ORIGINAL ARTICLE

Optimization of fermentation medium to maximize the production of recombinant human asparaginase in *Escherichia coli* through the statistical design of experiments

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Abstract

Aim: Recombinant human asparaginase (rhASP) from *Escherichia coli* is an important therapeutic enzyme used in the treatment of malignant cancers. Due to such a pivotal role, rhASP production in *E. coli* has drawn great attention of the biopharmaceutical market commercially. The present work aims at the optimization of fermentation medium for the production of rhASP in *E. coli*. **Materials and Methods:** The rhASP yield is optimized using sequential optimization designs comprising one variable at a time, Taguchi design, and central composite designs (CCDs). Taguchi design is used to select the effective variables such as soytone, sodium pyruvate, trace element solution, vitamin solution, and yeast extract, which are further optimized by CCD under response surface methodology. **Results and Discussion:** The CCD design developed a quadratic model with high adequacy for the prediction of rhASP yield with a statistically significant response (R^2 = 97.49% and $P < 0.0001$) toward the variables. **Conclusion:** The CCD results showed that the maximum rhASP yield of 38.4387 µg/mL was achieved with the optimized concentrations of media components comprising 9.0 g/L of soytone, 7.5 g/L of sodium pyruvate, 12.5 mL/L of trace element solution, 12.5 mL/L of vitamin solution, and 9.0 g/L of yeast extract.

Key words: Central composite design, fermentation, inclusion bodies, optimization, recombinant human asparaginase

INTRODUCTION

Recombinant human asparaginase (rhASP) is an important and best-selling chemotherapeutic drug in the world.[1] It (EC 3.5.1.1) is an effective antileukemic drug that catalysis hydrolysis of asparagine into L-aspartic acid and ammonium. This enzyme occurs in microbes, plants, and animals. It is used as a medication and in food manufacture. It is used in the treatment of acute lymphoblastic leukemia, acute myeloid leukemia, and Hodgkin's lymphoma.[2] Lack of the enzyme affects nucleic acid synthesis which is the cause for apoptotic cell death of cancer cell. In the food industry, this enzyme decreases acrylamide levels in fried foods maintaining food security. Considering the commercial importance of the protein, optimization of fermentation media for its production in *Escherichia coli* is the present day concern of the biopharmaceutical industry.

In the development of any fermentation process, fermentation medium is a crucial factor for the effective production of desired products.[3] In fact, the composition of fermentation medium greatly affects the biomass productivity and yield. Optimization of variables in the process is of primary

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importance as it affects the economy and feasibility of the process. Fermentation medium comprises several nutrient factors such as carbon, nitrogen, trace elements, and other nutritional factors. Thus, designing of fermentation medium occupies an important place to achieve target yield.

Statistical approaches offer ideal ways for process optimization studies in biotechnology.[4] They have been applied for the optimization of fermentation processes in the culture of bacteria, animal cell, and fungus. These experimental designs involve classical methods such as one variable at a time (OVAT) also called monothetic analysis where experiments are designed by testing one factor at a time instead of multiple factors simultaneously. It involves a single variable variation keeping the other factors constant. The orthogonal array designs (Taguchi design) accounts the "off-line quality control." It provides the initial information necessary for the primary selection of nutrient sources and best variables in the group. This method fixes a specific region of interest and studies the given system using a set of independent variables. Simultaneously, many more factors can be selected, and the required information can be obtained from very few experimental trials at ease.[5] The basic principle of this method serves as screening filters that examine the effects of many process variables and identify those factors that have major effects on the process.[6]

However, it is difficult to determine the key factors of the fermentation medium and to optimize the cultural conditions with single dimensional traditional methods as they are laborious, time consuming, and incapable of reaching the true optimal point due to the interaction between the variables. The response surface method (RSM) is one of the popularly used optimization procedures mainly developed based on full factorial central composite design (CCD). RSM is a better experimental strategy for seeking optimal conditions for multivariable system that is successfully employed for optimizing the media composition and operating conditions in many bioprocesses. Linear or square polynomial functions are employed to describe the system studied and to explore (modeling and displacing) the optimal experimental conditions.[7] Regression analysis is a modeling technique, which determines the relationship between a dependent (target) and independent variables (predictor). Using the regression methods, the experimental data thus obtained were utilized to build mathematical models which analyze the optimal conditions for the process.[8]

In the present work, optimization of rhASP yield using sequential statistical experimental designs was done for the first time. First (OVAT), the major sources such as carbon and nitrogen sources were screened for formulating the basic nutrient media. This is followed by Taguchi design where other media components which influence the yield were screened, and their effect on rhASP yield was determined. In the final attempt, the interactive effect of selected media components was analyzed, and the best fermentation media for the maximum production of rhASP were designed.

MATERIALS AND METHODS

Formulation of Fermentation Media

Soytone and yeast extract were procured from BD (NJ, USA). Ammonium sulfate ($(NH_4)_2$ SO_4), monopotassium phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), zinc chloride ($ZnCl_2$), cobalt chloride ($CoCl_2.2H_2O$), iron chloride ($FeCl2.6H_2O$), sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), copper sulfate pentahydrate ($CuSO_4.5H_2O$), calcium chloride dihydrate ($CaCl_2.2H_2O$), manganese chloride ($MnCl_2.4H_2O$), sodium citrate dehydrate ($Na_3C_6H_5O_7.2H_2O$), galactose amine ($C_6H_{13}NO_5$), glutamic acid ($C_5H_9NO_4$), and sodium hydroxide (NaOH) were procured from Merck Millipore (Massachusetts, USA). Kanamycin, dextrose, sodium citrate ($Na_3C_6H_5O_7$), pantothenic acid (Vitamin $B_{5)}$, nicotinic acid (Vitamin B_3), D-biotin (Vitamin B7), folic acid (Vitamin B_9), and riboflavin (Vitamin B_2) were procured from thermo fisher scientific (Waltham, Massachusetts, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), tryptone, sodium chloride (NaCl), bovine serum albumin (BSA), sodium pyruvate ($C_3H_3NaO_3$), and phenylmethylsulfonyl fluoride (PMSF) were obtained from HiMedia (Mumbai, India). Hypea is obtained from Kerry Inc., (Ireland). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to assess protein recovery for which the kit was procured from Bio-Rad Laboratory Inc., (California, USA).

Bacterial Strains and Maintenance of Culture

For the expression of the rhASP protein, *E. coli* BL 21 (DE3) pLysS bacterial strain was used. Initially, the gene encoding asparaginase was cloned into *E. coli* DH5 α cells. This increases the plasmid copy number. The cloned gene was subcloned into the expression vector pET 28 b (+) (Invitrogen, USA). For competency in *E. coli* (BL 21 [DE3] pLysS and DH5 alpha) cells, calcium chloride ($CaCl_2.2H_2O$) method was employed.[9] Transformation of the expression vector containing recombinant human ASP gene was done in *E. coli* BL 21 (DE3) pLysS competent cells using heat shock procedure.

Fermentation Process

LB medium (tryptone 1.0 g/L, yeast extract 0.5 g/L, and sodium chloride 1.0 g/L) was used for the inoculation of a single colony of screened recombinant *E. coli* with a protein of interest (rhASP) and grown overnight at 200 rpm and 37°C. The media were prepared as per the experimental design. The pH of the media is adjusted to 7.3 ± 0.1 with 5N sodium hydroxide solution. The overnight grown culture OD_{600} nm was measured and inoculated into the experimental flasks with equal volumes (1%). 1.5 mM IPTG was used for

the induction of all the flasks, and they were further incubated for 4–6 h at 37°C.

Isolation of Inclusion Bodies (IBs)

The cells from the fermented cultures were collected by centrifugation (Sorvall, Thermo Scientific, Massachusetts, USA) at 10°C, 45,000× g for 15 min and stored at −20°C for further analysis. For further use, the frozen cell pellets were thawed and resuspended in wash buffer (1:10). Wash buffer contained glycine 15.0 g/L pH 8.5, Triton X-100 0.5 g/L, ethylenediaminetetraacetic acid (EDTA) 2.0 g/L2.0 g/L sucrose, and NaCl 10.0 g/L. The suspension was then further homogenized at 3000 rpm for 30 min at 30°C, and the pellet was collected by centrifugation at 4°C, 48,000× g for 30 min. The pellets obtained were thoroughly resuspended in lysis buffer (1:10) containing, glycine 20.0 g/L pH 8.5, EDTA 2.0 g/L, NaCl 15.0 g/L, glycerol 0.5%, 1 Mm PMSF, and $Na_2HPO_4.2H_2O$ 0.2 g/L. They were further homogenized at 3000 rpm for 45 min at room temperature and subjected to the sonication (Lark Innovative Pvt. Ltd., India) procedure. Recovery of the IBs was done by the centrifugation at 4°C, 50,000× g for 50 min. The IBs were resuspended in sterile distilled water (500 µL) and separated into different vials for the SDS-PAGE analysis.

Analysis of Expressed rhASP by SDS-PAGE

The content of expressed rhASP was estimated by the Mini Protean II system (Bio-Rad Inc., CA). The gels (12% acrylamide SDS gel) were run at a constant voltage of 75 volts using bromophenol blue as an indicator for the progress of protein in the gel. The gel was washed twice with Milli-Q water (Merck Millipore, Massachusetts, USA) and stained by comassie brilliant blue R-250 for 2 h.[10] The band intensity of the rhASP protein against the standard concentrations of BSA was evaluated by Image J software v1.48.[11]

Statistical Software for Experimental Design and Optimization

The statistical software, Design Expert® v.11 (Stat-Ease, Inc. Minneapolis, USA), is used for the designing experiments and statistical analysis. The CCD design is used to identify how significant factors affect the response and to improve the composition of the medium. In this study of optimization, the selection of factors affecting the yield is demonstrated by the Taguchi design and the interaction effects of variables on the response are analyzed through the CCD.

Selection of Carbon and Nitrogen Sources

Carbon and nitrogen sources are the crucial primary nutrients for any kind of bacterial fermentation. Numerous experiments were carried out to select the best carbon and nitrogen source for the *E. coli* biomass. The effects of primary carbon and nitrogen sources in the medium on rhASP production are examined by the non-statistical approach, OVAT method where the sources were changed one at a time keeping the other growth media components constant. For selecting the carbon source dextrose, glycerol, sucrose, maltose, and fructose were used and for nitrogen source yeast extract, casamino acids, soytone, tryptone, and soy peptone were used. Experiments using various carbon (20 g/L) and nitrogen (5.0 g/L) sources were performed. The OVAT optimization method with various primary nutrient sources is represented in Table 1.

Sorting out of Initial Media Component using Taguchi Design

It is tenacious to identify the numerous variables that affect the response of any system studied. Screening designs help to identify several experimental variables and their interactions present more significant effects. Full or fractional two-level factorial designs are efficient and economical for this objective[12] which evaluates the initial component based on their effect over the yield. The Taguchi experimental design is a powerful tool for designing high-quality system. It ensures good performance in the design stage of processes. This method uses a special design of orthogonal arrays to study the entire parameter space with a small number of experiments.[13] A Taguchi design was preferred taking into consideration of L4 (2^3), where four indicates design run used to estimate main effects from a two level and three-factor design. Taguchi design is saturated orthogonal assay, which provides no interaction. In total 11 variables (sodium pyruvate, yeast extract, vitamin solution, soytone, trace element solution, glutamic acid, galactose amine, Hypea, and sodium citrate) were considered including two dummy variables with respect to 12 runs were performed with two levels [Table 2]. Each variable was designated at the high value (+) and the low value (−). All the experiments were carried out in triplicates, and the average of rhASP yield was used as the response. The effect of individual variables on the rhASP yield was evaluated by the following equation (Eq. 1).

$$E = (\sum M+ - \sum M-)/N \quad (1)$$

Where, E is the main effect of the variable on biomass, M + is the higher level, M is the lower level, and N represents the total number of trails, respectively.

Pre-assigned CCDs under RSM

A response surface design helps to understand and optimize a response. To identify the suitable concentrations of variables which enhance the rhASP yield experiments were preformed according to pre-assigned CCD under RSM.

RSM allows to optimize a response, which is influenced by several independent variables. In the present work, a CCD was performed to investigate the nature of the response surface in the optimum region, five level (relatively low, low, center, high, and relatively high) coded (−α, −1, 0, +1, and +α) was taken for the five factors with eight central point and axial points at a distance of α. Here, the distance of α from the central point to the axial point indicate that it an orthogonal CCD, which ensures no correlation between the responses. The experimental CCD consists of one replicate fractional and one replicate (axial star) point with a total of 50 runs with five factors was conducted [Table 3]. All the experiments were carried out in triplicate, and the average (rhASP) yield was considered as the response. The following polynomial equation (Eq. 2) explains the relationship between dependent variables and independent variables:

Where Y is the predicted dependent response, β_i is the linear coefficient, β_{ii} is a squared coefficient, β_{ij} is interaction coefficient, X_i^{th} independent variable, Xi^2 is squared effect, and $x_i x_j$ is interaction effects.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

RESULTS AND DISCUSSION

Selection of Primary Nutrients using OVAT Method

OVAT is a classical and non-statistical method to screen the factor that effects the response most. This method is used to select the best primary nutrient source (carbon and nitrogen) for good rhASP yield. Table 1 shows that the biomass yield values obtained with various carbon sources, namely dextrose, glycerol, sucrose, maltose, and fructose were found to be 12.01, 11.12, 10.61, 7.99, and 8.46 µg/mL, respectively, and biomass yields with various nitrogen sources such as yeast extract, casamino acids, soytone, tryptone, and soy peptone were found to be 10.12, 9.52, 11.09, 9.36, and 10.78 µg/mL, respectively. The results shown that dextrose from carbon source and soytone from nitrogen source are found to be effective and selected for further experimental studies.

Screening of Effective Variables using Taguchi Experimental Design

The Taguchi experimental design is a powerful tool for ensuring good performance in the design stage of processes. This method uses a special design of orthogonal arrays to

Table 1: Variables showing the primary nutrient components used in the one-variable-at-a-time method

Carbon source (20 g/L)	Carbon content (%)	Biomass (µg/mL)	Nitrogen source (5 g/L)	Nitrogen content (%)	Biomass (µg/mL)
Dextrose	40.00	12.01	Yeast extract	11.4	10.12
Glycerol	39.20	11.12	Casamino acids	8.0	9.52
Sucrose	42.10	10.61	Soytone	9.2	11.09
Maltose	42.10	7.99	Tryptone	13.5	9.36
Fructose	40.00	8.46	Soy peptone	10.0	10.78

Table 2: Experimental design using Taguchi showing actual values along with experimental results

Run	A	B	C	D	E	F	G	H	J	K	L	Response (µg/mL)
1	0.4	0.35	4.2	0.5	2.5	1.4	0.14	0.27	0.22	0.18	0.26	8.75
2	0.4	0.35	3.1	0.35	4	1.1	0.21	0.41	0.22	0.18	0.26	15.75
3	0.6	0.35	4.2	0.35	4	1.4	0.21	0.27	0.22	0.29	0.35	19.5
4	0.6	0.35	3.1	0.35	2.5	1.1	0.14	0.27	0.31	0.18	0.35	17
5	0.4	0.15	3.1	0.5	4	1.4	0.21	0.27	0.31	0.18	0.35	10.7
6	0.4	0.15	3.1	0.35	2.5	1.4	0.14	0.41	0.22	0.29	0.35	6
7	0.6	0.15	4.2	0.35	2.5	1.4	0.21	0.41	0.31	0.18	0.26	10.5
8	0.6	0.15	4.2	0.5	4	1.1	0.14	0.41	0.22	0.18	0.35	16
9	0.6	0.35	3.1	0.5	4	1.4	0.14	0.41	0.31	0.29	0.26	14.25
10	0.6	0.15	3.1	0.5	2.5	1.1	0.21	0.27	0.22	0.29	0.26	15.75
11	0.4	0.35	4.2	0.5	2.5	1.1	0.21	0.41	0.31	0.29	0.35	9.25
12	0.4	0.15	4.2	0.35	4	1.1	0.14	0.27	0.31	0.29	0.26	14.25

A to L A-sodium pyruvate, B-Hypea, C-dummy 1, D-vitamin solution, E-galactose amine, F-glutamic acid, G-soytone, H-yeast extract, J-trace element solution, K-dummy 2 and L-sodium citrate

Table 3: Central composite design and results with the actual and coded values

Run	Soytone (g/L)		Sodium pyruvate (g/L)		Trace element solution (mL/L)		Vitamin solution (mL/L)		Yeast extract (g/L)		rhASP yield (µg/mL)
	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual	
1	1	15	−1	3	1	20	−1	5	−1	3	27.67
2	1	15	1	12	−1	5	1	20	−1	3	28.67
3	1	15	−1	3	1	20	−1	5	1	15	26.45
4	1	15	−1	3	1	20	1	20	1	15	24.08
5	−1	3	−1	3	1	20	1	20	1	15	27.78
6	−1	3	−1	3	−1	5	1	20	−1	3	23.43
7	1	15	−1	3	−1	5	−1	5	−1	3	29.45
8	0	9	0	7.5	0	12.5	0	12.5	0	9	38.23
9	0	9	0	7.5	0	12.5	0	12.5	0	9	39.51
10	0	9	−2.37841	1.5	0	12.5	0	12.5	0	9	30.61
11	0	9	0	7.5	0	12.5	−2.37841	2.5	0	9	35.65
12	0	9	2.37841	18.2029	0	12.5	0	12.5	0	9	28.38
13	−1	3	−1	3	1	20	−1	5	1	15	25.76
14	−1	3	−1	3	1	20	−1	5	0	3	24.19
15	0	9	0	7.5	2.37841	30.338	0	12.5	0	9	28.78
16	1	15	−1	3	−1	5	−1	5	1	15	26.67
17	−1	3	1	12	1	20	−1	5	−1	3	27.12
18	−1	3	1	12	−1	5	1	20	−1	3	24.68
19	−1	3	−1	3	−1	5	−1	5	−1	3	24.77
20	0	9	0	7.5	0	12.5	0	12.5	2.37841	23.2705	21.53
21	−1	3	1	12	−1	5	−1	5	1	15	30.34
22	0	9	0	7.5	0	12.5	0	12.5	−2.37841	1.5	34.12
23	1	15	1	12	1	20	1	20	−1	3	28.34
24	−1	3	−1	3	−1	5	−1	5	1	15	30.32
25	0	9	0	7.5	0	12.5	0	12.5	0	9	37.34
26	1	15	1	12	1	20	−1	5	−1	3	32.89
27	−1	3	1	12	−1	5	1	20	1	15	26.95
28	0	9	0	7.5	0	12.5	0	12.5	0	9	38.84
29	0	9	0	7.5	−2.37841	2.5	0	12.5	0	9	37.22

(Contd..)

Table 3: *(Continued)*

30	1	15	1	12	−1	5	−1	5	−1	3	33.78
31	0	9	0	7.5	0	12.5	0	12.5	0	9	39.65
32	0	9	0	7.5	0	12.5	0	12.5	0	9	38.43
33	1	15	−1	3	1	20	1	20	−1	3	25.64
34	−1	3	1	12	1	20	−1	5	1	15	28.99
35	0	9	0	7.5	0	12.5	2.37841	30.3381	0	9	26.82
36	0	9	0	7.5	0	12.5	0	12.5	0	9	38.1
37	−1	3	1	12	1	20	1	20	1	15	27.43
38	−1	3	−1	3	1	20	1	20	−1	3	23.47
39	−1	3	1	12	−1	5	−1	5	−1	3	30.54
40	2.37841	23.2705	0	7.5	0	12.5	0	12.5	0	9	25.48
41	−1	3	−1	3	−1	5	1	20	1	15	26.51
42	0	9	0	7.5	0	12.5	0	12.5	0	9	39.54
43	1	15	1	12	−1	5	1	20	1	15	27.79
44	1	15	1	12	1	20	1	20	1	15	27.85
45	−1	3	1	12	1	20	1	20	−1	3	26.68
46	1	15	−1	3	−1	5	1	20	1	15	25.94
47	1	15	1	12	1	20	−1	5	1	15	27.29
48	−2.37841	2.5	0	7.5	0	12.5	0	12.5	0	9	33.45
49	1	15	−1	3	−1	5	1	20	−1	3	25.87
50	1	15	1	12	−1	5	−1	5	1	15	29.56

study the entire parameter space with a small number of experiments. Taguchi conceptualizes an experiment that derives maximum information in a short time especially in product development and industrial engineering. The experiment consisted of 11 variables including two dummy variables. The variables, which affected the rhASP yield (response) is found to be soytone, sodium pyruvate, trace element solution, vitamin solution, and yeast extract as shown in Table 2. The ANOVA results (data not shown) indicate the significant *F*-value (365.87) and "Prob>*F*" value (<0.05). Figure 1 shows the Pareto chart explained by Taguchi design and from the figure it can be seen that the five variables are projected more contribution toward the rhASP yield improvement when compared to the others. Therefore, the previously mentioned variables were taken forward for further optimization using CCD.

Optimization of Suitable Concentration and Implication of Variables on rhASP Yield using CCD

The selected variables from the Taguchi design are further optimized for a suitable concentration of variables using CCD. CCD is used to predict the best response and analyze the variance. The five variables such as soytone, sodium pyruvate, trace element solution, vitamin solution, and yeast extract were selected from the Taguchi design and further investigated for the maximum production of rhASP yield by the superior optimization stage, CCD. Table 3 presents the rhASP yield from the experiments of CCD, and the quadratic model is selected based on the results. Table 4 shows the degrees of freedom (df) for the quadratic model and recommended that the lack of fit test is valid with 22 lack of fit df and seven pure error df.

The second-order polynomial mathematical model (Eq. 3), empirically was related to rhASP yield with the all variables A, B, C, D, and E. The regression equation for rhASP yield in terms of coded factors can be used to make predictions about the response for given levels of each factor. The application of CCD is expressed as a coded equation, and it is useful for identifying the relative impact of the factors by comparing the factor coefficients.

$$\text{rhASP yield} = 38.4387 + 0.64349 \times A + 1.37596 \times B + -0.483213 \times C + -1.01836 \times D + 0.0450375 \times E + 0.246875 \times AB + -0.04375 \times AC + -0.14 \times AD + -1.12125 \times AE + 0.06875 \times BC + -0.29875 \times BD + -0.485 \times BE + 0.515625 \times CD + -0.101875 \times CE + 0.393125 \times DE + -2.57932 \times A^2 + -2.42205 \times B^2 + -1.4374 \times C^2 + -1.66342 \times D^2 + -2.96176 \times E^2 \quad (3)$$

Table 4: Degrees of freedom for the model

Model	20
Residuals	29
Lack of fit	22
Pure error	7
Corr. total	49

Where, rhASP is the response, and A, B, C, D, and E are the actual values of soytone, sodium pyruvate, trace element solution, vitamin solution, and yeast extract, respectively, whereas the variables AB for soytone – sodium pyruvate, AC for soytone – trace element solution, AD for soytone – vitamin solution, AE for soytone – yeast extract, BC for sodium pyruvate – trace element solution, BD for sodium pyruvate – vitamin solution, BE for sodium pyruvate – yeast extract, CD for trace element solution – vitamin solution, CE for trace element solution – yeast extract, and DE represents vitamin solution – yeast extract.

Table 5 represents the analysis of variance (ANOVA) and their mean square values of the rhASP. According to the result, the model-fitting step was carried out using the Design-Expert® software package, which employs the least squares procedure to compute the model coefficient. From Table 5, it can be seen that the model *F*-value of 56.21 implies the model is significant.

The greater *F*-value reflects an adequate variation in the mean data and that the estimated variable factor effects are real.[14] There are only a 0.01% chances that an F-value is large which could occur due to noise. The "Prob>*F*" P < 0.0500 indicate model terms are significant. In this case, A, B, C, D, AE, BE, CD, DE, A^2, B^2, C^2, D^2, and E^2 are significant model terms. The lack of fit *F*-value of 1.75 implies the lack of fit is not significant relative to the pure error. There is a 22.92% chance that a lack of fit *F*-value is large and could occur due to noise. The non-significant lack of fit is suggestable for the significant model, and therefore, the present selected quadratic model had insignificant lack-of-fit [Table 5].

The regression calculations were performed to fit the polynomial models based on the selected variables from response data. The summary of statistics and lack of a fit test for the response (rhASP) are presented in Table 6. The goodness-of-fit for the quadratic model is investigated by

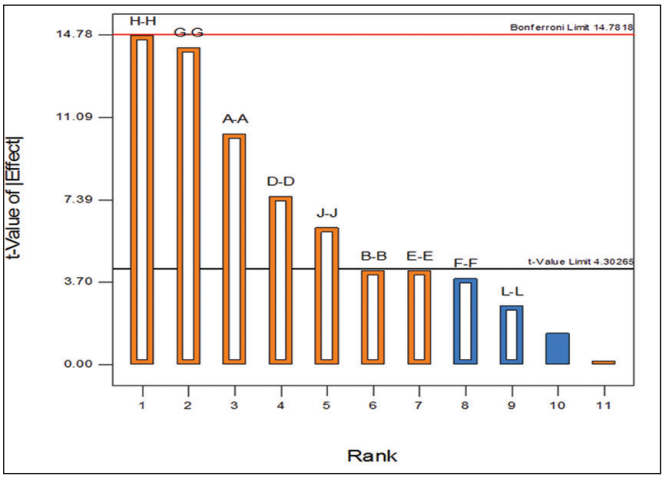

Figure 1: Pareto graph of the variables developed by the Taguchi design. H-H Yeast extract, G-G soytone, A-A sodium pyruvate, D-D vitamin solution, J-J trace element solution, B-B Hy-Pea, E-E galactose amine, F-F glutamic acid, and L-L sodium citrate

correlation coefficient R^2 = 0.9749, which indicates the model is reliable.

The fit statistics for the ANOVA model is presented in Table 7. From Table 7, it can be seen that the predicted $R^2$of 0.9230 is in reasonable agreement with the adjusted R^2 of 0.9575; i.e., the difference is <0.2. The Adeq precision measures the signal-to-noise ratio. A ratio >4 is desirable, and in the present study, the ratio of 24.8080 indicates an adequate signal. The coefficient of variation is 3.48% and this lower value indicates a better precision and reliability of the experimental design.

The predicted R^2 = 92.30% is in reasonable agreement with the adjusted R^2 = 95.75%, which indicates a high significance of the model.[15,16] Adjusted R^2 denotes the quantity of variations that can be explained by the model.

The residuals versus experimental run order provide the glance for hidden variables, which may have an effect on the response (rhASP yield) during the course of experiments [Figure 2]. The plot presents a random scatter and indicates a time-related variable lurking in the background. Nevertheless, the maximum yield of rhASP is found to be occupied in-between 1 and -1 of the extremely studentized residuals.

Table 5: ANOVA for response surface quadratic model (Type III– Partial)

Source	Sum of squares	df	Mean Square	*F*-value	*P* value	Significance
Model	1205.29	20	60.26	56.21	<0.0001	Significant
A-Soytone	14.49	1	14.49	13.52	0.0010	
B-Sodium pyruvate	68.81	1	68.81	64.18	<0.0001	
C-Trace element solution	8.49	1	8.49	7.91	0.0087	
D-Vitamin solution	37.69	1	37.69	35.15	<0.0001	
E-Yeast extract	0.0727	1	0.0727	0.0678	0.7964	
AB	1.95	1	1.95	1.82	0.1879	
AC	0.0612	1	0.0612	0.0571	0.8128	
AD	0.6272	1	0.6272	0.5850	0.4505	
AE	40.23	1	40.23	37.52	<0.0001	
BC	0.1513	1	0.1513	0.1411	0.7099	
BD	2.86	1	2.86	2.66	0.1135	
BE	7.53	1	7.53	7.02	0.0129	
CD	8.51	1	8.51	7.94	0.0086	
CE	0.3321	1	0.3321	0.3098	0.5821	
DE	4.95	1	4.95	4.61	0.0402	
A^2	209.93	1	209.93	195.81	<0.0001	
B^2	194.04	1	194.04	180.99	<0.0001	
C^2	68.34	1	68.34	63.74	<0.0001	
D^2	91.52	1	91.52	85.37	<0.0001	
E^2	284.61	1	284.61	265.47	<0.0001	
Residual	31.09	29	1.07			
Lack of Fit	26.30	22	1.20	1.75	0.2292	Insignificant
Pure Error	4.79	7	0.6839			
Cor. Total	1236.38	49				

Table 6: Summary of statistics for best fit models for the rhASP yield

Source	Standard deviation	R^2	Adjusted R^2	Predicted R^2	PRESS	Sum of squares	df	Mean square	*F*-value	*P* value
Linear	5.02	0.1024	0.0004	-0.0492	1297.24	1105.01	37	29.87	43.67	<0.0001
2FI	5.54	0.1567	-0.2153	-0.3583	1679.38	1037.82	27	38.44	56.21	<0.0001
Quadratic	1.04	0.9749	0.9575	0.9230	95.14	26.3	22	1.2	1.75	0.2292
Cubic	0.996	0.9896	0.9607			8.11	6	1.35	1.98	0.1968
Pure error						4.79	7	0.6839		

Figure 3 presents a plot between the predicted versus actual response in the experimental runs. The color points between the 21.53 and 39.65 µg/mL were scattered over the plot with predicted R^2 = 0.9230, which resembles the adequacy in the experimental results.

The three-dimensional (3D) response surface and two-dimensional (2D) contour plots were developed to find out the optimal concentrations of the factors and their synergistic effect on the response (rhASP yield). The 3D and 2D plots also provide the cyclic interactions between the two factors and additionally, individual response is generated to present the suitable concentration of variable (media component).

The 2D and 3D plots for the five variables and their response were presented in Figure 4a-j. The main aim of the response surface model is to track efficiently for the optimum concentration of the media components such that the response is maximized.[17] Each counter plot portrays an infinitive number of combinations of the two independent variables while the other variable is maintained at the center point.

Figure 4a presents the 2D and 3D plots from the interaction between the soytone and sodium pyruvate concentrations. The results indicate that with increase in the concentration of the soytone from 2.67703 g/L and sodium pyruvate from 1.56662 g/L, the rhASP yield also increased from 29.2141 µg/mL and the maximum rhASP protein yield of 37.4556 µg/mL were obtained at 13.1881 g/L of soytone and 10.8188 g/L of sodium pyruvate. The increment attributed the presence of secondary carbon source (sodium pyruvate) and nitrogen source (soytone) concentration in the culture medium, which facilitates the growth of the cells.

Figure 4b depicts the 2D and 3D plots of soytone and trace element solution. From the figure, it can be seen that the maximum rhASP yield of 37.0347 µg/mL was achieved with the concentrations of soytone and trace element solution of 12.4085 g/L and 17.3737 mL/L, respectively. The lowest rhASP yield of 16.5453 µg/mL was recorded with the higher concentrations of trace element solution (23.1042 mL/L) and soytone (29.9587 g/L).

Figure 4c illustrates the interaction between soytone and vitamin solution, which reported elliptical graphs with the maximum yield of 38.4387 µg/mL at the center of the elliptical boundaries with the soytone and vitamin solution concentrations of 9.0 g/L and 12.5 mL/L, respectively.

Table 7: Fit statistics for ANOVA

Standard deviation	1.04	R^2	0.9749
Mean	29.77	Adjusted R^2	0.9575
C. V. %	3.48	Predicted R^2	0.9230
		Adeq. Precision	24.8080

Figure 4d is the counter and response surface plots for the rhASP yield as a function of soytone and yeast extract. From the counterplot, it can be seen that the maximum production of rhASP was found to be 38.705 µg/mL with the similar concentrations (9.0 and 9.0 g/L) of soytone and yeast extract with strong positive interaction between the test variables. From the response plot, it can be seen that rhASP yield was increased with both concentrations and the maximum yield of 38.4 µg/mL was achieved at the intersection of 9.49 g/L of soytone and 9.18 g/L of yeast extract, respectively.

Figure 4e shows the effect of sodium pyruvate and trace element solution on rhASP yield. The results indicate that the maximum yield of 38.705 µg/mL was obtained with sodium pyruvate and trace element solution concentrations of 7.5 g/L and 12. 5 mL/L, respectively. The counter and response surface plots between the sodium pyruvate and vitamin solution represent an elliptical graph [Figure 4f], which indicates that the significant rhASP yield of 38.7944 µg/mL achieved with the sodium pyruvate and vitamin solution concentrations of 8.6 g/L and 9.2 mL/L, respectively.

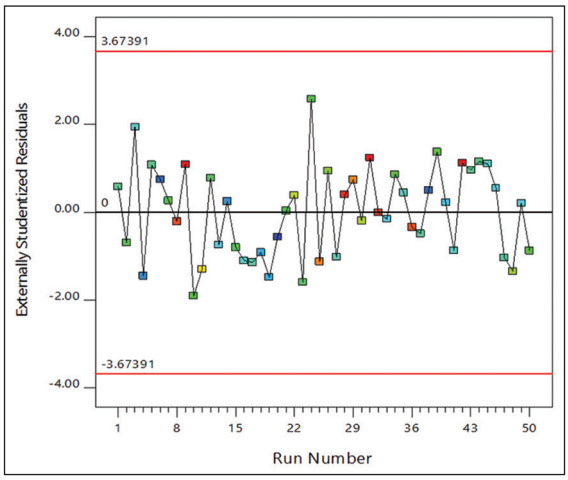

Figure 2: Residual plot against the experiment order

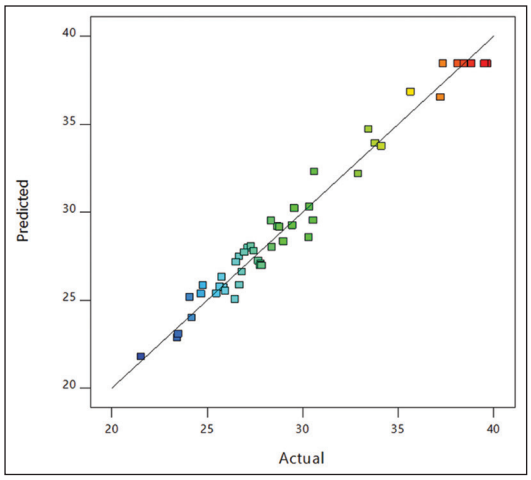

Figure 3: Predicted versus actual response plot with the experimental runs

The counter graph [Figure 4g] of the sodium pyruvate and yeast extract exhibited the maximum yield of 38.4387 µg/mL with the concentrations of 7.5 and 9.0 g/L. Figure 4h represents the elliptical response surface plot of the trace element solution and vitamin solution. From the plots, it can be observed that the lower concentrations of trace element (2.8 mL/L) solution and vitamin solution (2.7 mL/L) achieved the maximum rhASP yield of 36.0425 µg/mL. The suitable concentrations (9.7 and 7.04 mL/L) of both the solutions have achieved the highest rhASP yield of 38.4204 µg/mL. Nevertheless, the shape of the response surface curve shows a less significant interaction between the two tested variables.

The interaction between the trace element solution and yeast extract was presented in Figure 4i. The highest rhASP yield was attained with the optimal concentrations of trace element solution and yeast extract was found to be 12.5 mL/L and 9.0 g/L. The umbrella-shaped response curve [Figure 4j] also illustrates the strong positive interaction between the variables such as vitamin solution and yeast extract, reporting a higher rhASP yield of 38.4288 µg/mL with the suitable concentrations of 12.5 mL/L and 9.0 g/L.

The results of CCD optimization studies indicate that the rhASP yield of 38.7 µg/mL with optimized production media which is approximately two-fold higher than the yield achieved by the Taguchi design (19.5 µg/mL). The results achieved a significant rhASP yield with the CCD using suitable media component concentrations. This rhASP yield achievement is due to the increment in the cell biomass, as biomass is directly proportionate to the IBs, which resembles the product of interest. The results also revealed that the media components such as soytone, sodium pyruvate, trace element solution, vitamin solution, and yeast extract are essential media components for the growth and protein production as well.

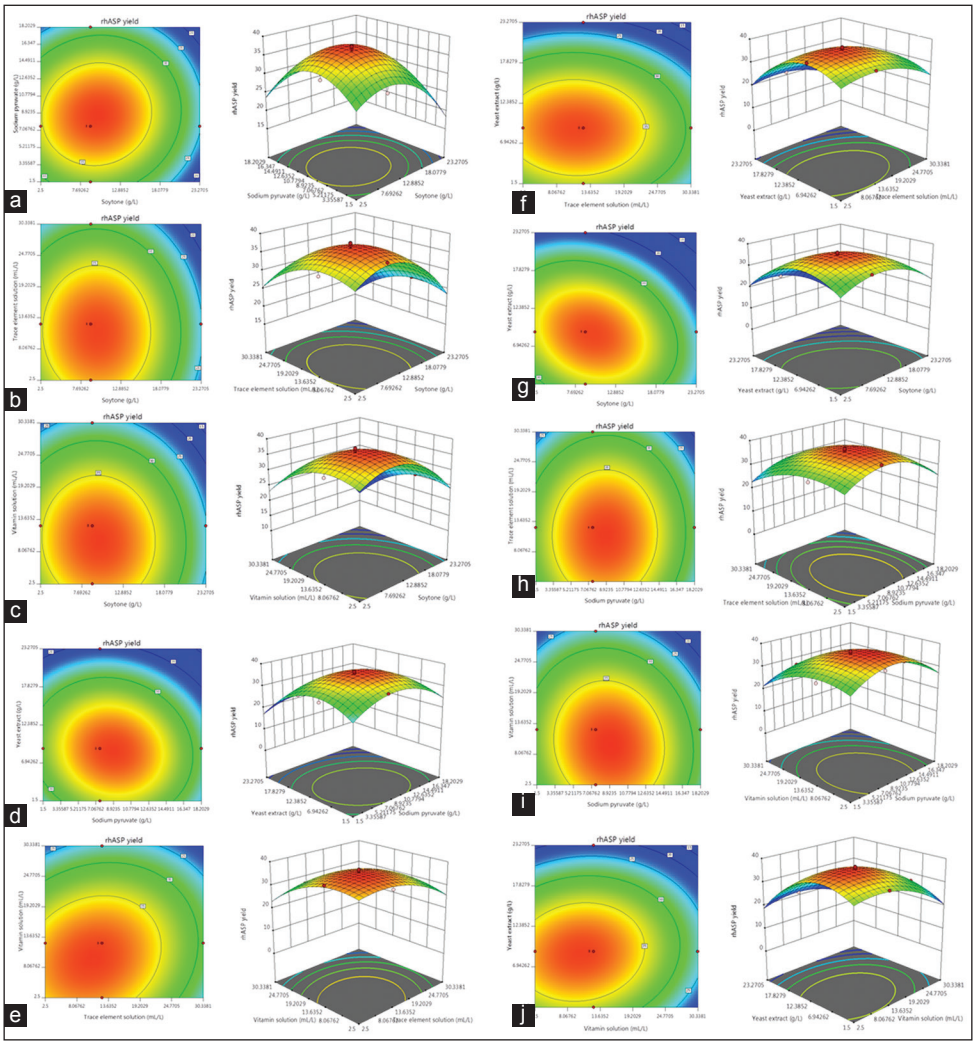

Figure 4: Two-dimensional (2D) and three-dimensional (3D) response plots showing the interaction of variables on rhASP production by *Escherichia coli*. (a) Interaction of soytone – sodium pyruvate (AB); (b) interaction of soytone – trace element solution (AC); (c) interaction of soytone – vitamin solution (AD); (d) interaction of soytone – yeast extract (AE); (e) interaction of sodium pyruvate – trace element solution (BC); (f) interaction of sodium pyruvate – vitamin solution (BD); (g) interaction of sodium pyruvate yeast extract (BE); (h) interaction of trace element solution – vitamin solution (CD); (i) interaction of trace element solution – yeast extract (CE); and (j) represents vitamin solution – yeast extract (DE)

VALIDATION EXPERIMENT

The response was set up to the maximum to confirm the predicted optimal response and level with the suitable concentrations of media components. The post-run analysis and confirmation run led to rhASP yield at the level of 38.4387 ± 1.0 µg/mL that was adequately closer to the predicted rhASP yield. Therefore, the current model is found to be acceptable.

CONCLUSION

In perspective to achieve the maximum rhASP yield from the fermentation of *E. coli*, various strategies have been adopted to optimize the fermentation medium. The present study describes the optimization of media for the maximum production of rhASP through OVAT, Taguchi and followed by CCD under RSM. The media components, namely soytone, sodium pyruvate, trace element solution, vitamin solution, and yeast extract are selected from the Taguchi design, and the effective concentrations of the media components have been optimized through CCD. The interaction effects of the media components are also demonstrated with counter and response surface models. The maximum predicted rhASP yield of 38.4387 µg/mL is achieved from the RSM. The optimized fermentation media would be useful for the effective production of rhASP from IBs at laboratory and industry as well.

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