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RESEARCH ARTICLES

Study of media optimization and kinetic modeling of L-methioninase from *Pseudomonas stutzeri*

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Abstract

In this study, *Pseudomonas stutzeri* has been explored as new bacterial strain for producing intracellular L-methioninase. Kinetic modeling and optimization of fermentation conditions are crucial parameters for improving production of L-methioninase. Response surface methodology has been employed as fast efficient technique for investigating the interactive effect of culture condition parameters for L-methioninase. Plackett–Burman design was first employed for screening significant variables for production media components including glucose, L-methionine, NaCl, malt extract and casein enzymic hydrolysate. The optimal level of these significant media components was further optimized using Central composite design. The high value of determination of coefficient ($R^2 = 0.95$) showed the good fitness of these statistical models for explaining the relationship between variables and response. The optimum values for the investigated variables were obtained as 5% glucose, 0.25% L-methionine, 0.2% malt extract, 1% casein enzymic hydrolysate and 1% NaCl. The maximum production of L-methioninase was obtained as 240.97 U/l after performing the experiments at these optimum data variables, which was increased by 1.61-fold in compare to classical method. The kinetic study was further performed at these optimum media components and the production of L-methioninase was found as non-growth associated behavior. Logistic equation and modified Luedeking–Piret equation were further employed to develop mathematical model for growth kinetic and production kinetic. These results would be significant for large scale production of these enzymes using bioreactors in industry.

Keywords L-Methioninase · *Pseudomonas stutzeri* · Media component · Statistical optimization · Kinetic modeling

Introduction

The industrial importance of microbial enzymes is always in demand in various sectors because of their high specificity for the catalyzing biochemical reaction to produce specific product (Sato and Nozaki 2009; Fogarty and Kelly 1990; Wriston and Yellin 1973). L-Methioninase/methionine lyase/methionine demythalase/methioninase/methionine-gamma-lyase (E.C.4.4.1.11) has currently wide application in pharmaceutical and food industry. In food industry, it imparts aroma to cheese which is a traditional fermented foods by degrading L-methionine which releases volatile sulfur compounds (VSCs) (Kharayat and Singh 2018). Methanethiol is the most common volatile sulfur compounds found in cheese, which is derived from the degradation of enzymes of the amino acid L-methionine (Khalaf and El-Sayed 2009; Weimer et al. 1999). L-Methioninase also acts as anticancer agent against various types of cell lines which includes breast, glioblastoma, colon, kidney and the lung (Sato and Nozaki 2009; Tan et al. 1998). L-Methioninase is pyridoxal-5′-phosphate (PLP) dependent enzyme which catalyzes the direct conversion of L-methionine ($C_5H_{11}NO_2S$) to methanethiol, α-ketobutyrate and ammonia (Tanaka et al. 1985). The production of L-methioninase is absent in mammalian cells and its production is reported as intracellular from many bacterial strains. Some fungal strains are also reported to produce it extracellularly and intracellularly (Sharma et al. 2014). L-Methionine is used as essential amino acids to regulate protein synthetic pathway and maintain cellular homeostasis process (Tanaka et al. 1985). Cancer cells lack the pathway to synthesize L-methionine and hence depend on the exogenous source for L-methionine supply (Cellarier et al. 2003; Pinnamaneni and Funderburgh 2012). Some microbial L-methioninase has been formulated as antitumoric drugs to inhibit the growth of L-methionine dependent

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tumor cells (Sato and Nozaki 2009; Cellarier et al. 2003). Hence, the exploitation of L-methioninase therapeutically to deplete plasma methionine has been extensively investigated (Pinnamaneni and Funderburgh 2012). The production of L-methioninase from different microbial strains would be effected by varying their fermentative conditions like inoculum age, inoculum volume, pH of media, incubation temperature, fermentation period, medium components and agitation speed. The growth of the microbial cell and production of specific metabolites are strongly influenced by medium composition such as carbon, nitrogen and mineral salts (Cutchins et al. 1952). Classical method is commonly used for optimization of fermentation condition by changing one variable at a time and keeping the other variables at fixed levels. Statistical approach has been currently preferred for optimization because of accounting interactive effects of the variables and screening of large experimental domains (Dutta et al. 2004; Ruchi et al. 2008). The modeling of kinetics is necessary for designing the bioreactors and scaling up the fermentation process. A kinetic model enables the bioengineers to operate, control and understand process of fermentation under optimized conditions (Subba Rao et al. 2009; Moser and Steiner 1975).

In this study, Statistical tools based on Plackett–Burman design (PBD) would be employed for screening of significant variables of media components of L-methioninase from *Pseudomonas stutzeri*. The optimum levels for these significant variables would be further optimized using central composite design to obtain high activity of L-methioninase. The kinetic models were developed for fermentation of L-methioninase using Logistic equation, Luedeking–Piret equation, and modified Luedeking–Piret equation for cell growth, substrate consumption, product formation, respectively. To the best of our knowledge, this is the first report on optimization of media components for the production of L-methioninase from *P. stutzeri* using PBD and RSM methods and kinetic modeling for fermentation process.

Materials and methods

Chemicals and microorganism

All the chemicals and reagents were of analytical grade and purchased from HiMedia Laboratories Pvt Ltd., Mumbai, India. The bacterial strain *P. stutzeri* (MTCC 101) was obtained from Microbial type culture collection (MTCC) and gene bank, IMTECH, Chandigarh, India.

Culture maintenance and conditions

The culture was maintained at − 80 °C by storing in media containing glycerol in equal proportions. Slants and petriplates were grown at 37 °C for 24 h and stored in refrigerator at 0–4 °C. The cells were grown in nutrient media (pH-7) containing beef extract (1 g/l), peptone (5 g/l), yeast extract (2 g/l) and 1% glucose and incubated at 37 °C for 18 h. Plackett–Burman design was applied for the production of L-methioninase consisting of glucose (5 g), malt extract (0.2 g), Casein enzymic hydrolysate (1 g), NaCl (1 g), KCl (1 g), $CaCO_3$ (0.2 g), K_2HPO_4 (0.2 g), KH_2PO_4 (0.2 g), $ZnSO_4$ (0.2 g), $CaCl_2$ (0.2 g), Na_2SO_4 (0.2 g), Na_2HPO_4 (0.2 g) and L-methionine (0.25 g) (Table 1). pH of the medium was adjusted to 7.5 by inoculating with 1% inoculums and incubating the flask at 37 °C for 20 h. Cell free broth was collected after centrifugation at 10,000 rpm at 4 ± 1 °C for 30 min. The supernatant was collected in other fresh tubes and used for the estimation on activity of L-methioninase. The pellet was resuspended in 0.5 M potassium phosphate buffer (pH 7.0) and was treated in ultra sonicator for three times for 3 min over 15 min at 20 Kc/s and at temperature 6 °C. This cell suspension was kept in alcohol: ice mixture throughout the sonic disruptions. The treated cells were centrifuged (Kreis and Hession 1973) at 10,000 rpm for 10 min in cold centrifuge.

Estimation of L-methioninase activity

The activity of L-methioninase was estimated by Nesslerization method (Pinnamaneni and Funderburgh 2012) with some modification. In this method, sample containing 1% of L-methionine, in 0.5 M phosphate buffer (pH-7.5), enzyme solution (0.5 ml), 100 mM pyridoxal-5′-phosphate was incubated at 37 °C for 1 h. Reaction was further stopped by adding 1.5 N Tricholoro-acetic acid (TCA) solution and solution was centrifuged 5000 rpm for 5 min. The reaction mixture with 3.7 ml of distilled water, 0.1 ml filtrate of above centrifuged mixture and 0.2 ml of Nessler's reagent was incubated for 20 min at room temperature and absorbance was noted at 480 nm. Blank were prepared without adding the enzyme solution.

Experimental design for statistical analysis

Plackett–Burman design (PBD)

Minitab 15 statistical tool was used for experimental design for optimization of variables. Plackett–Burman design (PBD) (Plackett and Burman 1946) with 2-level factorial design was employed to investigate significant variables for media components of glucose, malt extract, casein enzymic hydrolysate, NaCl, KCl, $CaCO_3$, K_2HPO_4, KH_2PO_4, $ZnSO_4$, $CaCl_2$, Na_2SO_4, Na_2HPO_4, and NaH_2PO_4. The experiments were carried out with incubating 100 ml of production media

Table 1 Plackett–Burman design for evaluation of input variables on the activity of L-methioninase

S. no.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	Exp. activity (U/l)	Pred. activity (U/l)
1	5	0.0	0.2	1	0	0	0	0	0.2	0	0.2	0	0.2	0.25	60.213	61.041
2	0	0.2	0.2	0	0	0	0	0.2	0	0.2	0	0.2	0.2	0.25	90.624	87.226
3	0	0.2	0.2	0	0.2	0.2	0	0	0	0	0.2	0	0.2	0.00	10.152	5.714
4	5	0.0	0.2	1	0.2	0.2	0	0	0.2	0.2	0	0.2	0.2	0.00	87.840	88.873
5	0	0.0	0	1	0	0.2	0	0.2	0.2	0.2	0.2	0	0.0	0.25	15.555	7.318
6	5	0.0	0.2	0	0.2	0.2	0.2	0.2	0	0	0.2	0.2	0.0	0.25	55.298	49.348
7	5	0.2	0.0	0	0	0	0.2	0	0.2	0	0.2	0.2	0.2	0.25	60.517	59.689
8	0	0.2	0.2	1	0.2	0	0	0.2	0.2	0	0.2	0.2	0.0	0.00	20.149	22.727
9	0	0.0	0.2	1	0	0.2	0.2	0	0	0	0	0	0.0	0.25	58.297	64.248
10	0	0.0	0.0	0	0.2	0	0.2	0	0.2	0.2	0.2	0.2	0.0	0.0	4.421	4.823
11	5	0.2	0.0	1	0.2	0	0	0	0	0.2	0	0	0.0	0.25	145.599	141.587
12	5	0.2	0.0	0	0.2	0.2	0	0.2	0.2	0	0	0	0.0	0.25	42.284	50.095
13	0	0.2	0.0	1	0	0.2	0.2	0.2	0.2	0	0	0	0.2	0.00	2.258	0.107
14	5	0.0	0.0	0	0	0.2	0	0.2	0	0.2	0.2	0.2	0.2	0.00	20.944	27.321
15	5	0.2	0.2	0	0	0.2	0.2	0	0.2	0.2	0	0	0.0	0.00	75.846	71.014
16	5	0.0	0.0	1	0.2	0	0.2	0.2	0	0	0	0	0.2	0.00	20.767	15.509
17	0	0.2	0.0	1	0.2	0.2	0.2	0	0	0	0.2	0.2	0.2	0.25	35.246	39.684
18	0	0.0	0.0	0	0	0	0	0	0	0	0	0	0.0	0.00	0.840	2.299
19	5	0.2	0.2	1	0	0	0.2	0.2	0	0.2	0.2	0.2	0.0	0.00	55.360	60.192
20	0	0.0	0.2	0	0.2	0	0.2	0.2	0.2	0.2	0	0	0.2	0.25	25.162	28.560

X_1 glucose, X_2 Malt extract, X_3 casein enzymic hydrolysate, X_4 NaCl, X_5 KCl, X_6 $CaCO_3$, X_7 K_2HPO_4, X_8 KH_2PO_4, X_9 $ZnSO_4$, X_{10} $CaCl_2$, X_{11} Na_2SO_4, X_{12} Na_2HPO_4, X_{13} NaH_2PO_4, *Exp.* experimental activity, *Pred.* predicted activity

in 250 ml Erlenmeyer flasks in orbital shaker at 37 °C, 110 rpm for 20 h for all experimental data. The activity of L-methioninase for each data was written as experimental activity in PBD design matrix table. The variables with confidence level greater than 90% were considered as more significant for L-methioninase production. Pareto Chart would also display the magnitude of each factor and the main effect was calculated between measurements at high level (+1), and low level (−1) for each factor.

Central composite design

The levels of screened five significant media components were optimized using central composite design (CCD), consisting a matrix of 32 experiments (Singh and Kharayat 2018). The effect of variables with upper and lower limits on the experimental activity of L-methioninase in CCD design matrix were glucose (0.5–9.5), L-methionine (0.15–0.35), NaCl (0.2–1.8),malt extract (0.1–0.3), casein enzymic hydrolysate (0.2–1.8). Central composite design (CCD) with 2^3 level factorial ($\alpha = 2.366$) had designed 32 experiments with seven replications at centre points.

The optimum level of cultural variables was obtained after regression analysis, ANOVA, contour and response surface plot. The following second order polynomial equation was adopted to study the effects of variables on the response:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \quad (1)$$

where, Y = predicted response, β_0 = intercept coefficient, β_i = linear coefficient, β_{ii} = quadratic coefficient, β_{ij} = interaction coefficient, X_i = i-th variable, X_j = j-th variable.

Development of kinetic modeling for L-methioninase

Microbial growth

Logistic equation was proposed for microbial fermentation process with non growth associated behavior. In this paper, L-methioninase production from *P. stutzeri* MTCC 101 was found to be substrate independent and hence, Logistic equation (Eq. 2) would be applied for growth kinetic study:

$$\frac{dX}{dt} = \mu_m \left(1 - \frac{X}{X_m}\right), \quad (2)$$

where, $\frac{dX}{dt}$ is cell growth (g cell biomass/h), μ_m is the maximum specific growth rate (/h), X is the biomass concentration, X_m is the maximum biomass concentration (g cell/l).

At the beginning of fermentation, when t = 0, the biomass concentration was given by the initial concentration value ($X = X_0$). The integrated form of Eq. (2) gives a sigmoid

variation of X as a function of t, which may represent both an exponential and a stationary phase:

$$X_t = \frac{X_o e^{\mu^t m^t}}{\left[1 - \left(\frac{X_o}{X_m}\right)\left(1 - e^{\mu^t m^t}\right)\right]}, \quad (3)$$

Where, m^t is maintenance coefficient at time interval, μ^t is specific growth rate constant at time interval, X_o is initial biomass concentration.

Product formation

The production of L-methioninase was observed maximum during late-log phase which showed its non growth associated behavior and hence Luedeking–Piret equation (Luedeking and Piret 1959) was applied:

$$r_p = \frac{dp}{dt} = \alpha\left(\frac{dx}{dt}\right) + \beta, \quad (4)$$

where α the growth is associated constant and β is the non-growth associated constants empirical constants, r_p is rate of product formation with time interval.

After integration following equation was obtained:

$$P_t = P_0 + \alpha A_t + \beta B_t, \quad (5)$$

where P_t is the product at time t and P_0 is the initial product concentration, and $A_t = X_0$:

$$B_t = \left(\frac{X_m}{\mu_m}\right)\left[\ln 1 - \left(\frac{X_o}{X_m}\right)\left(1 - e^{\mu^t m^t}\right)\right].$$

Substrate consumption rate

Kinetics for non-growth associated curve, where some substrate would be utilized for growth and some would be utilized for maintenance purpose.

Luedeking–Piret equation suggested for the consumption of substrate kinetics which include conversion of substrate into product and substrate consumption for cell maintenance:

$$\frac{-ds}{dt} = \left(\frac{1}{Y_{X/S}}\right)\frac{dX}{dt} + \left(\frac{1}{Y_{P/S}}\right) + m_s X, \quad (6)$$

where, $Y_{P/s}$ is product yield coefficient for substrate, $Y_{X/s}$ is cell yield coefficient for substrate and m_s is maintenance coefficient (g substrate * g cell/h).

Glucose consumption was obtained in this study after substituting and integrating $\frac{dX}{dt}$ from Eq. (2) in Eq. (6):

$$\frac{-ds}{dt} = \left(\frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}}\right) + \left(\frac{\beta}{Y_{P/S}} + m_s\right)X, \quad (7)$$

$$\frac{-ds}{dt} = \frac{\gamma dX}{dt} + \delta X, \quad (8)$$

where, $\gamma = 1/Y_{X/s} + \alpha/Y_{P/s}$ and $\delta = \beta/Y_{P/s} + m_s$.

The following equation was obtained, by integrating the equation:

$$S_t = S_o - \gamma C_t - \delta D_t, \quad (9)$$

whereas, S_t is residual glucose remained at time t, S_0 is initial amount of glucose at $t = 0$, C_t is the:

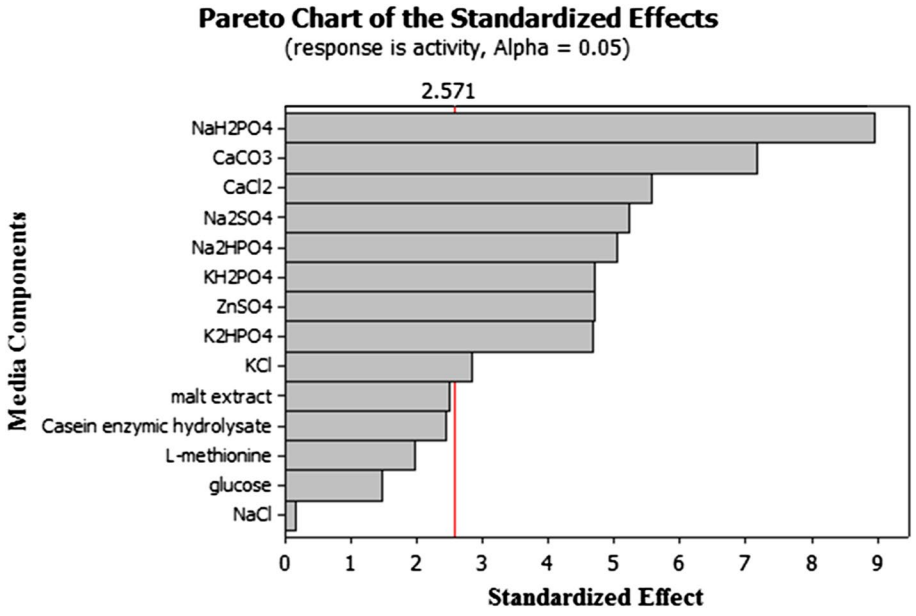

Fig. 1 Screening of significant media components for L-methioninase using Pareto chart of Plackett–Burman design

$$C_t = X_o\left[\frac{e^{\mu_m t}}{1-\left(\frac{X_o}{X_m}\right)(1-e^{\mu_m t})}-1\right],$$

$$D_t = \frac{X_m}{\mu_m}\left[\ln 1 - \frac{X_m}{\mu_m}(1-e^{\mu_m t})\right].$$

Results and discussion

Screening of significant variables using Plackett–Burman design (PBD)

Optimization of process variables is one of the essential prerequisite for industrial scale production. Currently statistical tools have been employed to achieve maximum production of some biomolecules by analyzing non linearity in terms of interactive effects of process variables on the response. Plackett–Burman Design (PBD) was applied to evaluate effect of 14 medium components on the production of L-methioninase by *P. stutzeri* MTCC 101. The variables within the range of α value were considered as significant variables and selected as optimal variables for further study (Hymavathi et al. 2010). Pareto chart (Fig. 1) shows the effects of variables on production of L-methioninase and screened the significant media components. In this study, pareto chart with α = 2.571 showed the factors like NaCl, glucose, L-methionine, casein enzymic

Table 2 Regression analysis data for different variables in terms of student "t" test value and probability (p) value in Plackett–Burman design

Term	Effect	Coef	SE Coef	t	p
Constant		44.37	2.023	21.93	0.000
X_1	36.20	18.10	2.023	8.95	0
X_2	18.87	9.43	2.023	4.66	0.006
X_3	19.05	9.53	2.023	4.71	0.005
X_4	11.52	5.76	2.023	2.85	0.036
X_5	0.65	0.32	2.023	0.16	0.879
X_6	−7.99	−4.00	2.023	−1.98	0.105
X_7	−10.10	−5.05	2.023	−2.50	0.068
X_8	−19.06	−9.53	2.023	−4.71	0.021
X_9	−9.89	−4.94	2.023	−2.44	0.058
X_{10}	22.58	11.29	2.023	5.58	0.450
X_{11}	−21.7	−10.58	2.023	−5.23	0.42
X_{12}	20.45	10.23	2.023	5.05	0.012
X_{13}	−5.99	−3.00	2.023	−1.48	0.199
X_{14}	29.02	14.51	2.023	7.17	0.001

X_1 Glucose, *X_2* Malt extract, *X_3* casein enzymic hydrolysate, *X_4* NaCl, *X_5* KCl, *X_6* CaCO3, *X_7* K_2HPO_4, *X_8* KH_2PO_4, *X_9* $ZnSO_4$, *X_{10}* $CaCl_2$, *X_{11}* Na_2SO4, *X_{12}* Na_2HPO_4, *X_{13}* NaH_2PO_4, *X_{14}* L-methionine

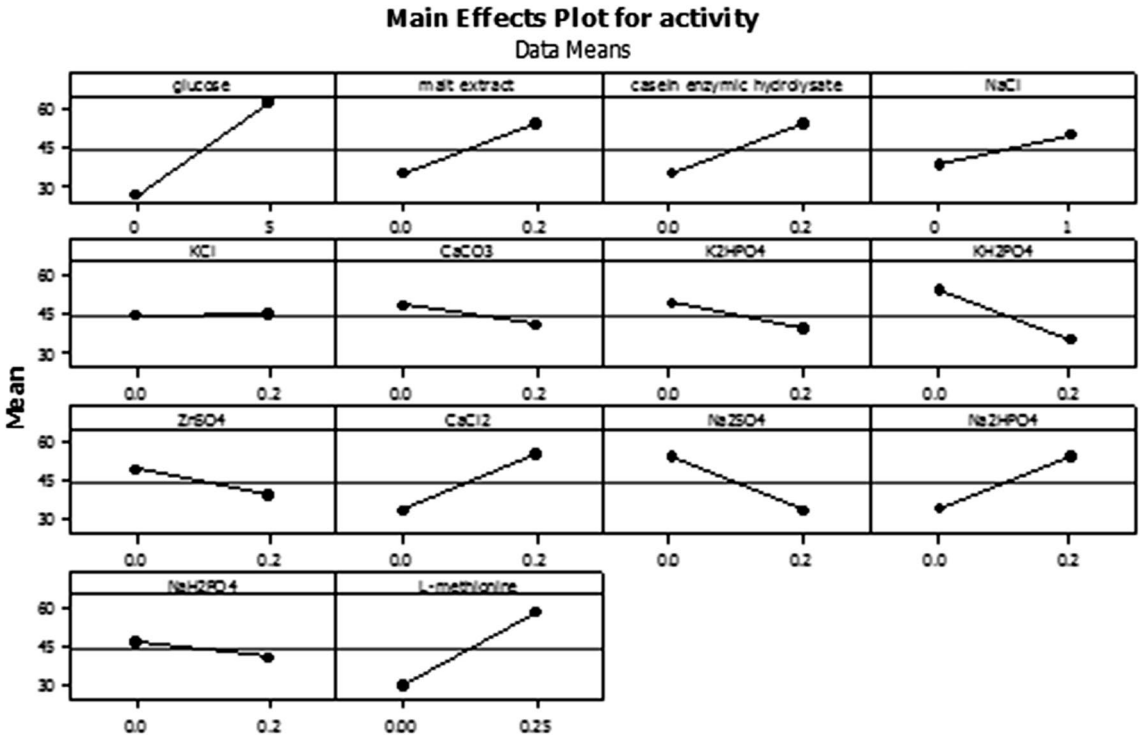

Fig. 2 Plot represents main effect of each media component variable on the activity of L-methioninase after applying Plackett–Burman design

Table 3 Experimental data for significant variables for activity of L-methioninase after applying central composite design (CCD)

Run order	X_1	X_2	X_3	X_4	X_5	Experimental activity (U/l)	Predicted activity (U/l)
1	5	0.05	1.0	0.2	1.0	190.200	190.723
2	5	0.25	0.6	0.2	1.0	198.038	202.358
3	5	0.25	1.0	0.2	1.0	240.970	240.193
4	9.5	0.35	1.8	0.3	1.8	150.690	150.311
5	5	0.25	2.6	0.2	1.0	185.808	186.151
6	9.5	0.35	1.8	0.1	0.2	177.280	177.280
7	0.5	0.35	1.8	0.1	1.8	188.000	187.339
8	5.0	0.25	1.0	0.2	1.0	240.970	240.193
9	0.5	0.15	0.2	0.3	0.2	194.320	192.287
10	5.0	0.25	1.0	0.4	1.0	205.770	208.263
11	5.0	0.45	1.0	0.2	1.0	189.280	190.420
12	0.5	0.35	0.2	0.3	1.8	210.250	208.514
13	9.5	0.15	1.8	0.1	1.8	183.670	182.776
14	9.5	0.15	0.2	0.1	0.2	169.360	167.770
15	5.0	0.25	1.0	0.2	1.0	240.970	240.193
16	5.0	0.25	1.0	0.0	1.0	186.465	188.635
17	9.5	0.15	0.2	0.3	1.8	199.380	197.411
18	0.5	0.15	1.8	0.3	1.8	202.350	201.012
19	4.0	0.25	1.0	0.2	1.0	198.840	201.897
20	5.0	0.25	1.0	0.2	2.6	201.380	204.308
21	0.5	0.15	0.2	0.1	1.8	187.870	185.619
22	9.5	0.35	0.2	0.3	0.2	190.360	189.285
23	5.0	0.25	1.0	0.2	1.0	240.970	240.193
24	0.5	0.35	1.8	0.3	0.2	189.580	189.137
25	14.0	0.25	1.0	0.2	1.0	180.917	180.917
26	5.0	0.25	1.0	0.2	0.6	190.015	190.015
27	5.0	0.25	1.0	0.2	1.0	240.970	240.193
28	9.5	0.35	0.2	0.1	1.8	183.200	181.907
29	0.5	0.35	0.2	0.1	0.2	177.280	175.923
30	0.5	0.15	1.8	0.1	0.2	169.390	168.432
31	9.5	0.15	1.8	0.3	0.2	178.280	177.603
32	5.0	0.25	1.0	0.2	1.0	240.970	240.193

X_1 glucose, X_2 L-methionine, X_3 NaCl, X_4 malt extract, X_5 casein enzymic hydrolysate

hydrolysate, malt extract as significant variables. The plot of main effect in Fig. 2 confirmed the significant effect of these variables on the production of L-methioninase. The variables were screened through PBD matrix with 90% confidence level on the basis of student's test (t) value, probability (p) value (Table 2). The variables with p value < 0.05 were considered as significant variables and after analysis of these data by PBD statistical tool, the significant effect was obtained for glucose, malt extract, casein enzymes hydrolysate, NaCl and L-methionine. The model was found to be significant because of high F value of 22.01 and low p value of 0.002.

Optimization of media components by using central composite design (CCD)

After the screening of significant variables, CCD was employed to obtain optimum level of screened significant variables (glucose, L-methionine, NaCl, malt extract, casein enzymic hydrolysate) along with their interactive effect on L-methioninase activity and results were shown in Table 3. Regression analysis of these CCD data (Table 4) showed the positive and negative effect of the significant variables on the production of L-methioninase. The higher magnitude of students 't' value and smaller 'p' value shows significant effect on the variables. The 'p' value delivers tool for checking the significance of each coefficients and is indicative of

Table 4 Regression analysis data for activity of L-methioninase after central composite designs (CCD)

Term	Coefficient	SE coefficient	t	p
Constant	240.193	1.194	201.51	0.000
X_1	−10.490	1.22	−8.583	0.000
X_2	−1.652	1.22	−1.351	0.204
X_3	−8.103	1.22	−6.630	0.000
X_4	9.814	1.22	8.030	0.000
X_5	7.147	1.22	5.847	0.000
X_1X_1	−48.786	2.211	−22.065	0.000
X_2X_2	−48.121	2.211	−21.764	0.000
X_3X_3	−45.938	2.211	−20.777	0.000
X_4X_4	−41.743	2.211	−18.880	0.000
X_5X_5	−43.031	2.211	−19.462	0.000
X_1X_2	−10.085	2.211	−3.369	0.000
X_1X_3	−7.995	2.994	−2.671	0.022
X_1X_4	−17.190	2.994	−5.742	0.00
X_1X_5	−14.060	2.994	−4.696	0.001
X_2X_3	−9.575	2.994	−3.198	0.008
X_2X_4	−12.230	2.994	−4.085	0.002
X_2X_5	−16.070	2.994	−5.368	0.000
$X3X_4$	−18.510	2.994	−6.183	0.000
X_3X_5	−9.800	2.994	−3.273	0.007
X_4X_5	−9.825	2.994	−3.282	0.007

X_1 glucose, X_2 L-methionine, X_3 NaCl, X_4 malt extract, X_5 casein enzymic hydrolysate

the interaction strength of each independent variable. Lower value of $p < 0.05$ indicates high significance response on the corresponding variables. In general, large 'T' value and small 'p' values indicates that the corresponding coefficient terms are significant (Li et al. 2008).

The fitness of model was analyzed statistically in terms of coefficient of determination (R^2) after applying analysis of variance (ANOVA) as shown in Table 5. Higher the value of coefficient of determination ($R^2 = 0.95$) and adjusted determination coefficient (Adj $R^2 = 0.93$) shows the good fitness of model for analyzing the experimental data. The slight variation in values of R^2 and Adj R^2 might be because of smaller the sample size and more number of terms in the model (Shanthi and Roymon 2015).

The effect of interaction on these variables for activity of L-methioninase was analyzed using graphical representations of the regression equation in terms of 3D response surface plot (Fig. 3) and 2D contour plot (Fig. 4). The greater significant effect of interactive variables like, casein enzymic hydrolysate and NaCl, Casein enzymic hydrolysate and L-methionine, L-methionine and Glucose, on the activity of L-methioninase enzyme had been observed in response surface plots of Fig. 3c, e, g, respectively. The response surface plots for NaCl and glucose (Fig. 3a), Malt extract and L-methionine (Fig. 3b), Malt extract and NaCl (Fig. 3d), Malt extract and Glucose (Fig. 3f), NaCl and L-methionine (Fig. 3h) were obtained with high degree of hill region indicating lower significant effect of these variables on activity of L-methioninase.

The regression equation is expressed graphically in the form of contour plots which depicts the interaction among the independent variables and their influence on enzyme production. The contour plots might be saddle points, elliptical mounds or rising ridges (Muralidhar et al. 2001). In this study, the elliptical nature of contour plot for the variables of NaCl versus glucose, Casein enzymic hydrolysate versus Malt extract and L-methionine versus Glucose (Fig. 4a, e, g) indicates their significant level of interactions. The contour plots with saddle points were obtained for the variables Malt extract versus L-methionine, Casein enzymic hydrolysate and NaCl, Malt extract versus NaCl, Malt extract versus Glucose, and NaCl versus L-methionine (Fig. 4b–d, f, h) which indicates lower level of significant interaction between these variables. These contour plots are generated by varying the levels of two factors while keeping the third one constant. The contour lines with elliptical shapes show lower effect of variables on response, whereas circular contour lies will show the optimum relationship between variables and response (Muralidhar et al. 2001). These results of contour plots

Table 5 Analysis of variance (ANOVA) for activity of L-methioninase

Sources	DF	Seq SS	Adj SS	Adj MS	F	p
Regression	20	17,972.5	17,972.5	898.62	100.27	0
Linear	5	1954.9	1954.9	390.99	43.63	0
Square	5	14,324.1	14,324.1	2864.81	319.64	0
Interaction	10	1693.5	1693.5	169.35	18.90	0
Residual error	11	98.6	98.6	8.96	*	*
Lack of fit	6	98.6	98.6	16.43		
Pure error	5	0	0	0		
Total	31	1807.1				

DF degree of freedom, *Adj SS* adjusted sum of squares, *Adj MS* adjusted mean squares, *F* Fischer's function, *p* probability value

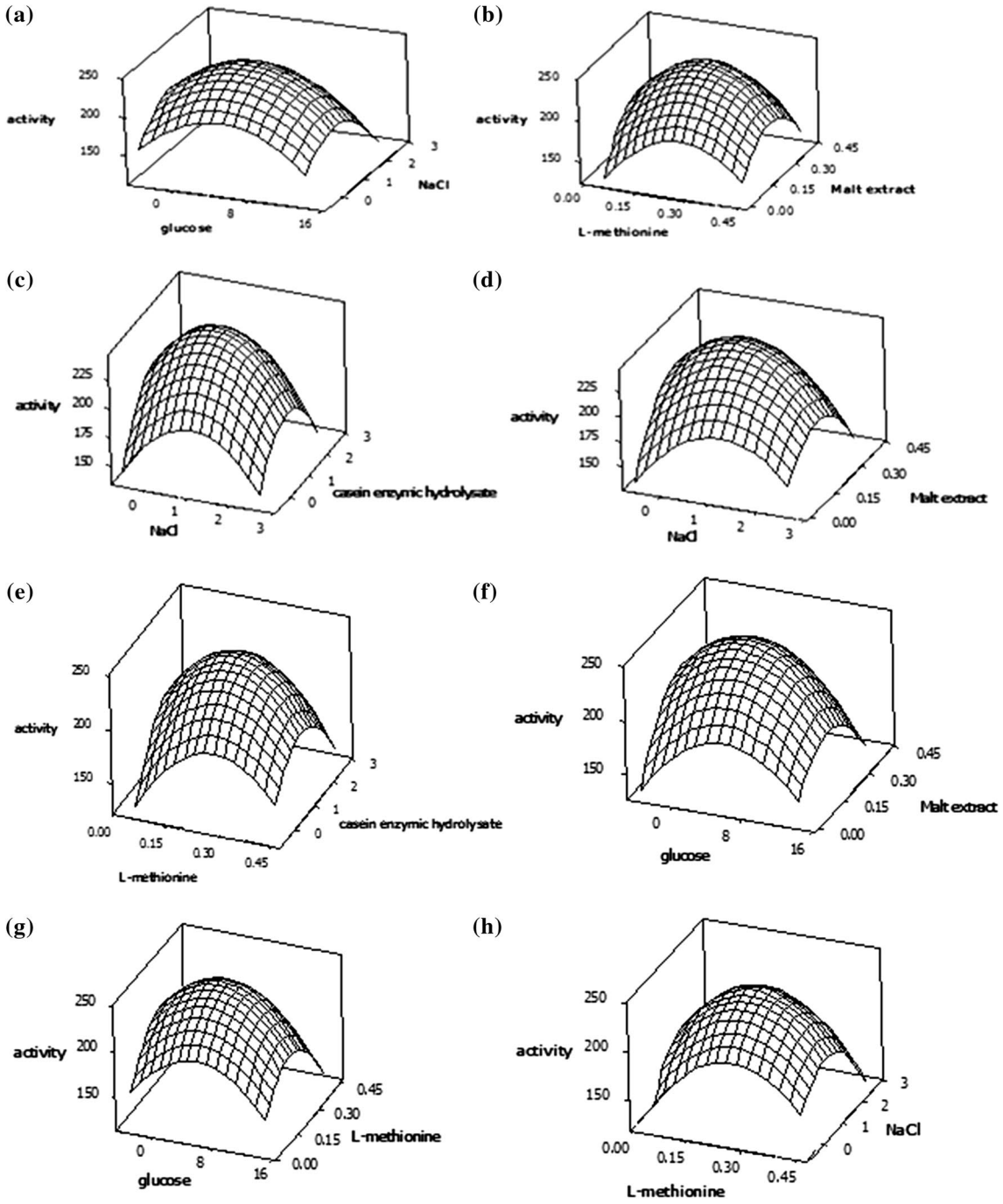

Fig. 3 Three dimensional Response surface plots for interactive effects of variables of media components on the activity of L-methioninase **a** NaCl and glucose, **b** malt extract and L-methionine, **c** casein enzyme hydrolysate and NaCl, **d** malt extract and NaCl, **e** casein enzyme hydrolysate and L-methionine, **f** malt extract and and glucose, **g** L-methionine and glucose, **h** NaCl and L-methionine

confirmed the adequacy of Plackett–Burman design (PBD) and response surface methodology (RSM) for optimization of the media components of L-methioninase. The study of statistical optimization using RSM has been already reported for other amidohydrolase enzyme like L-glutaminase (El-Naggar et al. 2015; Kiruthika et al. 2018; Ye et al. 2013) and L-asparaginase (Baskar and Renganathan 2012; El-Naggar et al. 2015; Varalakshmi and Raju 2013). In these studies, PBD has been employed for screening of media components and CCD has been used for estimation of their optimum values to achieve higher production of enzyme. The validation of experiment has been performed by carrying

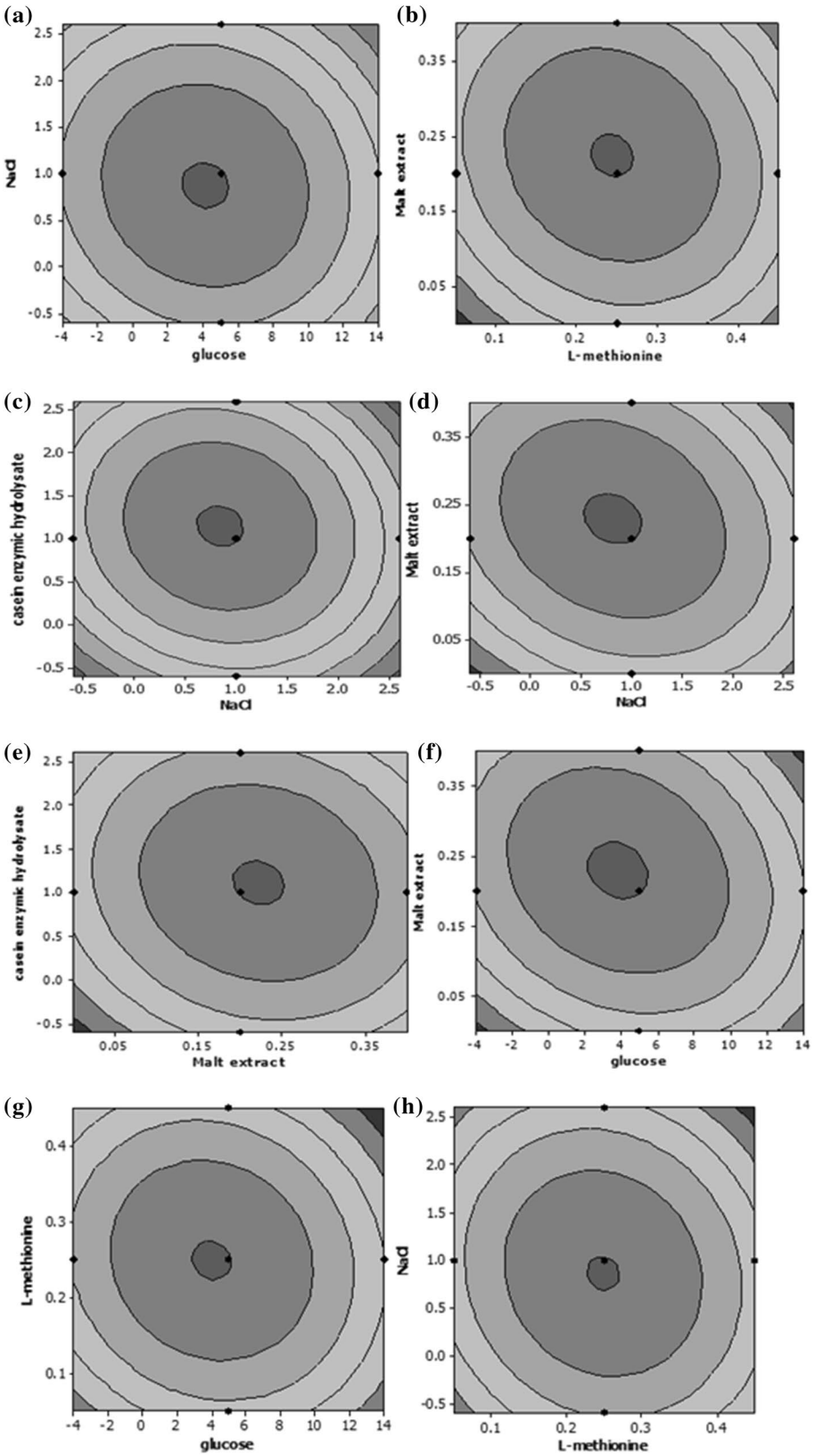

Fig. 4 Contour plots for two dimensional analysis of interactive effects of two variables on the activity of L-methioninase. **a** NaCl and glucose, **b** malt extract and L-methionine, **c** casein enzyme hydrolysate and NaCl, **d** malt extract and NaCl, **e** casein enzyme hydrolysate and L-methionine, **f** malt extract and glucose, **g** L-methionine and glucose, **h** NaCl and L-methionine

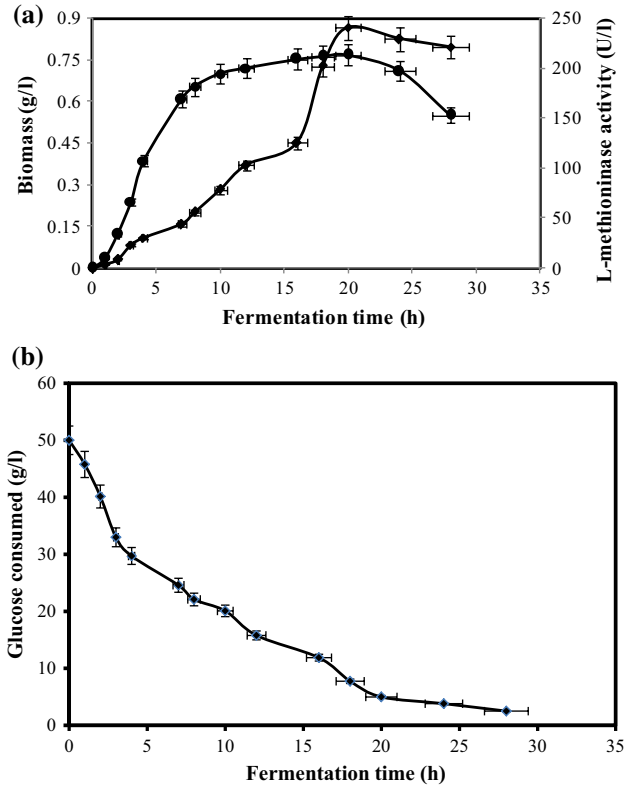

Fig. 5 Time period of submerged fermentation of L-methioninase from *Pseudomonas stutzeri*. **a** Biomass (filled circle) for experimental data and activity (filled square) of L-methioninase, **b** glucose consumption rate profile

out experiment with the optimum level of screened media components. The experimental activity has been obtained as 240.97 U/l after conducting the experiments at 5% glucose, 1% casein enzymic hydrolysate, 0.2% malt extract, 1% NaCl and 0.25% L-methionine. This experimental activity is quite close to the predicted value 240.193 U/l which indicates a strong agreement between predicted data and validated data. The activity of L-methioninase from *P. stutzeri* (MTCC 101) has been obtained as 148.91 by classical method. The statistical optimization strategy results in increment of activity of the L-methioninase by 1.61-fold in compare to classical methodology. These showed the successful implication of these RSM methodologies to analyze the effect of media component on their activity.

The study of kinetic modeling show that the production of L-methioninase has been non growth associated in case of *P. stutzeri* MTCC 101. These microbial cells have not followed the classical bacterial growth pattern for production of L-methioninase. The growth kinetic model showed that these microbes exhibit short lag phase of 6 h, exponential phase up to 20 h and stationary phase up to 28 h under the optimized fermentation condition. The study of production profile (Fig. 5a) and substrate utilization profile (Fig. 5b) for L-methioninase from *P. stutzeri* MTCC 101also validated these experimental results of growth kinetics The effect of fermentation time on the production of L-methioninase was also studied in Fig. 5a and maximum production of L-methioninase was observed at 20 h of fermentation period. The equation of Luedeking–Piret was used for developing model for production kinetic. The fermentation kinetic study of L-methioninase shows that the increase in the biomass concentration leads to the decrease in residual glucose concentration, which implies the utilization of glucose as a substrate for cell growth and their maintenance. These results show that product formation, substrate utilization and kinetic model for biomass provides a good clarification for the fermentation process of L-methioninase.

Conclusion

The study of mathematical modeling for growth kinetic of *P. stutzeri* MTCC 101 and production kinetic of L-methioninase has showed the non growth associated behavior of production of these enzymes.The optimization strategies of Plackett–Burman design (PBD) and central composite design (CCD) on the fermentation conditions has improved the production of L-methioninase by 1.61-fold in compare to classical methodology. These studies confirmed the adequacy of these RSM tools for improving production of L-methioninase which will be relevant study for carrying their plant scale study in bioreactor.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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