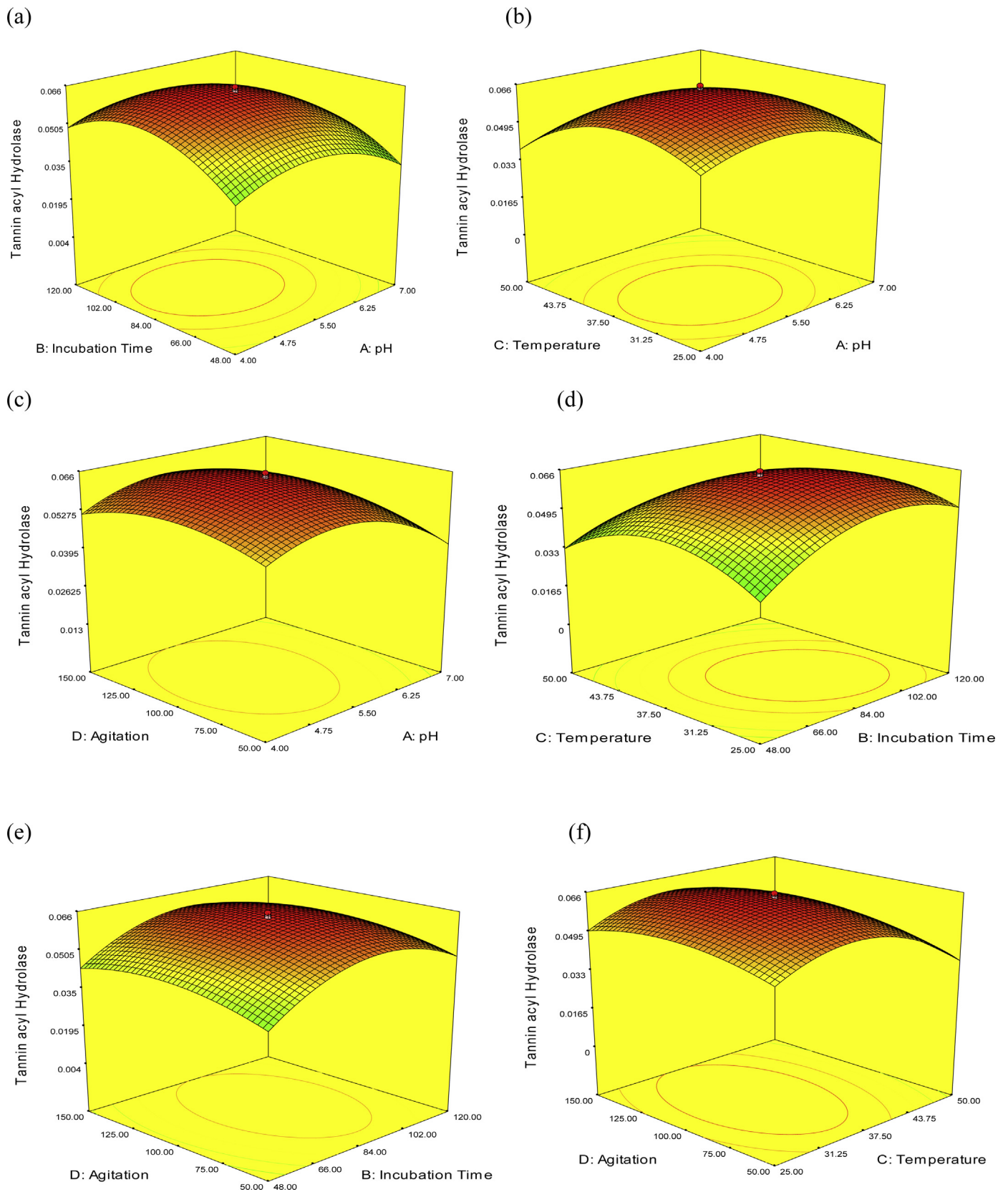
Fig. 2. (a) Effect of pH and incubation time on the production of tannase keeping temperature and agitation rate at zero level (coded), (b) effect of pH and temperature on the production of tannase. Time and agitation rate were held at zero level (coded), (c) effect of pH and agitation speed on the production of tannase. Other variables pH and temperature were kept at zero level (coded), (d) effect of incubation time and temperature on the production of tannase keeping pH and agitation rate at zero level (coded), (e) effect of agitation speed and incubation time on the production of tannase. Other variables pH and temperature were held at zero level (coded) and (f) effect of agitation rate and temperature on the production of tannase. Other variables pH and incubation time were held at zero level (coded).

decreased in the alkaline range [21,26]. The effect of pH on the activity of tannase may be due to the protonation or deprotonation of its amino acids and active site; in addition, it may attributed to the conformational changes induced by amino acids ionization [27]. The optimum conditions for tannase production obtained in the present investigation are in agreement with the findings of Mohan et al. [28] who reported 97 h of incubation period, 35.5 °C temperature and pH 5.5 as optimum conditions for maximum tannase from *Aspergillus foetidus* MTCC 3557 using response surface methodology. However, Kar and Banerjee [29] reported 48 h as optimum incubation period for the maximum tannase production. Sivashanmugam and Jayaraman [30] observed that *K. pneumoniae* MTCC 7162 produced maximum tannase at 37 °C and 100 rpm which are very close to the optimized values obtained in the present investigation. Similar results have also been reported for RSM mediated tannase production using *Bacillus sphaericus* [21] and co-culture of *Aspergillus awamori* and *Aspergillus heteromorphus* [11]. A tannase titer of 0.356 IU/ml has been reported from *B. licheniformis* KBR 6 after using a statistical method for optimization of the production process [28]. However, Raghuwanshi et al. [21] obtained comparatively a very high tannase titer of 11.2 IU/ml in 48 h.

In conclusion, the present study reports the isolation of tannase producing bacterium from soil (identified as *K. pneumoniae* GenBank Accession Number KP715242) and optimization of various physical parameters for maximum tannase production by this strain. Optimal tannase activity was obtained at 5.2 pH, 34.97 °C temperature, 103.34 rpm agitation speed and 91.34 h of incubation time. After optimization, the bacterium yielded 0.065 U/ml of tannase which is 3.25 fold higher than the 0.02U/ml of tannase initially produced under un-optimized conditions. The optimal pH obtained in the present study lies in the acidic range and may find its applications in food-processing industry such as clarification of acidic beverages. The low rpm may also help in enhancing the survival rate of the bacterium against shear stress generated due to high speed. In addition, the optimized temperature and incubation period might lower the production cost at commercial level. Moreover, the application of central composite design assisted in understanding the interaction between different physiological factors that lead to maximal bacterial tannase yield and for better understanding of the processes which may lead to enhanced production at industrial scale.

Acknowledgements

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