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Full Length Research Paper

Media optimization for extra cellular tannase production by *Klebsiella pneumoniae* MTCC 7162 using response surface methodology

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Klebsiella pneumoniae **MTCC 7162, isolated from tannery effluent was used for the production of extracellular tannase. Central composite design (for nutritional parameters) and Box Behnken design (for physicochemical parameters) were used to arrive at conditions for maximal tannase production. Box Behnken design was statistically significant and indicated that pH is a significant factor for tannase production. A 6.5 fold increase of extra cellular tannase (22.7 U / ml) production was observed at the optimized conditions. The yield of extra cellular tannase obtained in this study, from a bacterial source, is the highest compared to that of the earlier reports.**

Key words: *Klebsiella pneumoniae*, extra cellular tannase, central composite design, Box Behnken design, response surface methodology.

INTRODUCTION

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly referred as tannase, is an inducible and hydrolytic enzyme. Tannase has been known to hydrolyze the ester and depside linkages of hydrolysable tannins into gallic acid and glucose. Tannase is produced by bacteria, yeasts, and fungi. The major applications of this enzyme are in the production of gallic acid, which is used for the manufacture of an antimalarial drug, trimethoprim (Aguilar et al., 2001), and in the synthesis of esters, such as propyl gallate, used as antioxidants in the food industry. The enzyme also has its applications in the production of beer and fruit juices as a clarifier, in the manufacture of instant tea, and in the treatment of wastewater contaminated with polyphenolic compounds (Lekha et al., 1997). In spite of these wide applications, studies on tannase production by bacteria under submerged fermentation are very obscure. Filamentous fungi of the *Aspergillus* (Lekha et al., 1994; Bradoo et al., 1997; Seth and Chand, 2000; Aguilar et al., 2001; Sharma et al., 2007; Naidu et al., 2008; Beniwal et al., 2010), *Penicillium* (Batra and Saxena, 2005), *Rhizopus* (Kar et al., 2002; Panda et al., 2009), *Paecilomyces* (Macedo et al., 2007) genus and bacteria of the *Bacillus* (Mondal et al., 2001) and *Lactobacillus* (Sabu et al., 2006) genus have been investigated for tannase production.

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MATERIALS AND METHODS

"One-at-a-time" procedures used to find the desired media components in process optimization face limitations when a large number of factors have to be investigated, as the statistical interactions between factors could not be obtained (Hounsa et al., 1996). Hence, statistically based experimental designs are preferred to evaluate the influence of medium components in batch fermentations for the production of industrially important enzymes. Optimization studies for the production of tannase using statistical methods have been reported using either solid state fermentation or submerged cultures (Lekha et al., 1994; Seth et al., 2000; Sharma et al., 2007; Macedo et al., 2007; Naidu et al., 2008). However, very few investigations have exploited the use of bacterial sources for extra cellular tannase production.

We have earlier reported that *Klebsiella pneumoniae* MTCC 7162, isolated from tannery effluents of leather industries located at Ranipet, India, can produce extra cellular tannase (Karthikeyan and Jayaraman, 2011). In the present study we have used the statistical methods, central composite design (CCD) and Box Behnken design, to arrive at conditions for maximal tannase production. The models indicated that pH plays significant role for the production of extra cellular tannase.

Table 1. Levels of chemical parameters used in CCD and Box Behnken design for maximum tannase production from *K. pneumoniae* MTCC 7162.

Factor	Name	Units	Type	Low actual	High actual	Low coded	High coded
			Central composite design				
A	Tannic acid	%	Numeric	1	5	-1	1
B	Urea	Grams	Numeric	0.1	0.4	-1	1
C	Glucose	ml	Numeric	0.25	1	-1	1
D	CSL	Grams	Numeric	0.1	0.3	-1	1
			Box Behnken design				
A	pH	Range	Numeric	5.5	7.5	-1	1
B	Innoculum	% (v/v)	Numeric	1	5	-1	1
C	RPM	rpm	Numeric	100	250	-1	1

RESULTS AND DISCUSSION

K. pneumoniae MTCC 7162, isolated from tannery effluent of leather industries located at Ranipet (India), was observed to produce extra cellular tannase (3.46 U/ml) within 21 h under submerged fermentation condition with minimal medium containing 2% tannic acid (Karthikeyan and Jayaraman, 2011). Fermentation studies were carried out in 250 ml Erlenmeyer flasks containing 50 ml of production medium by batch mode with agitation of 150 rpm at 37°C. Optimum physical and nutritional parameters obtained from "one-at-a-time" approach using minimal medium was further selected for statistical studies using approaches of CCD and Box Behnken designs under response surface methodology. CCD design was used for varying the chemical components such as tannic acid, urea, glucose and corn steep liquor concentrations in addition to the minimal media components ($NaNO_3$ 3.0% w/v, KCl 0.05% w/v, $MgSO_4$ 0.05% w/v, K_2HPO_4 0.1% w/v). Box Behnken design was carried out for studying the impact of physical parameters on tannase production with varying values of pH, inoculum and agitation rate. These optimization experiments led to significant increase in the yield of extra cellular bacterial tannase production under submerged fermentation condition within 21 h.

In CCD experiments, the aforesaid variables viz, tannic acid, glucose, urea and corn steep liquor showing higher impact on tannase production in "one-at-a-time" was selected as the central point and accordingly the ranges for each of these factors was chosen (Table 1). CCD results indicated that there was a 4 fold increase (3.46 to 15.94 U/ml) in tannase production for the components chosen. The model could be summarized using the following quadratic equation:

$$\text{Tannase activity (U/ml)} = 8.35 - 0.28\,A - 1.10\,B - 0.24\,C + 0.35\,D + 1.29\,A^2 - 0.013\,B^2 - 3.29\,C^2 + 2.02\,D^2 - 0.69\,A\,B - 1.29\,A\,C - 1.89\,A\,D - 1.55\,B\,C + 2.46\,B\,D + 3.91\,C\,D$$

where, A- Tannic acid; B- Urea; C- Glucose; D- Corn steep liquor.

However, the F-value was found to be 1.06, which is much lower than the critical value (52.11) of F at 0.05 levels, indicating that the obtained model is not adequate and therefore was deemed to be insignificant.

Subsequently media optimization was carried with a minimal media designed from the results analyzed from CCD (MMT media containing 5% w/v tannic acid, 0.2% w/v urea). Box Behnken design was used to study the impact of three different physicochemical parameters with varying ranges of pH, inoculum and agitation rate. A set of 17 experiments were carried out under submerged fermentation condition for 21 h. After the desired incubation period, extra cellular tannase was extracted and were analyzed for tannase activity. The results obtained from Box Behnken experiments are given in Table 2.

The data obtained could be fit using a second order polynomial equation, which explains the tannase activity as the function of coded levels of pH (A), inoculum (B) and agitation rate (C):

$$\text{Tannase Activity (U / ml)} = 1.22 + 6.79\,A - 0.62\,B - 0.053\,C + 7.21\,A^2 - 3.04\,B^2 + 4.24\,C^2 - 0.14\,A\,B - 2.44\,A\,C + 1.82\,B\,C$$

It is evident that the pH range taken for the study interacts negatively with agitation and inoculum values, while interactions between agitation and inoculum range had a positive response on enzyme production. From the model, it is clear that the linear coefficients A, quadratic term A^2, C^2 and cross product term BC are significant andtherefore contributes to the model. Statistical analysis of this model reveals that this second order equation is significant at the 87% level (R^2 = 94%). This shows that the model is an adequate predictor of the experimental conditions. In the present work, only the estimates with significant levels higher than 90% were included in the

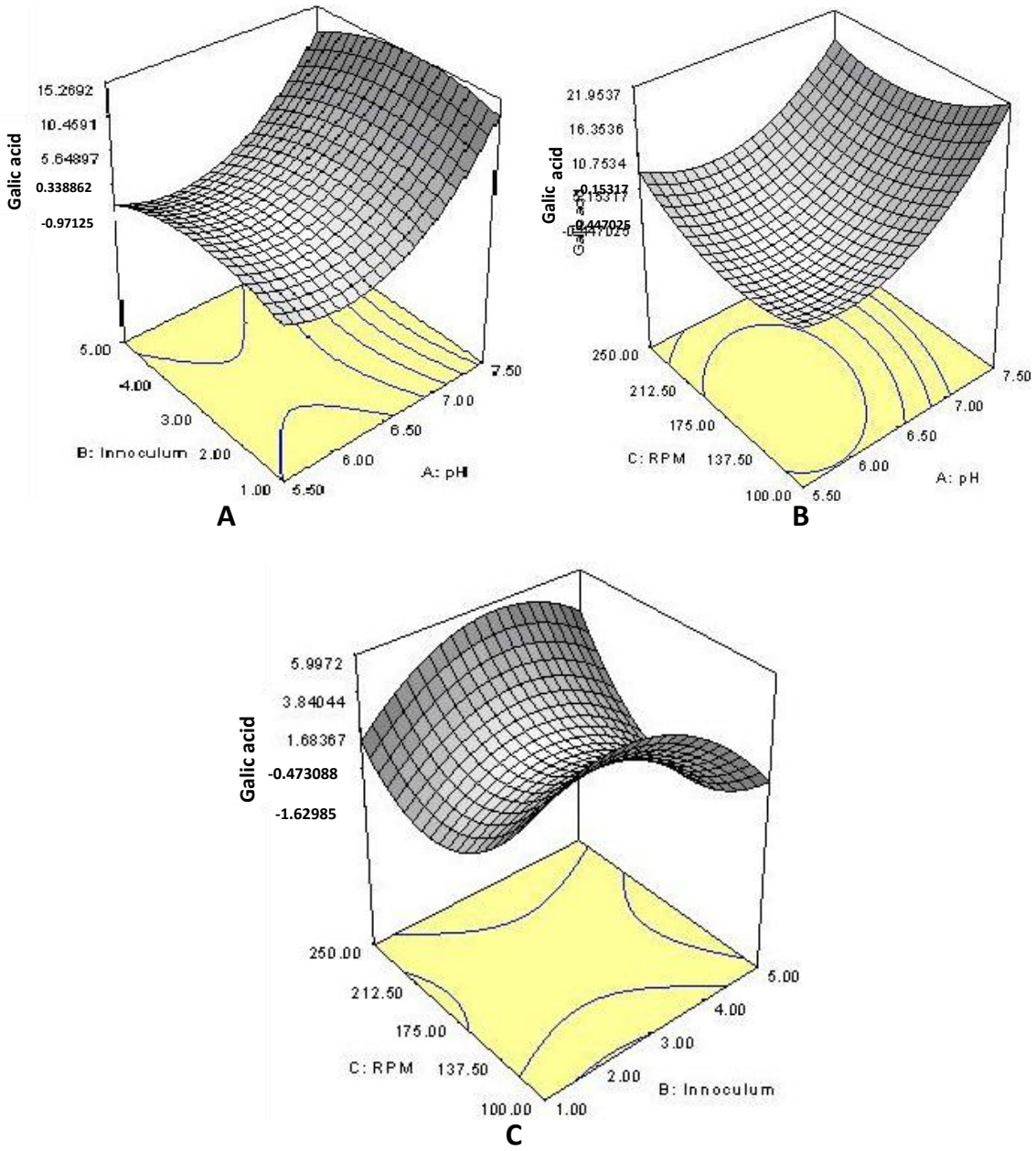

Figure 1. Three dimensional plot of tannase activity by *Klebsiella pneumoniae* MTCC 7162 – (a) as a function of pH (A) and Innoculum (B) at agitation rate of 175 rpm at constant. Negative interaction between the two factors is evident leading to reduced tannase production**; (b)** as a function of pH (A) and agitation rate (C) at inoculum concentration of 3 % v/v. Both the factors resulted in positive interaction with optimal conditions of 7.5 and at agitation rate at 100 rpm; (c) as a function of agitation rate (A) and innoculum (B), at pH of 6.5. The factors, inoculum and agitation rate at constant pH of 6.5, had negative interactions. The tannase activity was estimated as described in Karthikeyan and Jayaraman, 2011. The experimental designs, ANOVA and 3D graphs were created using Design Expert (Version 6.0) by Stat-Ease Inc., Minneapolis, MN, U.S.A.

final model. In addition, the F-value (12.34) and P-value (0.0016) obtained proved the significance of the model. It is evident from the Figure 1A, B and C that the factors chosen interact with each other (positive and negative).

The results from these independent experiments coincided with the estimated value and thus the model was proven to be adequate.

A comprehensive review of various microbial sources used for the production of tannase and its process optimization studies using statistical methods is presented in Table 3. Although most of the reports show optimization process for fungi, only a few had reported

Table 2. Box Behnken Design matrix of the variables for *Klebsiella pneumoniae* MTCC 7162 with experimental tannase activity as response.

Std	Run	pH	Inoculum	Agitation	Actual value	Predicted value	Residual activity
11	1	6.5	1	250	0.4	1.16	- 0.76
9	2	6.5	1	100	6.5	4.9	1.59
12	3	6.5	5	250	1.96	3.55	-1.60
13	4	6.5	3	175	1.45	1.22	0.23
5	5	5.5	3	100	0.38	3.48	-3.11
7	6	5.5	3	250	7.52	8.26	-0.75
10	7	6.5	5	100	0.79	0.02	0.76
3	8	5.5	5	175	0.45	-1.89	2.34
8	9	7.5	3	250	20.07	16.96	3.10
16	10	6.5	3	175	0.2	1.22	-1.02
17	11	6.5	3	175	0.2	1.22	-1.02
15	12	6.5	3	175	1.47	1.22	0.25
4	13	7.5	5	175	9.91	11.42	1.52
1	14	5.5	1	175	0.6	-0.91	1.52
2	15	7.5	1	175	10.6	12.94	-2.34
6	16	7.5	3	100	22.7	21.95	0.75
14	17	6.5	3	175	2.78	1.22	1.56

Table 3. Review of statistical experimental design employed for the production of tannase by different microbial sources.

Tannase producing microbial sources	Design of experiment	Fermentation conditions [Time (h), Type]	Tannase produced (Fold increase)	References
Aspergillus nigerPKL 104	RSM	72, SSF	1.34	Lekha and Lonsane (1994)
Aspergillus awamori	Box Behnken	60, SSF	24	Seth and Chand (2000)
Penicillium variable	D- Optimal and CCD	72, SSF	2.4	Batra and Saxena (2005)
Lactobacillus sp. ASR-S1	'One-at-a- time'	72, SSF	3.2	Sabu et al. (2006)
Aspergillus niger	CCD	48, SMF	2.0	Sharma et al. (2007)
Paecilomyces variotii	RSM	120, SSF	8.6	Macedo et al. (2007)
Aspergillus foetidus MTCC 6322	Plackett Burman and CCD	72, SSF	2.0	Naidu et al. (2008)
Bacillus licheniformis KBR6	Taguchi orthogonal array	36, SMF	2.18	Das et al. (2009)
Rhizopus oryzae NRRL 21498	EVOP and RSM	72, MSSF	4.3	Kar et al. (2002) and Panda et al. (2009)
Aspergillus awamori MTCC 9299	RSM	48, SSF	2.0	Beniwal et al. (2010)
Klebsiella pneumoniae MTCC 7162	Box Behnken	21, SMF	6.5	Present study

* h – Hours ; SSF – Solid State Fermentation ; SMF – Submerged Fermentation ; MSSF – Modified Solid State Fermentation ; CCD – Central Composite Design ; RSM – Response Surface Methodology ; EVOP – Evolutionary Operation Factorial Design.

tannase production by bacteria. Ashok et al. (2006) used statistical design for *Lactobacillus* sp. ASR-S1 under solid state fermentation and had shown a 3.5 fold increase of tannase yield for over a period of 72 h. Subsequently, another bacterial tannase production by *Bacillus licheniformis* KBR6 was reported by Das et al. (2007) under submerged fermentation using Taguchi orthogonal array resulting in 2.18-fold (from 0.163 to 0.356 U/ml) increase of tannase from its unoptimized condition.

Conclusion

this is the first report of bacterial tannase production by *K. pneumoniae* with distinct characteristics: (a) Maximum enzyme production within a short period of cultivation (21 h); (b) maximum fold (6.5) increase observed for production of bacterial tannase using statistical optimization procedure; (c) high yield of 22.7 U/ml from bacterial source; (d) optimum tannase production at alkaline pH (7.5), when compared to most of the acidic tannase reported.

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