

## Assessment of Physical Process Conditions for Enhanced Production of Novel Glutaminase-Free L-Asparaginase from *Pectobacterium carotovorum* MTCC 1428

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Received: 21 October 2009 / Accepted: 12 July 2010 /  
Published online: 29 July 2010  
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**Abstract** Statistically based experimental design was applied to maximize the production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. The effect of physical process parameters (initial pH of the medium, temperature, rpm of the shaking incubator, and inoculum size) on the production of L-asparaginase from *P. carotovorum* MTCC 1428 was studied using central composite design technique. The individual optimum levels of initial pH of the medium, temperature, rpm of shaking incubator, and inoculum size were found to be 6.90, 29.8 °C, 157 rpm, and 2.61% (v/v), respectively, for the production of L-asparaginase. After physical process parameters optimization, the production and productivity of L-asparaginase was enhanced by 26.39% (specific activity) and 10.19%, respectively. Maximization of L-asparaginase production was achieved at 12 h under optimal levels of physical process parameters in shake flask level.

**Keywords** *Pectobacterium carotovorum* · L-asparaginase · Physical process parameters · Central composite design · Response surface methodology

### Introduction

L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) is one of the most important agent used in multidrug chemotherapy regimens in the treatment of malignancies, which derive from lymphoid system, acute lymphoblastic leukemias, and non-Hodgkin lymphoma [1, 2]. However, the presence of partial glutaminase activity in L-asparaginase is known to be the main cause of the various side effects associated with this drug [3, 4]. Therefore, glutaminase-free L-asparaginase is essentially required for successful clinical studies. Moreover, L-asparaginase is also used in food industry for the production of acrylamide-free food [5], model enzyme for the development of new drug delivery system [6], and

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L-asparagine biosensor for leukemia [7]. L-asparaginase enzymatically cleaves L-asparagine (amino acid essential for lymphoblasts' growth) to ammonia and L-aspartic acid. The depletion of L-asparagine in serum and cerebrospinal fluid leads to destruction of lymphoblasts, as they are unable to build their proteins due to the inability of endogenic L-asparagine production [1, 8].

The production of L-asparaginase has been studied in *Serratia marcescens* [9], *Erwinia carotovora* [10], *Escherichia coli* [11], *Enterobacter aerogenes* [12], *Bacillus circulans* [13, 14], *Pseudomonas aeruginosa* [15], *Bacillus subtilis* [16], and *Aspergillus* species [17] with various carbon and nitrogen sources under both aerobic and fermentative conditions. The synthesis of L-asparaginase by Gram-negative bacteria is influenced significantly by process parameters such as pH, temperature, dissolved oxygen, incubation time, inoculum volume, aeration, agitation, and medium constituents [9, 12].

Process optimization plays a vital role in industrial production of any metabolites in which even small improvements would be decisive for commercial success. In any bioprocess, the improvement in productivity of any metabolite would be achieved through manipulation of nutritional and physical process parameters. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum levels of parameters for desirable responses [18]. This method has been successfully applied in many areas of biotechnology such as bioconversion of cheese whey to mycelia of *Ganoderma lucidum* [19], enzyme production [20], enzyme kinetics [21], and bacteriocin production [22].

In this study, we report the optimization of physical process parameters viz. initial pH of the medium, temperature, rpm of the shaking incubator, and inoculum size to maximize the production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. The optimization of these parameters for enhanced production of L-asparaginase was carried out by central composite design (CCD). The optimal levels of physical process parameters predicted by the model were verified both in shake flask and bioreactor.

## Material and Methods

### Microorganism and Culture Conditions

The bacterium used throughout the study, *P. carotovorum* MTCC 1428, was procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This organism was grown on the medium containing (g/l): beef extract 1.0, yeast extract 2.0, NaCl 5.0, peptone 5.0, and agar 15.0 (pH 7.0) at 30 °C. The organism was sub-cultured every month and maintained at 4±1 °C. The production of L-asparaginase have been studied in the modified basal semisynthetic medium containing (g/l): carbon source 3; Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 6.0; KH<sub>2</sub>PO<sub>4</sub>, 3; NaCl, 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.015; yeast extract 1.0; peptone, 1.0; and initial pH of 6.5 [23]. The inoculum was prepared by adding a loop full of freshly prepared 24 h old pure culture grown on slant into 50 ml of autoclaved above said medium containing glucose as carbon source in a 250-ml shake flask and incubated at 30 °C and 180 rpm in a shaking incubator for 10–12 h (to reach culture OD at 600 nm: 0.6~0.8). A 2% of inoculum from the above seed culture was added to 50 ml of the medium in 250 ml shake flasks. The flasks were incubated in a shaking incubator at 30 °C and 180 rpm. Samples were withdrawn at regular interval of time and measured for L-asparaginase production. Experiments were conducted in duplicates, and enzymatic assay was performed in duplicates for each sample.

### Assay for L-Asparaginase and L-Glutaminase Activity

Samples were centrifuged at 10,000×g for 10 min at 4±1 °C and washed twice with 0.05 M Tris–HCl buffer (pH 8.6). Washed cells were ultrasonicated (Model VC 505 Microprocessor based cell Ultrasonic processor, Sonics & Materials Inc, CT, USA) at 20 MHz, 35% amplitude, four cycles (2 min per cycles with 1.5 s on and 0.5 s off), and the contents were centrifuged at 20,000×g for 10 min (4±1 °C). The supernatant was analyzed for intracellular L-asparaginase activity. The activity of L-asparaginase was measured by modified Nessler's method described by Kumar et al. [24]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μM of ammonia per min at 37 °C. Specific activity is expressed as units per milligram of protein. Glutaminase activity was determined by direct nesslerization as described by modified method of Mashburn and Wriston [25] using L-glutamine as substrate.

### Protein Concentration

The total protein contents of the samples were determined according to the method described by Lowry et al. [26] using bovine serum albumin (Sigma) as standard.

### Optimization of the Physical Process Parameters by CCD

The initial pH of medium, temperature, rpm of the shaking incubator, and inoculum size were considered in the present optimization process. The production of L-asparaginase (specific activity) by *P. carotovorum* MTCC 1428 was taken as the response. The physical process parameters were optimized using RSM [27]. The minimum and maximum ranges of the variables with regard to their values in actual and coded form are provided in Table 1. The CCD with four variables at three levels was employed in this regard using statistical software package MINITAB® Release 15.1, PA, USA [28]. According to CCD, the total number of treatment combinations was 30 ( $= 2^k + 2k + 6$ ), where  $k$  is the number of independent variables [29]. Twenty-four experiments were augmented with six replications at the center points to evaluate the pure error (Table 2). The relationship among the variables, i.e., initial pH of the medium, temperature, rpm of the shaking incubator, and inoculum size was expressed mathematically in the form of a quadratic model (Eq. 1), which gave the response as a function of relevant variables.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (1)$$

**Table 1** Ranges and levels of the independent variables for CCD matrix.

Variables	Symbol coded	Range and levels				
		-2	-1	0	+1	+2
Initial pH	<i>A</i>	6.0	6.5	7	7.5	8.0
Temperature	<i>B</i>	24	28	32	36	40
RPM	<i>C</i>	100	140	180	220	260
Inoculum size	<i>D</i>	1	2	3	4	5

**Table 2** A 2<sup>4</sup> full-factorial CCD matrix in real values and coded units (in parenthesis) with experimental and predicted values of L-asparaginase production.

Run order	Uncoded and coded levels				Specific activity (U/mg)		Enzyme activity <sup>a</sup> (U/ml)	Cell growth <sup>a</sup> (g/l)
	A	B	C	D	Observed <sup>a</sup>	Predicted		
1	6.5 (-1)	28 (-1)	140 (-1)	2 (-1)	34.10±0.25	33.62	17.45±0.42	1.17±0.02
2	7.5 (+1)	28 (-1)	140 (-1)	2 (-1)	33.85±0.29	32.71	17.23±0.37	1.12±0.03
3	6.5 (-1)	36 (+1)	140 (-1)	2 (-1)	25.42±0.42	24.39	14.17±0.51	1.01±0.06
4	7.5 (+1)	36 (+1)	140 (-1)	2 (-1)	24.35±0.22	24.24	13.98±0.27	0.96±0.04
5	6.5 (-1)	28 (-1)	220 (+1)	2 (-1)	25.38±0.18	25.92	14.18±0.42	1.02±0.01
6	7.5 (+1)	28 (-1)	220 (+1)	2 (-1)	24.36±0.29	23.65	14.28±0.25	0.97±0.03
7	6.5 (-1)	36 (+1)	220 (+1)	2 (-1)	16.67±0.12	17.24	9.40±0.51	0.65±0.05
8	7.5 (+1)	36 (+1)	220 (+1)	2 (-1)	15.42±0.28	15.74	9.31±0.19	0.62±0.04
9	6.5 (-1)	28 (-1)	140 (-1)	4 (+1)	27.30±0.22	27.30	15.38±0.22	0.99±0.07
10	7.5 (+1)	28 (-1)	140 (-1)	4 (+1)	28.55±0.51	26.54	16.49±0.59	1.05±0.08
11	6.5 (-1)	36 (+1)	140 (-1)	4 (+1)	17.41±0.16	16.68	10.60±0.36	0.75±0.05
12	7.5 (+1)	36 (+1)	140 (-1)	4 (+1)	16.91±0.89	16.69	9.61±0.53	0.69±0.04
13	6.5 (-1)	28 (-1)	220 (+1)	4 (+1)	25.36±0.30	24.03	14.75±0.93	1.02±0.01
14	7.5 (+1)	28 (-1)	220 (+1)	4 (+1)	20.57±0.28	21.92	12.20±0.26	0.86±0.03
15	6.5 (-1)	36 (+1)	220 (+1)	4 (+1)	12.52±0.43	13.97	7.02±0.39	0.49±0.04
16	7.5 (+1)	36 (+1)	220 (+1)	4 (+1)	13.58±0.29	12.62	7.87±0.88	0.53±0.03
17	7.0 (0)	32 (0)	180 (0)	3 (0)	33.42±0.42	34.60	17.38±0.59	1.13±0.02
18	7.0 (0)	32 (0)	180 (0)	3 (0)	33.40±0.16	34.60	17.24±0.73	1.08±0.01
19	7.0 (0)	32 (0)	180 (0)	3 (0)	33.65±0.48	34.60	17.33±0.46	1.08±0.03
20	7.0 (0)	32 (0)	180 (0)	3 (0)	33.51±0.03	34.60	17.31±0.70	1.09±0.02
21	6.0 (-2)	32 (0)	180 (0)	3 (0)	26.63±0.28	26.56	14.03±0.63	0.98±0.03
22	8.0 (+2)	32 (0)	180 (0)	3 (0)	23.13±0.01	24.30	12.69±0.26	0.97±0.02
23	7.0 (0)	24 (-2)	180 (0)	3 (0)	22.19±0.03	23.52	12.34±0.14	0.95±0.07
24	7.0 (0)	40 (+2)	180 (0)	3 (0)	5.21±0.76	4.99	2.37±0.59	0.31±0.04
25	7.0 (0)	32 (0)	100 (-2)	3 (0)	22.97±0.62	25.26	11.85±0.41	0.96±0.04
26	7.0 (0)	32 (0)	260 (+2)	3 (0)	14.67±0.32	13.49	8.21±0.57	0.54±0.03
27	7.0 (0)	32 (0)	180 (0)	1 (-2)	21.31±0.14	21.76	11.44±0.30	0.86±0.02
28	7.0 (0)	32 (0)	180 (0)	5 (+2)	11.67±0.34	12.33	6.05±0.38	0.43±0.04
29	7.0 (0)	32 (0)	180 (0)	3 (0)	33.70±0.28	31.30	17.32±0.57	1.11±0.03
30	7.0 (0)	32 (0)	180 (0)	3 (0)	33.35±0.21	31.30	17.39±0.48	1.07±0.02

<sup>a</sup>The observed values of L-asparaginase specific activity, enzyme activity and cell growth were the mean values of duplicates with standard deviation (mean±SD)

Where,  $Y$  is the response (enzyme production),  $\beta_0$  is the constant coefficient,  $X_i$  ( $i=1-4$ ) are non-coded variables (initial pH of medium: A, temperature: B, rpm of the shaking incubator: C and inoculums size: D),  $\beta_i$  are the linear,  $\beta_{ii}$  are the quadratic, and  $\beta_{ij}$  ( $i$  and  $j=1-4$ ) are the second-order interaction coefficients. The statistical software package, MINITAB® Release 15.1, PA, USA, was used for the regression analysis of the experimental data and also to plot the response surface graphs. The  $F$  test was used to evaluate the significance of the model.

## Validation of the Experimental Model

In order to validate the model, experiments were performed at optimal levels of physical process parameters in pre-optimized medium in shake flask and 7 L bioreactor (Biostat B plus, Sartorius, Germany). The bioreactor was operated at optimal levels of physical process parameters and aeration rate of 1.5 vvm with uncontrolled pH. The samples were drawn at regular interval of time and specific activity was measured in duplicates. All experiments in shake flasks were conducted in duplicates and averages of the results were taken as response.

## Results and Discussion

### Production of L-Asparaginase

Initially basal medium containing glucose was used for the production of L-asparaginase from *P. carotovorum* MTCC 1428. Under un-optimized levels of medium components the production of intracellular L-asparaginase was found to be 3.35 U/mg of protein. The production of L-asparaginase in the optimized medium was found to be 27.88 U/mg of protein under un-optimal levels of physical process parameters in shake flask level [24]. There was no trace of extracellular L-asparaginase observed in the culture filtrates. It was reported that the glutaminase-free L-asparaginase have shown less allergic reactions [3]. Hence, the existence of glutaminase activity in the samples of L-asparaginase from *P. carotovorum* MTCC 1428 was tested. The L-asparaginase from *P. carotovorum* MTCC 1428 was shown high specificity towards its natural substrate, L-asparagine, and no activity of L-glutaminase was observed in the samples [30]. We have isolated the crude cytosolic L-asparaginase and purified to homogeneity by three steps purification process and molecular weight of purified L-asparaginase was confirmed by Native PAGE, gel exclusion chromatography and MALDI-TOF (data not shown). The intact L-asparaginase was found to be homotetramer of size 144.42 kDa and its subunit size of 36.10 kDa was determined by MALDI-TOF mass spectrophotometry analysis.

Though L-asparaginase was produced by various microorganisms, L-asparaginase from various *E. coli* and *Erwinia* strains were used as a chemotherapy agent due to high catalytic activity and specificity towards L-asparagine [4]. Most of the L-asparaginases from various microorganisms possess glutaminase activity, which causes various side effects during therapy, hence glutaminase-free L-asparaginase will be advantageous and value added product [3].

### Optimization of Physical Process Parameters for Maximization of L-Asparaginase Production

According to the CCD, each physical process parameter was investigated at three levels to maximize the L-asparaginase production [31]. Experiments were performed according to the design matrix to optimize the levels of physical process parameters (initial pH of the medium, temperature, rpm of the shaking incubator, and inoculum size) using central composite experimental design. The design matrix and the corresponding results of observed and predicted responses (production of L-asparaginase) are shown in Table 2. The cell growth and L-asparaginase activity (U/ml) data inferred the growth-associated production of L-asparaginase (Table 2). The enzyme activity varies from 2.37 to 17.45 U/ml, where as cell growth from 0.31 to 1.17 g/l. Hence, optimization of levels of physical process parameters for the

production of L-asparaginase is required. Table 2 showed that the high agitation speed (220 and 260 rpm) has negative affect on the L-asparaginase production, which inferred that the production of L-asparaginase by *P. carotovorum* MTCC 1428 favor at low agitation speed. Geckil and Gencer [32] were also observed the similar effect on the production of L-asparaginase by *E. aerogenes*. Similarly, the cell growth was inhibited at high aeration rates. This might be due to the microorganism used more substrate for its maintenance than the growth, which leads to low level of enzyme production. The cell growth and L-asparaginase production by *P. carotovorum* MTCC 1428 was also significantly affected by temperature and less than 32 °C favors the cell growth and L-asparaginase production (Table 2). At an initial pH of 7.0 and low rpm favors the cell growth and production of L-asparaginase. By applying the multiple regression analysis on the experimental data, the following second-order polynomial equation (Eq. 2) was found to explain the production of L-asparaginase from *P. carotovorum* MTCC 1428.

$$Y_{\text{specific activity}} = -541.370 + 80.820A + 15.326B + 0.606C + 16.258D - 5.870A^2 - 0.266B^2 - 0.002C^2 - 3.564D^2 + 0.095AD - 0.017AB + 0.078AC + 0.001BC - 0.087BD + 0.028CD \quad (2)$$

Where,  $Y_{\text{specific activity}}$  is the response (enzyme production), A is initial pH of the medium, B is temperature, C is rpm of the shaking incubator, and D is inoculum size.

ANOVA gives the value of the model and can explain whether a more complex model is required for a better fit. If the  $F$  test for the model is significant at the 5% level ( $P < 0.05$ ), then the model is fit and can adequately account for the variation observed. The statistical significance of the second-order model equation was checked by an  $F$  test (ANOVA) as shown in Table 3. According to the ANOVA of the quadratic regression model, the model is highly significant, as is evident from the Fisher,  $F$  test (mean square regression: mean square residual is 123.19) with a very low probability value ( $P_{\text{model}} > F$  is 0.0001). The  $F$  value is the ratio of the mean square due to regression to the mean square due to error. In general, the calculated  $F$  value should be several times greater than the tabulated value for a good model. If the  $F$  value is greater than tabulated  $F$  value, the null hypothesis is rejected at  $\alpha$  level of significance and infers that the variation accounted for the model is significantly greater than the unexplained variation. In this case, the null hypothesis is rejected at  $P < 0.0001$  level of significance for specific activity of L-asparaginase. This shows that the squared regression was significant at the level of 97.61% for L-asparaginase

**Table 3** Analysis of variance (ANOVA) for quadratic model.

Source	DF	SS	MS	$F$ value	Prob. ( $P$ )> $F$
Block	1	145.55	145.55	71.83	0.000
Regression	14	3,494.76	249.62	123.19	0.000
Linear	4	1,728.00	91.98	45.39	0.000
Square	4	1,718.04	429.51	211.96	0.000
Interaction	6	48.73	8.12	4.01	0.003
Residual Error	44	89.16	2.02		
Lack-of-Fit	10	80.61	8.06	32.04	0.000
Pure Error	34	8.55	0.25		
Total	59	3,729.48			

$$R^2 = 97.61\%; \text{Adj } R^2 = 96.79\%$$

SS sum of squares, DF degrees of freedom, MS mean square

production. This indicates that the combined effects of all the independent variables significantly contributed to maximize the production of L-asparaginase. The goodness of the model was checked by coefficient of determination,  $R^2$ , implies that the sample variation of 97.61% for L-asparaginase production is attributed to the physical parameters and also only 2.39% of the total variation is not explained by the model. The Student  $t$  distribution and the corresponding  $P$  values, along with the parameter estimate are shown in Table 4. The  $P$  values of all linear and quadratic relationships between medium components and L-asparaginase production suggest that they are highly significant ( $P < 0.0001$ ). Also the interaction effect of rpm and inoculum size is highly significant on the production.

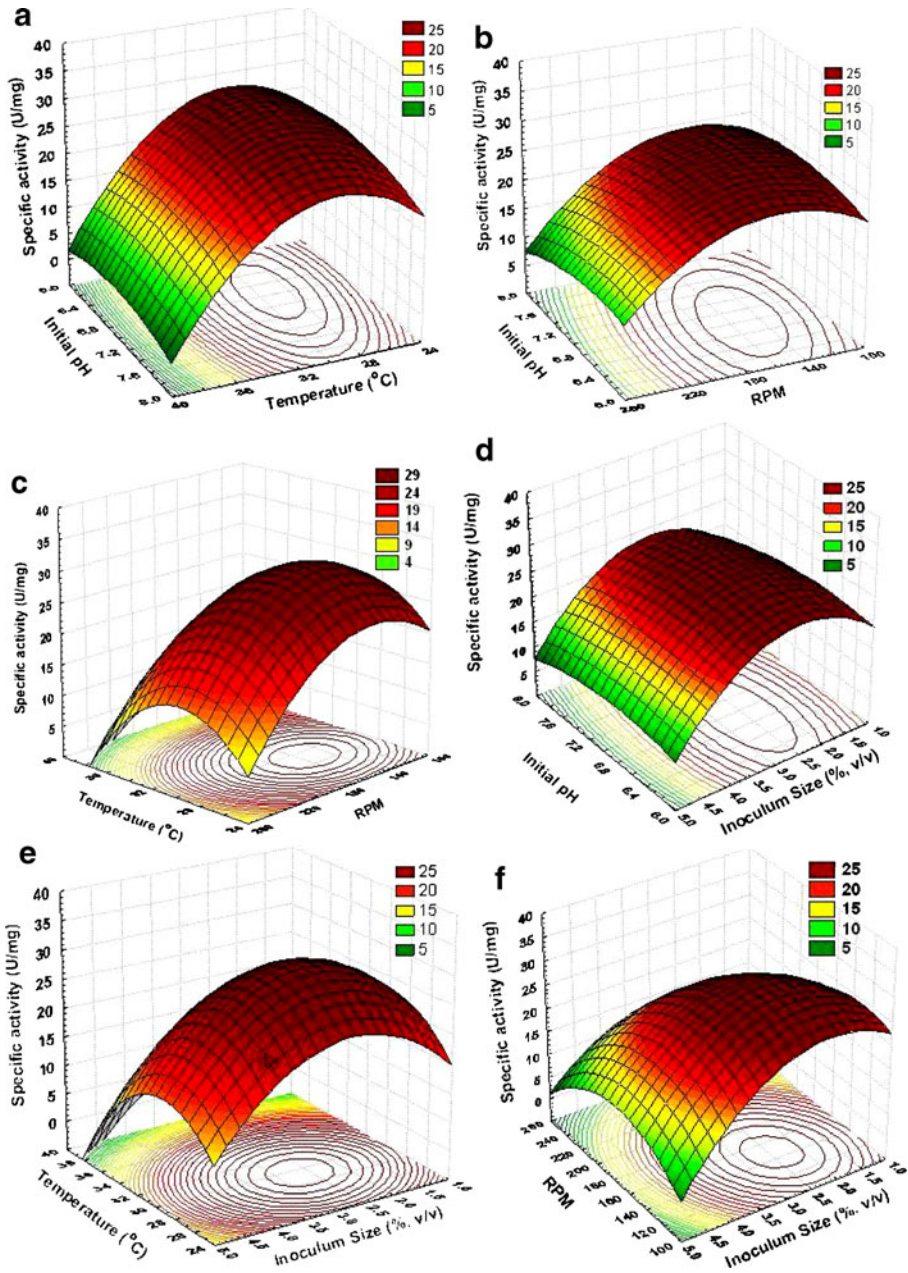
The three-dimensional response surface plots were constructed to predict the L-asparaginase production for different values of the tested variables and to analyze the interactions among the variables. The response surface plots were constructed by plotting the response (specific activity of L-asparaginase) on the  $z$ -axis against any two independent variables, while maintaining other variables at their optimal levels as shown in Fig. 1 (a–f). As shown in the Fig. 1 (a, c, and e), there was significant effect of temperature on the production of L-asparaginase. The maximum level of L-asparaginase production was observed at a range of 28–32 °C. However, effect of initial pH of the medium on L-asparaginase production with other physical parameters was not much significant in comparison to other interactions and achieved upper limit at a range of 2–3% ( $v/v$ ; Fig. 1 (a, b, and d)). The interaction of initial pH (Fig. 1 (a, b, and d),  $P > 0.184$ ) with other variables and temperature with rpm (Fig. 1 (c),  $P = 0.585$ ) were not prominent. The interaction of rpm and inoculum size ( $P = 0.000$ ) was very prominent as shown in Fig. 1 (f). Similar effect was observed for temperature with inoculum size (Fig. 1 (e)).

The optimum levels of initial pH of the medium, temperature, rpm of the shaking incubator, and inoculum size were determined by maximizing the regression equation and were found to be 6.9, 29.8 °C, 157 rpm, and 2.61% ( $v/v$ ), respectively. The parity plot (Fig. 2) showed a satisfactory correlation between the experimental and predicted values

**Table 4** Model coefficient estimated by multiple linear regressions.

Model term	Parameter estimate	SE coefficient	Computed $t$ value	$P$ value
Intercept	-541.370	55.4847	-9.757	0.000
Block	1.652	0.1949	8.475	0.000
$A$	80.820	11.8166	6.840	0.000
$B$	15.326	1.2187	12.576	0.000
$C$	0.606	0.1120	5.407	0.000
$D$	16.258	4.3725	3.718	0.001
$A^2$	-5.870	0.7688	-7.636	0.000
$B^2$	-0.266	0.0120	-22.175	0.000
$C^2$	-0.002	0.0001	-15.518	0.000
$D^2$	-3.564	0.1922	-18.542	0.000
$AD$	0.095	0.1258	0.759	0.452
$AB$	-0.017	0.0126	-1.350	0.184
$AC$	0.078	0.5033	0.155	0.877
$BC$	0.001	0.0016	0.550	0.585
$BD$	-0.087	0.0629	-1.377	0.175
$CD$	0.028	0.0063	4.407	0.000

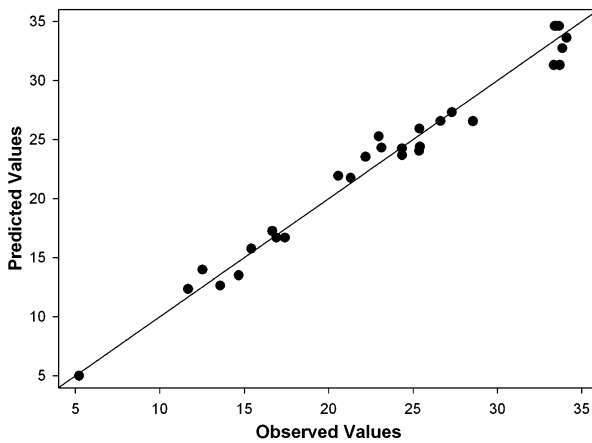




**Fig. 1** Three-dimensional response surface plot for L-asparaginase production showing the interactive effects of **a** initial pH and temperature, **b** initial pH and rpm, **c** temperature and rpm, **d** initial pH and inoculum size, **e** temperature and inoculum size, and **f** rpm and Inoculum size

(obtained from Eq. 2) of L-asparaginase production, wherein, the points cluster around the diagonal line which indicated the optimal fit of the model, since the deviation between the experimental and predicted values were minimum.





**Fig. 2** Parity plot showing the distribution of experimental vs. predicted values of L-asparaginase

CCD has been successfully applied in several similar studies to optimize the physical process parameters for the enhanced production of glucoamylase from *Aspergillus* species [33], bacteriocin from *Bacillus* sp. strain P34 [34], cephalosporin C from *Acremonium chrysogenum* [35] and citric acid from *Aspergillus niger* [36] etc. Recently, Rahulana et al. [37] successfully used central composite experimental design to optimize the heterogeneous process parameters for L-leucine amino peptidase production from *Streptomyces gedanensis* and achieved twofold enhancement in the production. To the best of our knowledge, we could not find any report in the literature on the optimization of physical parameters for the production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 in submerged fermentation. However, a few reports are available for improved production of L-asparaginase using statistical methods. Enhanced production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 using optimized medium has been reported by our group [24]. Abdel-Fattah and Olama [15] successfully used Plackett-Burman and Box-Behnken experimental designs for screening and optimization of process parameters to improve the production of L-asparaginase from *P. aeruginosa* in solid-state fermentation. Recently, Hymavathi et al. [13], Prakasham et al. [38], and Neto et al. [39] were also reported for the optimization of process parameters to maximize the production of L-asparaginase from *B. circulans*, *Zymomonas mobilis*, and *Staphylococcus* using CCD, Box-Behnken, and Taguchi experimental design techniques, respectively. Shake flask experiments have limitations to control pH and dissolved oxygen level in the broth. Earlier reports showed that the L-asparaginase production was affected by DO level and pH of the fermentation broth [12, 32]. Fermenter studies under batch and fed-batch mode could be better option to further optimize these physical parameters for improvement of productivity.

In this current investigation, the highest production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 was found to be 35.24 U/mg of protein. This is very much comparable with the reported values. The range of specific activity of L-asparaginases from different microorganisms and production time were 0.1–35.0 U/mg of protein and 36–120 h, respectively [3, 10, 15, 38, 40–42]. Productivity of L-asparaginase from *P. carotovorum* MTCC 1428 was higher due to short duration of fermentation time (12 h) than reported values (above 16 h). This study indicates that the large amount of the enzyme with higher activity would obtain in a short period under optimal conditions. In addition, L-asparaginase

from *P. carotovorum* MTCC 1428 also displays comparable catalytic activity, making it attractive for further screening as a potential anticancer agent.

### Verification of Model

Experiments were performed at the optimal levels of physical process parameters and production (specific activity) of L-asparaginase was found to be 35.24 U/mg of protein, and it was in very good correlation with the predicted value (35.55 U/mg of protein). The production of L-asparaginase under un-optimal levels of physical parameters was found to be 27.88 U/mg of protein [24], and an enhancement of 26.39% was achieved under optimal levels of physical parameters (Table 5). The verification revealed a high degree of accuracy of the model of more than 99.0%, which is an evidence for the model validation under the investigated conditions. The production of L-asparaginase was slightly higher (35.71 U/mg of protein) in the fermenter as compared with shake flask experiment. Maximization of L-asparaginase production was achieved at 12 h both in shake flask and bioreactor. The overall productivity of L-asparaginase was increased from 1,350.83 to 1,404.16 U/l/h (Table 5).

### Conclusion

In this study, RSM was applied to optimize the levels of physical process parameters for the production of glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428. The effect of physical process parameters viz. initial pH of the medium, temperature, rpm of the shaking incubator, and inoculum size on the production of glutaminase-free L-asparaginase was studied using CCD. The optimum levels of initial pH of the medium, temperature, rpm of shaking incubator, and inoculum size were found to be 6.90, 29.8 °C, 157 rpm, and 2.61% (v/v), respectively. The production of L-asparaginase (specific activity) was enhanced by 26.39% under optimal levels of physical process parameters. In the present investigation, novel glutaminase-free L-asparaginase from *P. carotovorum* MTCC 1428 was obtained in a short period and displayed comparable catalytic activity with the literature reports.

**Table 5** Comparison of L-asparaginase production in the initial and different optimization stages.

Process conditions for media	Level of scale	L-asparaginase production <sup>a</sup>		Enhanced Production (fold)	Productivity <sup>a</sup> (U/l/h)	References
		Enzyme activity (U/ml)	Specific activity (U/mg)			
Before optimization	Shake flask	0.76±0.01	3.35±0.32	–	63.33±0.85	Kumar et al. [24]
RSM—chemically optimized	Shake flask	14.71±0.53	27.88±0.46	8.32	1,225.83±44.23	
RSM—chemically optimized	Bioreactor	15.39±0.33	28.87±0.37	8.62	1,282.50±27.50	
RSM—process optimized	Shake flask	17.81±0.67	35.24±0.71	10.52	1,350.83±56.16	In this study
RSM—process optimized	Bioreactor	17.95±0.76	35.71±0.82	10.66	1,404.16±63.35	

<sup>a</sup> Mean values of duplicates with standard deviation (mean±SD)

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