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Quality by design approach implementation in media development for the production of therapeutic enzyme

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ABSTRACT

L-asparaginase is a highly demanding therapeutic enzyme. This enzyme has wide applications in food, therapeutic, and biosensor industries. Since L-asparaginase is a chemotherapeutic agent/enzyme, it is important to maintain the final product quality. Nowadays, FDA and other regulatory authorities emphasize having quality by design (QbD) based product development. Because of this requirement, QbD principles were implemented in this study for optimization of the L-asparaginase production by *Bacillus subtilis* THARAKA. QbD was implemented through the design of the experiment strategy. Initially, the Plackett–Burman design (PBD) was used to screen the nutrients which have a significant effect on the enzyme. Among 11 studied nutrients, 3 components glucose, L-asparagine, and $(NH_4)_2SO_4$ were screened by using PBD, and their concentrations were further optimized by using response surface methodology. Screened nutrients and process parameters were optimized using the central composite design (CCD). By sequential optimization methods such as PBD followed by CCD a 28% L-asparaginase production was enhanced.

1. INTRODUCTION

L-Asparaginase (EC 3.5.1.1; L-asparagine amino hydrolase) is an enzyme that hydrolyzes the L-asparagine to L-aspartic acid and ammonia [1,2]. It belongs to the amidase group. Currently, L-asparaginase has gained industrial attention due to its potent market in various sectors. This enzyme has been widely used in the medical as well as food industry [3]. L-asparaginase has a high therapeutic value as a chemotherapeutic agent and represented a milestone in the field of medicine due to the ratio of acute lymphoblastic leukemia pediatric patients who achieve complete remission after treatment incorporating L-asparaginase (93%) and due to its selectivity against the tumor cells [4]. It is also being administered to patients to treat Hodgkin's disease, melanosarcoma, etc. as an individual medicine or in combination with other medicines to improve the therapeutic efficacy and suppress the deleterious effects caused by drugs [3,5,6]. The neoplastic cells in the body consume a high amount of L-asparagine from the bloodstream for their growth [7]. Usually, the cancer cells lack the L-asparagine synthetase (EC 6.3.5.4), which is a key enzyme for the synthesis of L-asparagine [8,9]. On the other hand, normal healthy cells have L-asparagine synthetase and can synthesize the L-asparagine. By intravenous administration of L-asparaginase, it breaks downs the free L-asparagine in the bloodstream which tremendously reduces the L-asparagine concentration in the blood [10]. Due to the unavailability of L-asparagine, the cancer cells undergo starvation which compromises their cellular functions and leads to cell death [11]. The selective death of the neoplastic cells by administering L-asparaginase makes this enzyme of higher importance in the treatment of acute lymphocytic leukemia.

In the food industry, L-asparaginase is used to reduce the acrylamide (carcinogenic toxicant) formation in baking and French fries [12]. At high temperatures, L-asparagine reacts with carbohydrates and forms acrylamide [13]. The addition of L-asparaginase to starchy foods before processing reduces the L-asparagine levels and subsequently inhibits acrylamide formation [12]. Recent studies have shown that L-asparaginase has good antioxidant properties [14]. Based on the applications of

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this enzyme, it is gaining industrial attention, and in near future, several folds of L-asparaginase production could increase to meet the market demands [15].

L-asparaginase could be obtained from various biological sources, however, microbial specifically bacterial L-asparaginases gained industrial attention because of their ease of cultivation and large-scale production in a short period [16]. Generally, at an industrial scale, L-asparaginase was produced by submerged fermentation (SMF) [1,17]. In SMF, the composition of medium and culture conditions such as temperature, pH, mixing, etc., play a vital role in bacterial growth as well as production formation [18]. Currently, there is no definitive medium for L-asparaginase production so it is important to the development of a balanced nutrient media as well as the setting of environmental conditions is mandatory to achieve a higher amount of L-asparaginase from particular bacteria [19].

Selection of proper nutrients and their concentrations as well as environmental factors are major challenges for scientists and industrial persons in the upstream process [17]. Media plays a vital role in the growth of bacteria, process productivity, and final product quality. Optimization of media and USP conditions is necessary for achieving the goals. One-factor-at-a-time (OFAT) is a traditional strategic method of optimization. In this method, one factor changes and remaining the factors are kept constant [4]. OFAT has many drawbacks so nowadays this method is less preferred. Design of experiments (DOE) is a statistical method, which has many advantages over the OFAT method [17]. Nowadays, many industries are following the DOE method of optimization [20]. DOE is extensively using the procedure for the employment of the "Quality by design (QbD)" approach in Pharma and Bio-pharma industries [21]. According to regulatory authorities, the FDA also suggests QbD implementation in the industries [22]. According to the ICH Q8 guidelines, QbD is a systematic approach, which emphasizes the building of product quality by better understanding the process itself [20,23]. QbD includes risk assessment, DOE, and process analysis tools, and it provides superior results with a smaller number of experiments [24]. The QbD includes screening as well as optimization designs [22]. With the help of QbD, individual variables' effects as well as their interaction influence on the product could be assessed [20,22]. This study aimed to screen the various nutrients that help to enhance L-asparaginase production and optimize the screened nutrients concentration along with environmental conditions based on QbD principles that could enhance the L-asparaginase production by isolated bacteria.

2. MATERIALS AND METHODS

2.1. Microorganisms and Cultural Conditions

Isolated *Bacillus subtilis* THARAKA was employed for this study. The microorganism isolation and cultural conditions were given in elsewhere. The culture was stored on M9 medium slants at 4°C, and every 7 days the culture was subcultured on fresh media [10].

2.2. Production of L-Asparaginase in SMF

After incubation, the L-asparaginase activity was determined in cell-free broths. All experiments were performed in triplicate (three aliquots). The data presented in this investigation were the average results of all the above experimentations.

SMF experiments were carried out in 250 ml conical flasks which contain 100 ml of sterile M9 medium. The media composed of (g/l) glucose-2.0; L-asparagine-5.0; Na_2HPO_4-6.0; KH_2PO_4-3.0; NaCl-0.5; $CaCl_2$-0.12, and $MgSO_4$-2.46. Each sterile flask receives 2% of 24 hours aged inoculum having 0.8 absorbances at 600 nm. The inoculated flasks were incubated at 37°C in an orbital shaker at 200 rpm. After 24 hours of incubation, the samples were collected and centrifuged at 5,000 g, the supernatant was collected and analyzed for L-asparaginase activity. In subsequent experiments, the nutrients and culture conditions were changed as per DOE experimental plan.

2.3. Estimation of L-Asparaginase Activity

L-asparaginase activity was estimated by the spectrophotometric method developed by Hymavathi *et al* [10]. The enzyme activity was determined by measuring the amount of ammonia liberated during the reaction, and its absorbance was measured at 436 nm using a UV-Visible spectrophotometer. The assay mixture contains 0.1 ml of obtained enzyme solution, 1.0 ml of 0.5 M Tris buffer pH 8.6, and 183 mM of asparagine as a substrate in a final volume of 2.2 ml. The enzyme and substrate reaction mixture were incubated at 37°C for 30 minutes. After the incubation, the reaction was terminated by the addition of 0.1 ml of 15% trichloroacetic acid (TCA). A similar procedure follows to prepare the control where enzyme solution was added after terminating the reaction by TCA. In another tube, 4.3 ml of distilled water was taken, and to this, 0.2 ml of the above enzyme, a reaction mixture, was added and 0.5 ml of Nessler's reagent was added. With the addition of Nessler's reagent, yellowish-orange color was developed, and the color was measured at 436 nm against the control. One unit of enzyme is defined as 1 µmol of ammonia liberated per minute under assay conditions.

2.4. Media Development by QbD Approach

2.4.1. Step-1: screening of important nutrients by Plackett–Burman design (PBD)

A PBD was employed to screen the best suitable carbon and nitrogen sources for enhanced L-asparaginase production by *B. subtilis* THARAKA. A total of 11 various nutrients were selected and each parameter was tested at 3 levels. Table 1 shows the selected 11 parameters and their levels along with the 16 experimental PB designs used in this study. The obtained data were analyzed by the first-order model. The coefficient of each selected nutrient is calculated based on the following equation:

$$Y = \beta_0 + \sum \beta_i x_i (i = 1,2,3 - - - k) \quad (1)$$

where, Y is L-asparaginase activity (Response), β_0 is intercept, and β_i is the coefficient of variation (CV) (of a particular variable). A high coefficient value (either positive or negative) denotes that the corresponding variable has a major effect on enzyme yield. A lower p-value of selected nutrients indicates a significant effect on L-asparaginase production.

Table 1: PBD for screening of nutrients for L-asparaginase production by *B. subtilis* THARAKA.

S. no	Level	Yeast extract (mg)	Soya peptone (mg)	Glucose (mg)	Starch (mg)	Galactose (mg)	Dextran (mg)	L-asparagine (mg)	$NaNO_3$ (mg)	Urea (mg)	$(NH_4)_2SO_4$ (mg)	NH_4NO_3 (mg)	L-asparaginase activity (IU/ml)
	−1	50	50	50	50	50	25	25	25	5	5	5	
	0	75	75	75	75	75	50	50	50	10	10	10	
	1	100	100	100	100	100	75	75	75	15	15	15	
1		1 (100)	−1 (50)	1 (100)	−1 (50)	−1 (50)	−1 (25)	1 (75)	1 (75)	−1 (5)	1 (15)	1 (15)	208.85
2		1 (100)	1 (100)	−1 (50)	1 (100)	−1 (50)	−1 (25)	−1 (25)	1 (75)	1 (15)	1 (15)	−1 (5)	251.73
3		−1 (50)	1 (100)	−1 (50)	−1 (50)	1 (100)	−1 (25)	1 (75)	1 (75)	1 (15)	−1 (5)	1 (15)	259.39
4		1 (100)	−1 (50)	−1 (50)	1 (100)	−1 (50)	1 (75)	1 (75)	−1 (25)	1 (15)	−1 (5)	1 (15)	263.41
5		1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	−1 (25)	−1 (25)	−1 (25)	−1 (5)	−1 (5)	1 (15)	69.68
6		1 (100)	1 (100)	−1 (50)	−1 (50)	1 (100)	1 (75)	1 (75)	−1 (25)	−1 (5)	1 (15)	−1 (5)	216.89
7		−1 (50)	1 (100)	1 (100)	1 (100)	−1 (50)	1 (75)	1 (75)	1 (75)	−1 (5)	−1 (5)	−1 (5)	185.88
8		−1 (50)	−1 (50)	1 (100)	1 (100)	1 (100)	−1 (25)	1 (75)	−1 (25)	1 (15)	1 (15)	−1 (5)	162.52
9		−1 (50)	−1 (50)	−1 (50)	1 (100)	1 (100)	1 (75)	−1 (25)	1 (75)	−1 (5)	1 (15)	1 (15)	247.90
10		1 (100)	−1 (50)	1 (100)	−1 (50)	1 (100)	1 (75)	−1 (25)	1 (75)	1 (15)	−1 (5)	−1 (5)	123.85
11		−1 (50)	1 (100)	1 (100)	−1 (50)	−1 (50)	1 (75)	−1 (25)	−1 (25)	1 (15)	1 (15)	1 (15)	197.55
12		−1 (50)	−1 (50)	−1 (50)	−1 (50)	−1 (50)	−1 (25)	−1 (25)	−1 (25)	−1 (5)	−1 (5)	−1 (5)	81.17
13		0 (75)	0 (75)	0 (75)	0 (75)	0 (75)	0 (50)	0 (50)	0 (50)	0 (10)	0 (10)	0 (10)	197.55
14		0 (75)	0 (75)	0 (75)	0 (75)	0 (75)	0 (50)	0 (50)	0 (50)	0 (10)	0 (10)	0 (10)	205.21
15		0 (75)	0 (75)	0 (75)	0 (75)	0 (75)	0 (50)	0 (50)	0 (50)	0 (10)	0 (10)	0 (10)	209.04
16		0 (75)	0 (75)	0 (75)	0 (75)	0 (75)	0 (50)	0 (50)	0 (50)	0 (10)	0 (10)	0 (10)	197.55

2.4.2. Step-2: enhancement of L-asparaginase production by response surface methodology (RSM)

Based on preliminary studies and PBD experiments, important parameters were selected for L-asparaginase production by *B. subtilis* THARAKA. Table 2 depicts the six selected parameters and their levels. Each parameter was studied at five levels. To optimize the selected parameters, a central composite design (CCD) with 50 experiments was employed. The 50 runs CCD plan was presented in Table 2. All parameter's real values were converted to coded values by using the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1,2,3.....k \qquad (2)$$

where x_i is the coded value of the selected parameter, X_0 actual value of the selected parameter at a central point, X_i actual value of the selected parameter, and ΔX_i is the step change. According to Table 2, row-wise experiments were conducted and the obtained L-asparaginase activity was recorded as a response. To find the correlation between the studied parameter and L-asparaginase yield, a second-order polynomial model was fitted. The general form of the second-order polynomial equation is

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_i x_i^2 + \sum_{i}^{i(} \sum_{j}^{j} \beta_{ij} x_i x_j + e \qquad (3)$$

where, Y_i is the predicted response viz L-asparaginase yield, $x_i x_j$ are studied variables that affect the L-asparaginase production by *B. subtilis* THARAKA, β_0 is the offset term, β_i is linear, β_{ii} is the quadratic coefficients of *i*th term, β_{ij} the interaction coefficient of *i*th and *j*th terms, and "*e*" is the error.

Statistical tests such as analysis of variance (ANOVA), lack of fit test, and correlation coefficient (R^2) were performed to check the obtained data and constructed model. The R^2-value was used to determine the percentage of variability in the selected parameters and their levels in the model. To understand the interactive influence of selected parameters on L-asparaginase production by *B. subtilis* THARAKA, surface (3D) and contour (2D) plots were drawn.

3. RESULTS AND DISCUSSION

3.1. Screening of Important Nutrients by PBD

In this study, four carbon sources and seven nitrogen sources that can affect L-asparaginase production by *B. subtilis* THARAKA were evaluated by PBD. All selected components were considered at three levels. Table 1 depicts the designated components and their levels along with the L-asparaginase obtained. It was noticed that the fourth experimental run had the highest amount of enzyme production (263.41 IU/ml) and the fifth run has the lowest L-asparaginase activity (69.68 IU/ml) in the entire study (Table 1). From Table 1, it can be noticed that the studied compounds and their levels govern the variation of enzyme production.

The regression coefficients were calculated by taking the L-asparaginase as a dependent variable. Table 3 shows the obtained coefficients and corresponding *p*-values. The coefficients which have a lower *p*-value ($p < 0.05$) are considered significant coefficients. A first-order polynomial equation [Eq. (4)] was constructed by using the obtained coefficients. The obtained higher correlation coefficient value ($R^2 = 0.9876$) indicates the goodness of the experiments carried out. Further, the higher value of adjusted R^2 (0.9628) which is nearer to the calculated R^2 indicates that the constructed Equation (4) could be used to predict L-asparaginase yield from *B. subtilis* THARAKA.

L-asparaginase activity (IU/ml) = 192.39 − 0.00 * Yeast Extract + 7.78 * Soya Peptone − 31.01 * Glucose + 7.79 * Starch − 9.03 * Galactose + 16.85 * Dextran + 27.09 * L-Asparagine + 23.86 * $NaNO_3$ + 20.67 * Urea + 25.17 * $(NH_4)_2SO_4$ + 18.73 NH_4NO_3 (4)

Based on this, in this study, glucose followed by L-asparagine showed the highest effect on L-asparaginase production by *B. subtilis* THARAKA. Complex nitrogen sources such as yeast extract and soy peptone were found insignificant for enzyme production. Similarly, starch and galactose are insignificant for L-asparaginase production by isolated bacterial strains. From Table 3, it can be observed that the complex carbon and nitrogen sources are not suitable for L-asparaginase production by this isolated bacterium. According to Sathish and Prakasham [17] and Sarquis *et al.* [25], the polysaccharides and complex nitrogen sources could induce other metabolic enzymes than desired amidases.

The negative sign of the glucose coefficient (−31.01) designates that low concentrations of glucose could yield higher amounts of L-asparaginase by *B. subtilis* THARAKA. L-asparagine and $(NH_4)_2SO_4$ have positive coefficients specifying that these nutrients should add higher concentrations to achieve higher amounts of L-asparaginase production by isolated bacterial strains. Based on PBD, it was observed that carbon source was the most influencing compound for L-asparaginase production by *B. subtilis* THARAKA. L-asparagine and $(NH_4)_2SO_4$ are next to the glucose.

The obtained results are in agreement with the reports of Sukumaran *et al.* [26] and Boeck and Ho [27] where the authors observed that glucose enhanced L-asparaginase production in *Serratia marcescens* and *Escherichia coli* respectively. However, De Jong [28] and Heinemann and Howard [29] noticed that glucose was a repressor for L-asparaginase production in *Streptomyces griseus*. Cedar and Schwartz [30] observed that galactose enhanced the production of L-asparaginase by *E. coli*, however, in this study galactose was found as a non-significant nutrient.

Abdel-Fattah and Olama [31] noticed that L-asparaginase production was enhanced with the supplement of corn steep liquor and casein as nitrogen sources in *Pseudomonas aeruginosa* fermentation. Similarly, Khan *et al.* [32] and Liu and Zajic [33] reported that yeast-extract addition in the media increased the amidase production in *S. marcescens* and *Erwinia aroideae,* respectively.

Peterson and Ciegler [34] documented that supplementation of tryptone and yeast extract increased the L-asparaginase production in *E. aroideae* NRRLB 138. These literature reports

Table 2: CCD for optimization of L-asparaginase production by *B. subtilis* THARAKA.

S. no	Temperature (°C)	pH	Agitation speed (rpm)	$(NH_4)_2SO_4$ (g/l)	Glucose (g/l)	L-asparagine (g/l)	L-asparaginase activity (IU/ml)		
							Experimental	Predicted	Error
1	−1 (35)	−1 (6.5)	−1 (175)	−1 (7.5)	−1 (7)	−1 (5)	212.19	208.28	3.91
2	−1 (35)	−1 (6.5)	−1 (175)	−1 (7.5)	1 (8)	1 (10)	201.14	195.90	5.24
3	−1 (35)	−1 (6.5)	−1 (175)	1 (12.5)	−1 (7)	1 (10)	225.61	242.70	−17.09
4	−1 (35)	−1 (6.5)	−1 (175)	1 (12.5)	1 (8)	−1 (5)	245.15	241.10	4.05
5	−1 (35)	−1 (6.5)	1 (225)	−1 (7.5)	−1 (7)	1 (10)	242.78	238.59	4.19
6	−1 (35)	−1 (6.5)	1 (225)	−1 (7.5)	1 (8)	−1 (5)	216.33	217.55	−1.22
7	−1 (35)	−1 (6.5)	1 (225)	1 (12.5)	−1 (7)	−1 (5)	266.47	261.79	4.68
8	−1 (35)	−1 (6.5)	1 (225)	1 (12.5)	1 (8)	1 (10)	302.99	298.76	4.23
9	−1 (35)	1 (7.5)	−1 (175)	−1 (7.5)	−1 (7)	1 (10)	251.67	247.58	4.09
10	−1 (35)	1 (7.5)	−1 (175)	−1 (7.5)	1 (8)	−1 (5)	146.66	147.68	−1.02
11	−1 (35)	1 (7.5)	−1 (175)	1 (12.5)	−1 (7)	−1 (5)	258.97	254.59	4.38
12	−1 (35)	1 (7.5)	−1 (175)	1 (12.5)	1 (8)	1 (10)	276.54	272.21	4.33
13	−1 (35)	1 (7.5)	1 (225)	−1 (7.5)	−1 (7)	−1 (5)	190.28	191.36	−1.08
14	−1 (35)	1 (7.5)	1 (225)	−1 (7.5)	1 (8)	1 (10)	272.79	275.02	−2.23
15	−1 (35)	1 (7.5)	1 (225)	1 (12.5)	−1 (7)	1 (10)	278.12	273.16	4.96
16	−1 (35)	1 (7.5)	1 (225)	1 (12.5)	1 (8)	−1 (5)	196.60	196.95	−0.35
17	1 (39)	−1 (6.5)	−1 (175)	−1 (7.5)	−1 (7)	1 (10)	212.19	212.31	−0.12
18	1 (39)	−1 (6.5)	−1 (175)	−1 (7.5)	1 (8)	−1 (5)	235.87	241.30	−5.43
19	1 (39)	−1 (6.5)	−1 (175)	1 (12.5)	−1 (7)	−1 (5)	272.79	271.03	1.76
20	1 (39)	−1 (6.5)	−1 (175)	1 (12.5)	1 (8)	1 (10)	247.92	247.30	0.62
21	1 (39)	−1 (6.5)	1 (225)	−1 (7.5)	−1 (7)	−1 (5)	210.81	215.60	−4.79
22	1 (39)	−1 (6.5)	1 (225)	−1 (7.5)	1 (8)	1 (10)	253.05	257.90	−4.85
23	1 (39)	−1 (6.5)	1 (225)	1 (12.5)	−1 (7)	1 (10)	179.42	178.87	0.55
24	1 (39)	−1 (6.5)	1 (225)	1 (12.5)	1 (8)	−1 (5)	226.99	231.55	−4.56
25	1 (39)	1 (7.5)	−1 (175)	−1 (7.5)	−1 (7)	−1 (5)	195.21	199.91	−4.70
26	1 (39)	1 (7.5)	−1 (175)	−1 (7.5)	1 (8)	1 (10)	217.72	222.87	−5.15
27	1 (39)	1 (7.5)	−1 (175)	1 (12.5)	−1 (7)	1 (10)	207.25	206.50	0.75
28	1 (39)	1 (7.5)	−1 (175)	1 (12.5)	1 (8)	−1 (5)	175.67	180.32	−4.65
29	1 (39)	1 (7.5)	1 (225)	−1 (7.5)	−1 (7)	1 (10)	172.91	177.43	−4.52
30	1 (39)	1 (7.5)	1 (225)	−1 (7.5)	1 (8)	−1 (5)	148.43	131.81	16.62
31	1 (39)	1 (7.5)	1 (225)	1 (12.5)	−1 (7)	−1 (5)	107.18	112.88	−5.70
32	1 (39)	1 (7.5)	1 (225)	1 (12.5)	1 (8)	1 (10)	180.80	185.18	−4.38
33	−2 (33)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	285.81	297.28	−11.47
34	2 (41)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	249.30	235.97	13.33
35	0 (37)	−2 (6)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	289.96	286.48	3.48
36	0 (37)	2 (8)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	224.23	225.84	−1.61
37	0 (37)	0 (7)	−2 (150)	0 (10)	0 (7.5)	0 (7.5)	276.73	273.16	3.57
38	0 (37)	0 (7)	2 (250)	0 (10)	0 (7.5)	0 (7.5)	253.05	254.76	−1.71
39	0 (37)	0 (7)	0 (200)	−2 (5)	0 (7.5)	0 (7.5)	254.43	254.84	−0.41
40	0 (37)	0 (7)	0 (200)	2 (15)	0 (7.5)	0 (7.5)	291.34	289.07	2.27
41	0 (37)	0 (7)	0 (200)	0 (10)	−2 (6.5)	0 (7.5)	247.92	244.50	3.42
42	0 (37)	0 (7)	0 (200)	0 (10)	2 (8.5)	0 (7.5)	249.30	250.86	−1.56

Continued

S. no	Temperature (°C)	pH	Agitation speed (rpm)	$(NH_4)_2SO_4$ (g/l)	Glucose (g/l)	L-asparagine (g/l)	L-asparaginase activity (IU/ml)		
							Experimental	Predicted	Error
43	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	−2 (2.5)	171.53	173.42	−1.89
44	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	2 (12.5)	230.74	226.99	3.75
45	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	321.54	313.54	8.00
46	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	312.26	313.54	−1.28
47	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	307.13	313.54	−6.41
48	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	315.42	313.54	1.88
49	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	309.50	313.54	−4.04
50	0 (37)	0 (7)	0 (200)	0 (10)	0 (7.5)	0 (7.5)	311.67	313.54	−1.87

Table 3: Effects, coefficients, and ANOVA for PBD.

Parameter	Effect	Coefficients	SS	df	MS	*t*-value	*F*-value	*p*-value
Mean/intercept	192.3856	192.3856	–	–	–	61.4540	–	0.0000
Yeast extract	0.0000	0.0000	0.00	1	0.00	0.0000	0.0000	1.0000
Soya peptone	15.5700	7.7850	727.27	1	727.27	2.1536	4.6380	0.0976
Glucose[a]	−62.0267	−31.0133	11,541.92	1	11,541.92	−8.5794	73.6060	0.0010
Starch	15.5700	7.7850	727.27	1	727.27	2.1536	4.6380	0.0976
Galactose	−18.0600	−9.0300	978.49	1	978.49	−2.4980	6.2401	0.0669
Dextran[a]	33.6900	16.8450	3,405.05	1	3,405.05	4.6599	21.7149	0.0096
L-asparagine[a]	54.1767	27.0883	8,805.33	1	8,805.33	7.4936	56.1540	0.0017
$NaNO_3$[a]	47.7300	23.8650	6,834.46	1	6,834.46	6.6019	43.5852	0.0027
Urea[a]	41.3467	20.6733	5,128.64	1	5,128.64	5.7190	32.7067	0.0046
$(NH_4)_2SO_4$[a]	50.3433	25.1717	7,603.35	1	7,603.35	6.9634	48.4887	0.0022
NH_4NO_3[a]	37.4567	18.7283	4,209.01	1	4,209.01	5.1809	26.8420	0.0066
Error	–	–	627.23	4	156.81	–	–	–
Total	–	–	50,588.03	15	–	–	–	–

SS = Sum of squares; MS = mean square; df = degree of freedom.
[a] Significant terms.

were contradicting the current experimental reports. Sarquis *et al.* [25] noticed that Proline was an inducer for the L-asparaginase in *Aspergillus terreus* and *Aspergillus tamari*. Current PBD represented $(NH_4)_2SO_4$ as an important nutrient is correlated with Paul and Cooksey's [35] reports.

3.2. Enhancement of L-Asparaginase Production by RSM

Based on the OFAT method and screening experiments (PBD), three environmental and three nutrient parameters were selected for further optimization by RSM. The selected parameters such as temperature (°C), pH, agitation speed (rpm), the concentration of $(NH_4)_2SO_4$ (g/l), glucose (g/l), and L-asparagine (g/l) were optimized by using the 50 experimental CCD for enhancement of L-asparaginase secretion by *B. subtilis* THARAKA. Table 2 depicts the engaged CCD matrix along with six selected parameters and their levels along with L-asparaginase activity noticed in corresponding experiments. From Table 2, it was noticed that the enzyme activity varied along with the concentration or level variations. During the experimentations, 107.18 U/ml as the lowest and 321.54 U/ml as the highest enzyme activity was observed, which indicates that selected parameters and their levels have a significant effect on L-asparaginase production by *B. subtilis* THARAKA. The obtained enzyme activity data were fitted by the linear regression method and obtained coefficients were verified for their significance.

The R^2-value was calculated as 0.9873, indicating that in the constructed model, only 1.27% of the variability in L-asparaginase production by selected parameters could not be explained. The adjusted R^2 (0.9617) and predicted R^2 (0.9259) were nearer to the R^2-value (0.9873) designating a higher significance of the model [1,36–38]. Less variation between experimental and predicted enzyme values signifies the accuracy of experiments conducted. The correlation between observed and predicted L-asparaginase yields are depicted in Figure 1. In the graph, it was noticed that all data points are closer to the fitted line, which infers that the model forecasted enzyme yields were similar to the experimental

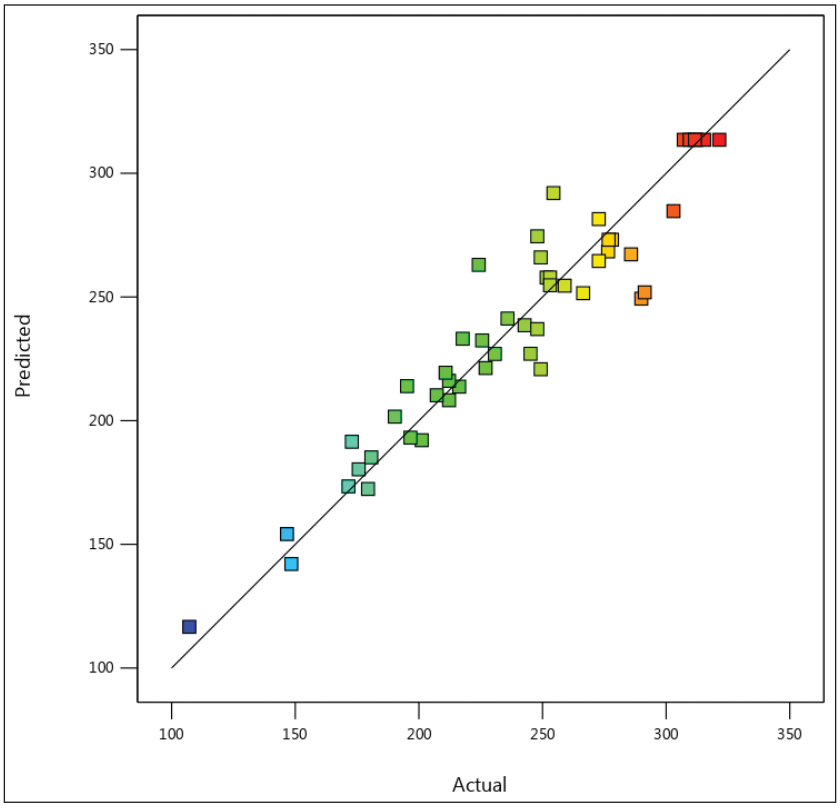

Figure 1: Correlation between the experimental and predicted L-asparaginase yield by *B. subtilis* THARAKA.

L-asparaginase yields. The lower CV value of 3.5% specifies that the experiments were carried out with better precision and reliability.

A second-order regression equation [Eq. (5)] was constructed based on L-asparaginase activity as a function of studied parameters. Equation (3) represents an empirical relationship between the enzyme production by *B. subtilis* THARAKA and selected factors. The constructed equation could be used to predict the enzyme yields for given levels of each factor. Based on factor coefficients, the impact of corresponding factors on response could be estimated.

L-asparaginase activity (IU/ml) = 313.5414 − 15.3275 * Temperature − 15.159 * pH − 4.599 * Agitation speed + 8.5565 * $(NH_4)_2SO_4$ + 1.589 * Glucose + 13.393 * L-Asparagine − 11.7296 * Temperature * Temperature − 14.3446 * pH * pH − 12.3958 * Agitation speed * Agitation speed − 10.397125 * $(NH_4)_2SO_4$ * $(NH_4)_2SO_4$ − 16.4658 * Glucose * Glucose − 28.3346 * L-Asparagine * L-Asparagine − 12.2762 * Temperature * pH − 13.5456 * Temperature * Agitation speed − 11.3993 * Temperature * $(NH_4)_2SO_4$ + 6.1431 * Temperature * Glucose − 6.8962 * Temperature * L-Asparagine − 7.1425 * pH * Agitation speed − 3.0475 * pH * $(NH_4)_2SO_4$ − 4.79875 * pH * Glucose (g/l) + 14.384375 * pH * L-Asparagine (g/l) − 6.439375 * Agitation speed * $(NH_4)_2SO_4$ + 7.4756 * Agitation speed * Glucose + 6.945 * Agitation speed * L-Asparagine + 1.6531 * $(NH_4)_2SO_4$ * Glucose − 3.7387 * $(NH_4)_2SO_4$ * L-Asparagine + 9.5375 * Glucose (g/l) * L-Asparagine (5)

The obtained *t*, *F*, and *p*-values of the selected parameters linear, square, and interaction terms are presented in Table 4. The results of ANOVA are presented in Table 4. The terms which have a lesser *p*-value and more *F*-value ($p < F$) are considered significant terms and the term which has a *p*-value of more than 0.05 ($p > 0.05$) is considered insignificant term [38]. Based on this, it was noticed that the quadratic term of L-asparagine (−56.6693) followed by glucose (−32.9318) has the highest effect among all selected parameters. The linear term of glucose is insignificant; however, its quadratic term is significant indicating that glucose is one of the important parameters for L-asparaginase production by *B. subtilis* THARAKA. L-asparagine, $(NH_4)_2SO_4$, and agitation speed have more effect on quadratic terms than linear terms (Table 4) which indicates that these variables have the highest effect on enzyme production and small variations in their levels could significantly affect the L-asparaginase production by *B. subtilis* THARAKA. Among all interaction terms, pH with L-Asparagine has the highest effect (−28.7688) followed by temperature with agitation speed (−27.0913). The interaction terms of $(NH_4)_2SO_4$ with pH and glucose were insignificant and the remaining interaction terms were significant.

To understand the interaction influence of two selected parameters on L-asparaginase production by *B. subtilis* THARAKA, surface plots (3D) with contours (2D) were generated by using Equation (5). In this equation, two-parameter values were changed, and remaining all other variables were kept constant at the central value. These graphs are also useful for predicting the enzyme yield by a given set of conditions also. Figure 2 depicts the

Table 4: Effects, coefficients, and ANOVA for CCD.

Factor	Effect	Coefficients	SS	df	MS	*t*-value	*F*-value	*p*-value
Mean/intercept	313.5414	313.5414				100.7090		0.000000
Temperature	−30.6550	−15.3275	9,397.3	1	9,397.29	−11.6503	135.7304	0.000000
pH	−30.3180	−15.1590	9,191.8	1	9,191.81	−11.5223	132.7625	0.000000
Agitation speed	−9.1980	−4.5990	846.0	1	846.03	−3.4957	12.2197	0.002045
$(NH_4)_2SO_4$	17.1130	8.5565	2,928.5	1	2,928.55	6.5037	42.2987	0.000002
Glucose	3.1780	1.5890	101.0	1	101.00	1.2078	1.4588	0.239953
L-asparagine (g/l)	26.7860	13.3930	7,174.9	1	7,174.90	10.1799	103.6311	0.000000
Temperature * Temperature	−23.4593	−11.7296	4,402.7	1	4,402.69	−7.9744	63.5906	0.000000
pH * pH	−28.6893	−14.3446	6,584.6	1	6,584.58	−9.7522	95.1049	0.000000
Agitation speed * Agitation speed	−24.7918	−12.3959	4,917.0	1	4,917.05	−8.4273	71.0197	0.000000
$(NH_4)_2SO_4$ * $(NH_4)_2SO_4$	−20.7943	−10.3971	3,459.2	1	3,459.21	−7.0685	49.9633	0.000000
Glucose (g/l) * Glucose (g/l)	−32.9318	−16.4659	8,676.0	1	8,676.00	−11.1943	125.3124	0.000000
L-asparagine (g/l) * L-asparagine (g/l)	−56.6693	−28.3346	25,691.2	1	25,691.23	−19.2633	371.0730	0.000000
Temperature * pH	−24.5525	−12.2763	4,822.6	1	4,822.60	−8.3460	69.6556	0.000000
Temperature * Agitation speed	−27.0913	−13.5456	5,871.5	1	5,871.49	−9.2090	84.8052	0.000000
Temperature * $(NH_4)_2SO_4$	−22.7988	−11.3994	4,158.3	1	4,158.26	−7.7498	60.0602	0.000000
Temperature * Glucose (g/l)	12.2863	6.1431	1,207.6	1	1,207.62	4.1764	17.4423	0.000392
Temperature * L-asparagine (g/l)	−13.7925	−6.8963	1,521.9	1	1,521.86	−4.6884	21.9812	0.000112
pH * Agitation speed	−14.2850	−7.1425	1,632.5	1	1,632.49	−4.8558	23.5790	0.000075
pH * $(NH_4)_2SO_4$	−6.0950	−3.0475	297.2	1	297.19	−2.0718	4.2925	0.050206
pH * Glucose (g/l)	−9.5975	−4.7988	736.9	1	736.90	−3.2624	10.6434	0.003566
pH * L-asparagine (g/l)	28.7688	14.3844	6,621.1	1	6,621.13	9.7792	95.6327	0.000000
Agitation speed * $(NH_4)_2SO_4$	−12.8788	−6.4394	1,326.9	1	1,326.90	−4.3778	19.1651	0.000240
Agitation speed * Glucose (g/l)	14.9513	7.4756	1,788.3	1	1,788.32	5.0823	25.8297	0.000043
Agitation speed * L-asparagine (g/l)	13.8900	6.9450	1,543.5	1	1,543.46	4.7215	22.2930	0.000104
$(NH_4)_2SO_4$ * Glucose (g/l)	3.3062	1.6531	87.5	1	87.45	1.1239	1.2631	0.273180
$(NH_4)_2SO_4$ * L-asparagine (g/l)	−7.4775	−3.7388	447.3	1	447.30	−2.5418	6.4607	0.018586
Glucose (g/l) * L-asparagine (g/l)	19.0750	9.5375	2,910.8	1	2,910.85	6.4841	42.0430	0.000002
Error			1,523.2	22	69.23			
Total			119,867.3	49				

interaction influence of selected parameters on L-asparaginase production by *B. subtilis* THARAKA. Figure 2a and b show the temperature interaction with pH and L-asparagine. In these graphs, the contours are circular and elliptical which indicates that the temperature has no interaction with pH and L-asparagine. Figure 2a and b also show that a temperature of 35°C–39°C is suitable for L-asparaginase production. The influence of pH on agitation is shown in Figure 2c. From this figure, it can be noticed that agitation speed is independent of pH. From Figure 2d, it can be noticed that $(NH_4)_2SO_4$ concentration was dependent on the glucose, however, the L-asparagine concentration was independent of glucose concentration (Fig. 2e). The interaction between the two nitrogen sources was depicted in Figure 2f, from which it can be observed that $(NH_4)_2SO_4$ concentration is influenced by the L-asparagine concentration.

By using Equation (5), the optimum conditions were predicted. The anticipated conditions were temperature of 33.5°C, pH of 7.2, agitation speed of 214 rpm, $(NH_4)_2SO_4$ concentration of 12.7 g/l, glucose concentration of 7.5 g/l, and L-asparagine concentration of 8.8 g/l. Under these conditions, the experiments were conducted to check the accuracy of the model. The obtained L-asparaginase activity was 340 ± 10.53 IU/ml which is nearer to the predicted one of 332 IU/ml. The obtained experimental values and software-predicted values were closer indicating that the constructed model is useful to predict the L-asparaginase yield by *B. subtilis* THARAKA.

Similar modeling studies for L-asparaginase optimization were reported in the literature. Kumar and Verma [39] and Meena *et al.* [15] used the Box–Behnken design, and Shakambari *et al.* [40], Morales-Gonzalez *et al.* [14], Mangamuri *et al.* [41], Rajamanickam *et al.* [42], and El-Naggar *et al.* [4] used the CCD for optimization of amidase production by various microorganisms. Prakasham *et al.* [43] used an orthogonal array-based optimization method for the enhancement of L-asparaginase production. These reports

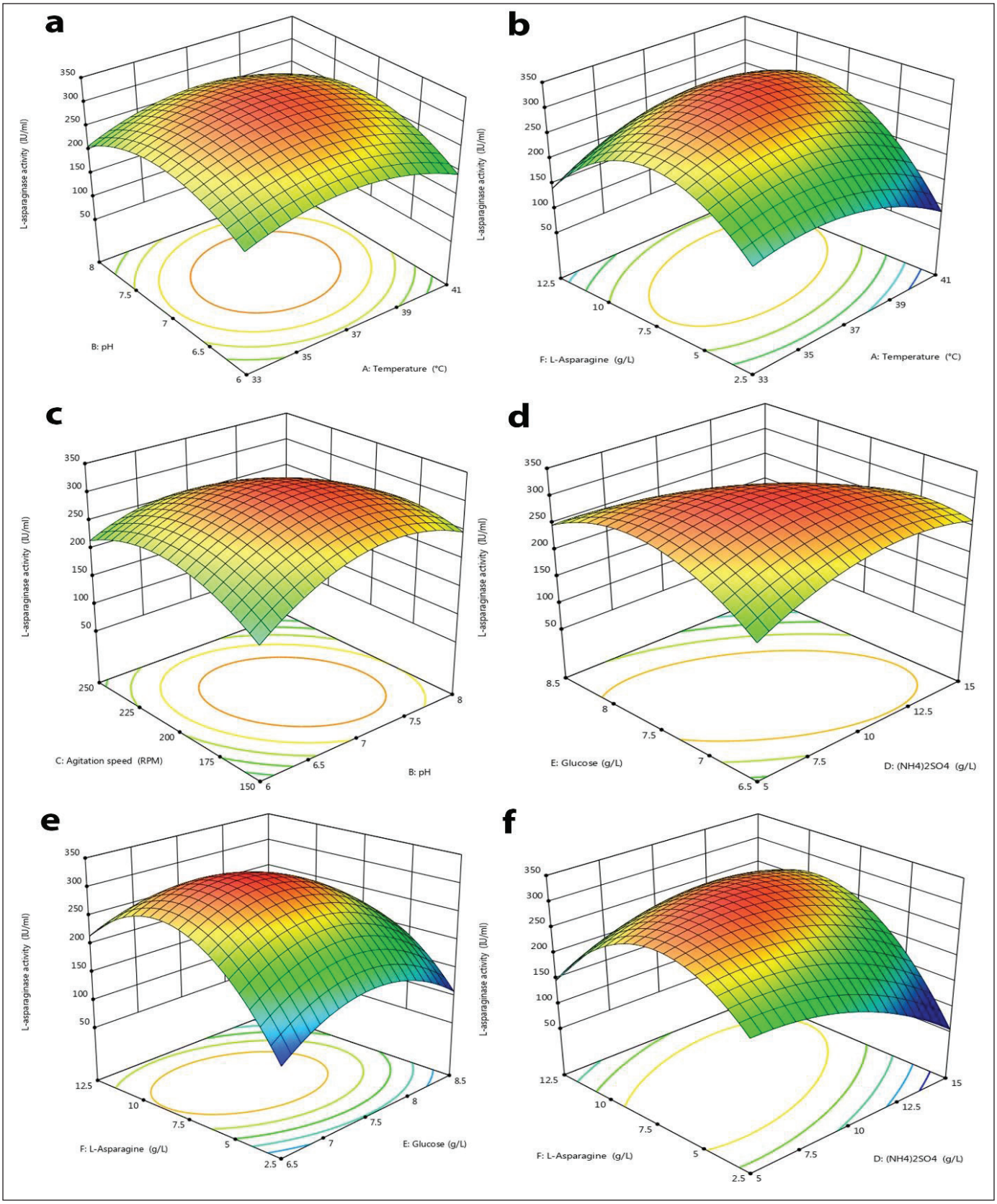

Figure 2: Interaction influence of selected parameters on L-asparaginase production by *B. subtilis* THARAKA. (a) Temperatures versus pH; (b) temperature versus L-asparagine concentration; (c) pH versus agitation speed; (d) $(NH_4)_2SO_4$ concentration versus glucose concentration; (e) glucose concentration versus L-asparagine concentration; and (f) $(NH_4)_2SO_4$ concentration versus L-asparagine concentration.

indicate that depending on their laboratory conditions and need, various authors used different statistical methods of optimization for the enhancement of L-asparaginase production.

4. CONCLUSION

This study deals with the QbD approach in enzyme production. By using the sequential design space such as PB followed by CCD, L-asparaginase production was enhanced. Based on this QbD approach, the yield was enhanced as well as a control strategy on variables and products was also developed.

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6. CONFLICTS OF INTEREST

We declare "no conflicts of interest."

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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