

## Chapter 4

### L-asparaginase production using *Bacillus indicus* bacteria

---

#### 4.1 Introduction

The L-asparagine depletion by the L-asparaginase leads to starvation of the leukemic lymphoblast cells in therapeutic applications and also prevents the formation of acrylamide neurotoxin in food processing applications (Hendriksen *et al.*, 2009; Sharma *et al.*, 2019). However, the usage of current therapeutic formulations derived from *Escherichia coli* and *Erwinia chrysanthemi* are accompanied by several immunological side issues that occurs due to (a) the accompanying high L-glutaminase activity of both the L-asparaginase preparations which lead to the hydrolysis of glutamine and further causes neurotoxicity and hypersensitivity reactions. (Shrivastava *et al.*, 2016; Nguyen *et al.*, 2016; Fonseca *et al.*, 2021) (b) Moreover, the treatment regimen requires multiple different types of L-asparaginases as the enzyme preparation from the single microorganism will be rapidly cleared during the subsequent doses by the host immune system (Ceconello *et al.*, 2020). Hence, these pitfalls of the commercial L-asparaginase preparations necessitated exploring alternative new asparaginase preparations that show high specificity to L-asparagine resulting in low/negligible glutaminase coactivity (Costa *et al.*, 2022; Uber *et al.*, 2022). In addition, the new L-asparaginases must show the capability to cause apoptosis in leukemia cells and also possess different physico-chemical properties.

*Bacillus* species are long known as safe microbial workhorses for the production and secretion of extracellular enzymes. As the L-asparaginase is present in diverse groups of microorganisms, only a few possess anti-leukemic properties. Therefore, the production of secretory L-asparaginase having anticancer properties and low/negligible glutaminase activity

is an attractive approach. A safe spore-forming *Bacillus* species that has gained recent attention is *Bacillus indicus* due to its utilization as a probiotic. Studies on the in vitro and in vivo safety assessment for the use of *Bacillus indicus* as probiotics were performed by several researchers. No toxicity issues were observed in animal models and the *Bacillus* produced no enterotoxins (Hong *et al.*, 2008). In a recent study, dark chocolates were enriched with the *Bacillus indicus* microorganism to produce novel probiotic chocolates with enhanced antioxidant properties (Erdem *et al.*, 2014). In this perspective, the L-asparaginase preparation from *Bacillus indicus* should be free from health concerns and safe enough for utilization as an effective anticancer agent and food processing agent.

Screening and selection of crucial nutritional components are a requisite for developing an effective and economical culture medium for bioprocesses (Reddy *et al.*, 2003). To achieve the goal, a number of screening methods including the Taguchi orthogonal array, and Plackett Burman design can be employed to discern the role of each factor on the product formation (Singh *et al.*, 2021). The next step involves the optimization using response surface methods in which multiple factors are varied simultaneously to identify the optimum concentration and also the interaction effects among the nutritional parameters (Sharma and Mishra, 2021). The present chapter describes the production process for a new L-asparaginase preparation from *Bacillus indicus* MTCC 4374 in submerged fermentation using cost-effective and readily available carbon and nitrogen sources. The concentration of these cheaper constituents was then optimized making it highly useful for the industrial production of the L-asparaginase at low cost.

## 4.2 Materials and Methods

### 4.2.1 Microorganism growth parameters and screening for amidohydrolase activity

The bacterial culture used in this study, *Bacillus indicus* MTCC 4374 or *Metabacillus indicus* Sd/3 (Equivalent number: DSM 15820, LMG 22858) was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The culture medium with the following composition (g/L): peptone (10), beef extract (10), NaCl (5) and agar (20), was utilized for the growth and maintenance of the bacterial culture (pH 7) at 37° C. Initially, screening for asparaginase enzyme activity was carried out using the basal M-9 medium of composition (g/L): Na<sub>2</sub>HPO<sub>4</sub> (6), glucose (3), L-asparagine (3), KH<sub>2</sub>PO<sub>4</sub> (2), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.5), NaCl (0.5), agar (20) and cresol red dye as pH indicator in plate assay. Stock solution (2.5%) of cresol red dye was constituted and 0.009% was added into the above-said M-9 medium. The agar medium was then autoclaved and the plates were prepared after the pH adjustment of the medium to 7.0. The color of the cresol red dye changes from yellow at neutral pH to red at alkaline pH (Mihooliya *et al.*, 2017; Sousa *et al.*, 2021).

### 4.2.2 Assay for the enzymatic activity of L-asparaginase

Production samples were centrifuged at 10,000xg for 10 min at 4±1 °C and the supernatant was investigated for L-asparaginase enzyme activity. 189 mM L-asparagine was prepared in 0.05 M Tris buffer (pH 8.6) and used as the substrate for the enzyme. The reaction mixture containing the substrate, Tris-HCl buffer and enzyme preparation was incubated at 37 °C for 30 min. The quantification of ammonia released was performed colorimetrically at 436 nm (Shifrin and Parrott, 1974). The calibration curve was prepared by using ammonium sulfate as standard. One International unit (IU) of enzyme activity of L-asparaginase is defined as the amount of a particular enzyme required to form one µmole of ammonia/mL/minute at pH 8.6 and a temperature of 37 °C.

### 4.2.3 Screening of medium components using Plackett Burman design (PBD)

Eight medium components in a modified M-9 medium were chosen as input. A 12-run PBD for screening the influential medium components affecting the enzyme production was utilized. The components utilized were sucrose (A), L-asparagine (B),  $\text{Na}_2\text{HPO}_4$  (C), corn steep liquor (D),  $\text{KH}_2\text{PO}_4$  (E),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (F), NaCl (G) and  $\text{CaCl}_2$  (H). The chosen variables together with their coded and real concentrations are depicted (**Table 4.1**). PBD is a robust and widely utilized screening design that efficiently determines the independent effects of medium components involved in the production process (Plackett and Burman, 1946; Kasemiire *et al.*, 2021). The experimental runs were performed in triplicates and the average of each run was used as a response.

**Table 4.1:** Plackett-Burman screening design (two levels) matrix depicting concentration of Modified M-9 medium components accompanying the obtained L-asparaginase enzyme activity (The experimental values obtained were mean values of triplicates  $\pm$  standard deviation).

Run	Variables and their levels (g/L)								Enzyme Activity (IU/mL)	Cell Growth (g/L)
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)		
1	4	1	10	5	1	0.4	0.6	0.08	5.94 $\pm$ 0.06	0.69 $\pm$ 0.03
2	4	8	1	15	1	0.4	0.1	0.08	9.78 $\pm$ 0.08	1.09 $\pm$ 0.07
3	1	8	10	5	5	0.4	0.1	0.03	6.47 $\pm$ 0.09	0.71 $\pm$ 0.04
4	4	1	10	15	1	1.0	0.1	0.03	8.48 $\pm$ 0.11	0.98 $\pm$ 0.04
5	4	8	1	15	1	0.4	0.6	0.03	9.97 $\pm$ 0.12	1.13 $\pm$ 0.04
6	4	8	10	5	5	1.0	0.1	0.08	10.58 $\pm$ 0.14	1.27 $\pm$ 0.06
7	1	8	10	15	1	1.0	0.6	0.03	7.74 $\pm$ 0.07	0.83 $\pm$ 0.03
8	1	1	10	15	5	0.4	0.6	0.08	4.12 $\pm$ 0.04	0.61 $\pm$ 0.03
9	1	1	1	15	5	1.0	0.1	0.08	6.91 $\pm$ 0.07	0.76 $\pm$ 0.04
10	4	1	1	5	5	1.0	0.6	0.03	6.70 $\pm$ 0.08	0.73 $\pm$ 0.04
11	1	8	1	5	1	1.0	0.6	0.08	6.76 $\pm$ 0.06	0.75 $\pm$ 0.05
12	1	1	1	5	1	0.4	0.1	0.03	3.59 $\pm$ 0.04	0.55 $\pm$ 0.02

A- Sucrose, B- L-asparagine, C- Na<sub>2</sub>HPO<sub>4</sub>, D- corn steep liquor, E- KH<sub>2</sub>PO<sub>4</sub>, F-MgSO<sub>4</sub>, G- NaCl, H- CaCl<sub>2</sub>

#### 4.2.4 Process modeling using central composite design of experiments (CCD)

The production studies for L-asparaginase enzyme were carried out on the 4 significant medium components obtained from previously performed Plackett Burman design for screening of significant medium components. The concentrations of the following constituents (g/L): sucrose (X<sub>1</sub>), L-asparagine (X<sub>2</sub>), corn steep liquor (X<sub>3</sub>) and MgSO<sub>4</sub>.7H<sub>2</sub>O

( $X_4$ ) were then varied according to the central composite experimental design at five distinct coded levels (-2, -1, 0, +1, +2) in thirty experimental runs. Erlenmeyer flasks (250 mL) having 50 mL of the production medium were inoculated with 5% (v/v) inoculum and incubated at 37 °C and 200 rpm for 36 hours. Experiments were carried out in triplicates to determine the enzyme activity and dry cell weight measurements.

The experimental response in terms of enzyme activity (IU/mL) was evaluated using the regression analysis in Minitab® 18.0 statistical software package. A second-order quadratic model was obtained and the regression equation was then used to depict the linear and two-way interactions between the operating variables and their impact on L-asparaginase production (response).

$$y = \beta_0 + \sum_{a=1}^n \beta_a X_a + \sum_{a=1}^n \beta_{aa} X_a^2 + \sum_a \sum_b \beta_{ab} X_a X_b \quad (1)$$

Where  $y$  indicates the response,  $n$  depicts the number of factors,  $\beta_0$  is the constant,  $\beta_a$  is the linear coefficient,  $\beta_{aa}$  is the quadratic (second order) coefficient and  $\beta_{ab}$  denotes the interaction coefficient connecting the factors  $X_a$  and  $X_b$ .

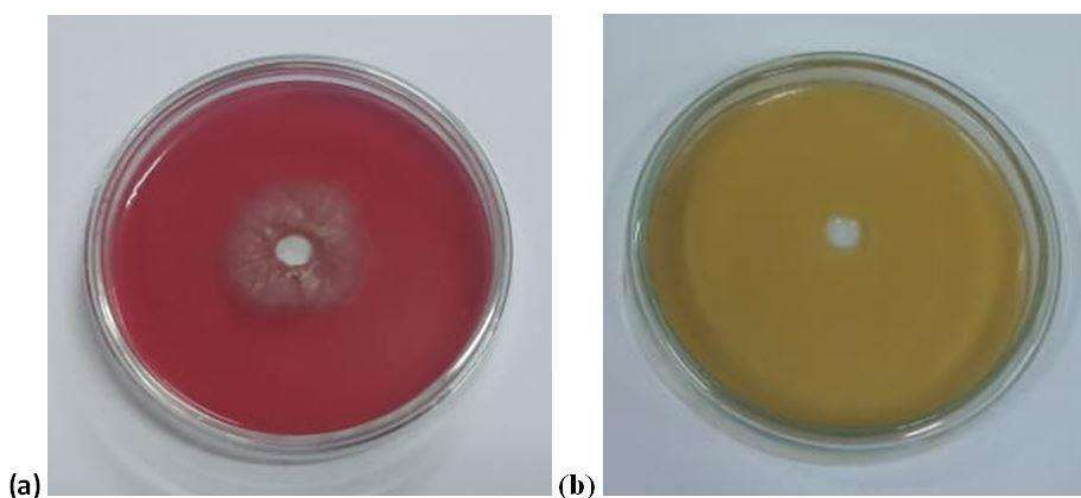
#### 4.2.5 Bioreactor operation for model validation and scale-up

The data in the form of optimized values from the CCD were further validated by conducting experimental runs in both shake flask and bioreactor conditions. A 3.7 L bioreactor (Bioengineering KLF) of 2.5 L processing capacity was utilized in batch mode. The optimized medium was in-situ sterilized and inoculated with 5% (v/v) culture of *Bacillus indicus* MTCC 4374 bacteria. Optimal operating conditions of 37°C temperature, and 200 rev/min were maintained. The pH of the medium at the start was 7.0 and the reactor was allowed to run at uncontrolled pH. Samples were withdrawn every 3 h intervals and analyzed for enzyme activity and biomass concentration.

### 4.3 Results and discussion

#### 4.3.1 Plate assay based screening for enzyme activity

The microorganism *Bacillus indicus* MTCC 4374 was initially screened for the presence of extracellular L-asparaginase enzyme. Initially, the active growing culture was spread on the plate and incubated for L-asparaginase enzyme production. The microorganism depicted a sharp region of hydrolysis of substrate L-asparagine as indicated by the appearance of a red color on medium containing L-asparagine after the incubation time of 24 h (**Figure 4.1a**). The red zone indicated the release of ammonia by the catalytic L-asparaginase reaction mechanism. The ammonia release further causes a change in the pH from neutral to alkaline resulting in color change of cresol red dye from yellow to red. A zone index (ratio of zone diameter to colony diameter) of 2.44 was obtained, which is consistent with other efficient L-asparaginases. *Aspergillus* sp. and *Curvularia* sp. L-asparaginases showed a zone index of 2.40 and 2.06, respectively using bromothymol blue as an indicator (Doriya and Kumar, 2016). Thus, the outcome obtained distinctly demonstrated the significant L-asparaginase activity. The uninoculated plate served as the control (**Figure 4.1b**).



**Figure 4.1:** Plate assay for screening of L-asparaginase enzyme activity by the bacterium

*Bacillus indicus* MTCC 4374. (a) Petri plate containing L-asparagine as substrate; and (b) Uninoculated plate as control (Sharma and Mishra, 2023).

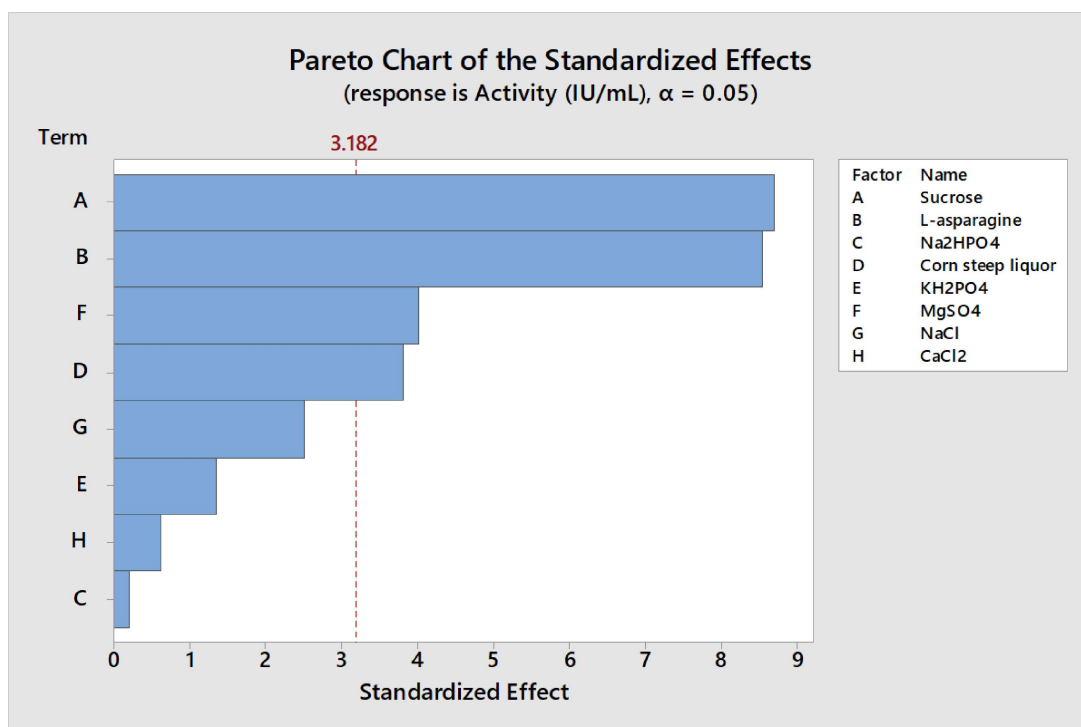
### 4.3.2 Screening of significant medium constituents affecting L-asparaginase production (Plackett Burman design)

Experiments were performed in line with the PBD to screen significant medium constituents (Table 4.1). The L-asparaginase activity varied in the range from  $3.59 \pm 0.04$  IU/ml to  $10.58 \pm 0.15$  IU/ml. The cell growth varied from  $0.55 \pm 0.02$  g/l to  $1.27 \pm 0.06$  g/l and showed correlation with L- asparaginase activity. The screening of significant components was performed based on a p-value below 0.05. The four variables out of eight components viz. sucrose (A), L-Asparagine (B), corn steep liquor (D) and  $MgSO_4 \cdot 7H_2O$  (F) were found to be significant based on analysis of variance (ANOVA) studies (Table 4.2). The remaining components consisting of  $Na_2HPO_4$  (C),  $KH_2PO_4$  (E), NaCl (G) and  $CaCl_2$  (H) had p-values above 0.05, thus considered insignificant.

**Table 4.2:** Statistical evaluation of PBD matrix depicting t values and p values for each variable in L-asparaginase production.

Variable	Coded Symbol	Effect	Coefficient	t-value	p-value
Constant			7.257	47.79	<0.0001
Sucrose	A	2.641	1.321	8.70	0.003 <sup>S</sup>
L-Asparagine	B	2.593	1.296	8.54	0.003 <sup>S</sup>
$Na_2HPO_4$	C	-0.062	-0.031	-0.21	0.851 <sup>NS</sup>
Corn steep liquor	D	1.160	0.580	3.82	0.032 <sup>S</sup>
$KH_2PO_4$	E	0.411	0.205	1.35	0.269 <sup>NS</sup>
$MgSO_4 \cdot 7H_2O$	F	1.218	0.609	4.01	0.028 <sup>S</sup>
NaCl	G	-0.764	-0.382	-2.51	0.087 <sup>NS</sup>
$CaCl_2$	H	0.191	0.095	0.63	0.575 <sup>NS</sup>

<sup>S</sup> denotes significant values, <sup>NS</sup> denotes non-significant values.



**Figure 4.2:** Pareto chart indicating the effect of medium components on the L-asparaginase enzyme production.

The Pareto chart is a diagrammatic approach to visualize the results of Plackett Burman screening design (**Figure 4.2**). It allows the identification of those factors that are majorly responsible for the design process. The reference line was used to indicate that the effect of variables would be significant if they passed through the bar with a specific value of  $\alpha$ . Based on the PBD results, it can be inferred that the sucrose acted as the carbon source, corn steep liquor (CSL) as a source of nitrogen, L-asparagine as the inducer and Mg<sup>2+</sup> acted as an essential growth element that led to the enhanced L-asparaginase production.

### 4.3.3 Production and optimization using central composite design of experiments (CCD)

The influence of the medium components was investigated with the aim to enhance the L-asparaginase production in a cost-effective manner using cheaper and widely available

substrates (sucrose and corn steep liquor). Corn steep liquor is a cheaper low-cost nitrogen source that can be efficiently employed to replace the expensive yeast extract and peptones. Experiments were carried out according to the CCD matrix and the resulting responses in terms of observed and predicted values of L-asparaginase activity (IU/mL) are revealed in **Table 4.3**. Variation in enzyme activity was observed in the range from  $6.81 \pm 0.09$  IU/mL to  $15.34 \pm 0.24$  IU/mL. The cell growth of *Bacillus* varied from  $0.74 \pm 0.03$  g/L to  $1.57 \pm 0.02$  g/L. The enzyme activity observed in the present investigation was comparatively higher than other reported studies, viz 1.03 IU/mL was reported in *Escherichia coli* ATCC 11303,  $3.35 \pm 0.11$  IU/mL in *Pseudomonas plecoglossicida* RS1, 3.66 IU/mL in *Erwinia aroideae* utilizing lactose and 3.25 IU/mL in *Pectobacterium carotovorum* MTCC 1428 (Kenari *et al.*, 2011; Shakambari *et al.*, 2015; Liu and Zajic, 1972; Sanjeeviroyar *et al.*, 2010).

A polynomial equation was obtained that describes the response in terms of L-asparaginase enzyme production using the cost-effective industrial substrates in the present study.

$$Y_{\text{activity}} = 15.01 + 0.709 * X_1 + 0.393 * X_2 - 0.202 * X_3 + 0.303 * X_4 + 0.435 * X_1 X_2 - 0.358 * X_1 X_3 - 0.191 * X_1 X_4 - 0.291 * X_2 X_3 - 0.258 * X_2 X_4 - 0.15 * X_3 X_4 - 1.72 * X_1^2 - 1.472 * X_2^2 - 1.0116 * X_3^2 - 0.813 * X_4^2 \quad (2)$$

Where  $X_1$  is sucrose,  $X_2$  is L-asparagine,  $X_3$  is corn steep liquor and  $X_4$  is  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

The evaluation of the experimental outcomes was performed using ANOVA (Analysis of variance) studies (**Table 4.4**). An important output of the regression analysis is  $R^2$  value or coefficient of determination which is used to check the closeness of the model. The  $R^2$  value of 0.9832 indicated that 98.32% of the variation in the test samples was due to medium constituents in the model. The Lack of fit (LOF)  $p$ -value of the optimized model was 0.157 (insignificant) which further proving the regression-based model to be an appropriate fit and can sufficiently explains the relation among the selected factors and the response.

**Table 4.3:** Central composite experimental design matrix specifying four significant factors and the output in the form of experimental values of L-asparaginase production.

Run	Concentration and levels of media components				L-asparaginase activity (IU/mL)		Cell growth (g/L)
	(X <sub>1</sub> ) (g/L)	(X <sub>2</sub> ) (g/L)	(X <sub>3</sub> ) (g/L)	(X <sub>4</sub> ) (g/L)	Experimental values	RSM predicted Values	
1	-1 (2)	-1(3)	-1 (6)	-1 (0.5)	8.09 ± 0.20	7.98	0.87 ± 0.02
2	1 (4)	-1 (3)	-1 (6)	-1 (0.5)	9.49 ± 0.18	9.62	1.03 ± 0.03
3	-1 (2)	1 (7)	-1 (6)	-1 (0.5)	8.76 ± 0.16	8.99	1.0 ± 0.03
4	1 (4)	1 (7)	-1 (6)	-1 (0.5)	12.15 ± 0.17	12.38	1.35 ± 0.05
5	-1 (2)	-1 (3)	1 (14)	-1 (0.5)	8.64 ± 0.21	9.17	0.95 ± 0.04
6	1 (4)	-1 (3)	1 (14)	-1 (0.5)	9.05 ± 0.13	9.38	0.97 ± 0.04
7	-1 (2)	1 (7)	1 (14)	-1 (0.5)	8.81 ± 0.11	9.02	1.03 ± 0.04
8	1 (4)	1 (7)	1 (14)	-1 (0.5)	10.61 ± 0.09	10.98	1.26 ± 0.03
9	-1 (2)	-1 (3)	-1 (6)	1 (1.1)	9.89 ± 0.11	9.78	1.12 ± 0.04
10	1 (4)	-1 (3)	-1 (6)	1 (1.1)	10.74 ± 0.12	10.67	1.29 ± 0.04
11	-1 (2)	1 (7)	-1 (6)	1 (1.1)	9.95 ± 0.22	9.76	1.13 ± 0.04
12	1 (4)	1 (7)	-1 (6)	1 (1.1)	12.66 ± 0.21	12.39	1.38 ± 0.03
13	-1 (2)	-1 (3)	1 (14)	1 (1.1)	10.46 ± 0.16	10.38	1.23 ± 0.03
14	1 (4)	-1 (3)	1 (14)	1 (1.1)	9.79 ± 0.23	9.82	1.08 ± 0.04
15	-1 (2)	1 (7)	1 (14)	1 (1.1)	9.06 ± 0.16	9.19	0.96 ± 0.03
16	1 (4)	1 (7)	1 (14)	1 (1.1)	10.12 ± 0.15	10.38	1.18 ± 0.04
17	-2 (1)	0 (5)	0 (10)	0 (0.8)	6.81 ± 0.09	6.72	0.74 ± 0.03
18	2 (5)	0 (5)	0 (10)	0 (0.8)	9.85 ± 0.21	9.56	1.11 ± 0.04
19	0 (3)	-2 (1)	0 (10)	0 (0.8)	8.44 ± 0.14	8.33	0.98 ± 0.03
20	0 (3)	2 (9)	0 (10)	0 (0.8)	10.18 ± 0.17	9.91	1.20 ± 0.05
21	0 (3)	0 (5)	-2(2)	0 (0.8)	11.06 ± 0.21	11.37	1.27 ± 0.04
22	0 (3)	0 (5)	2 (18)	0 (0.8)	11.24 ± 0.22	10.56	1.28 ± 0.03
23	0 (3)	0 (5)	0 (10)	-2 (0.2)	11.89 ± 0.16	11.15	1.32 ± 0.03
24	0 (3)	0 (5)	0 (10)	2 (1.4)	11.99 ± 0.18	12.36	1.33 ± 0.03
25	0 (3)	0 (5)	0 (10)	0 (0.8)	15.34 ± 0.24	15.01	1.57 ± 0.02
26	0 (3)	0 (5)	0 (10)	0 (0.8)	14.75 ± 0.19	15.01	1.48 ± 0.04
27	0 (3)	0 (5)	0 (10)	0 (0.8)	15.16 ± 0.17	15.01	1.51 ± 0.04
28	0 (3)	0 (5)	0 (10)	0 (0.8)	14.93 ± 0.14	15.01	1.50 ± 0.05
29	0 (3)	0 (5)	0 (10)	0 (0.8)	14.58 ± 0.18	15.01	1.44 ± 0.05
30	0 (3)	0 (5)	0 (10)	0 (0.8)	15.28 ± 0.19	15.01	1.55 ± 0.03

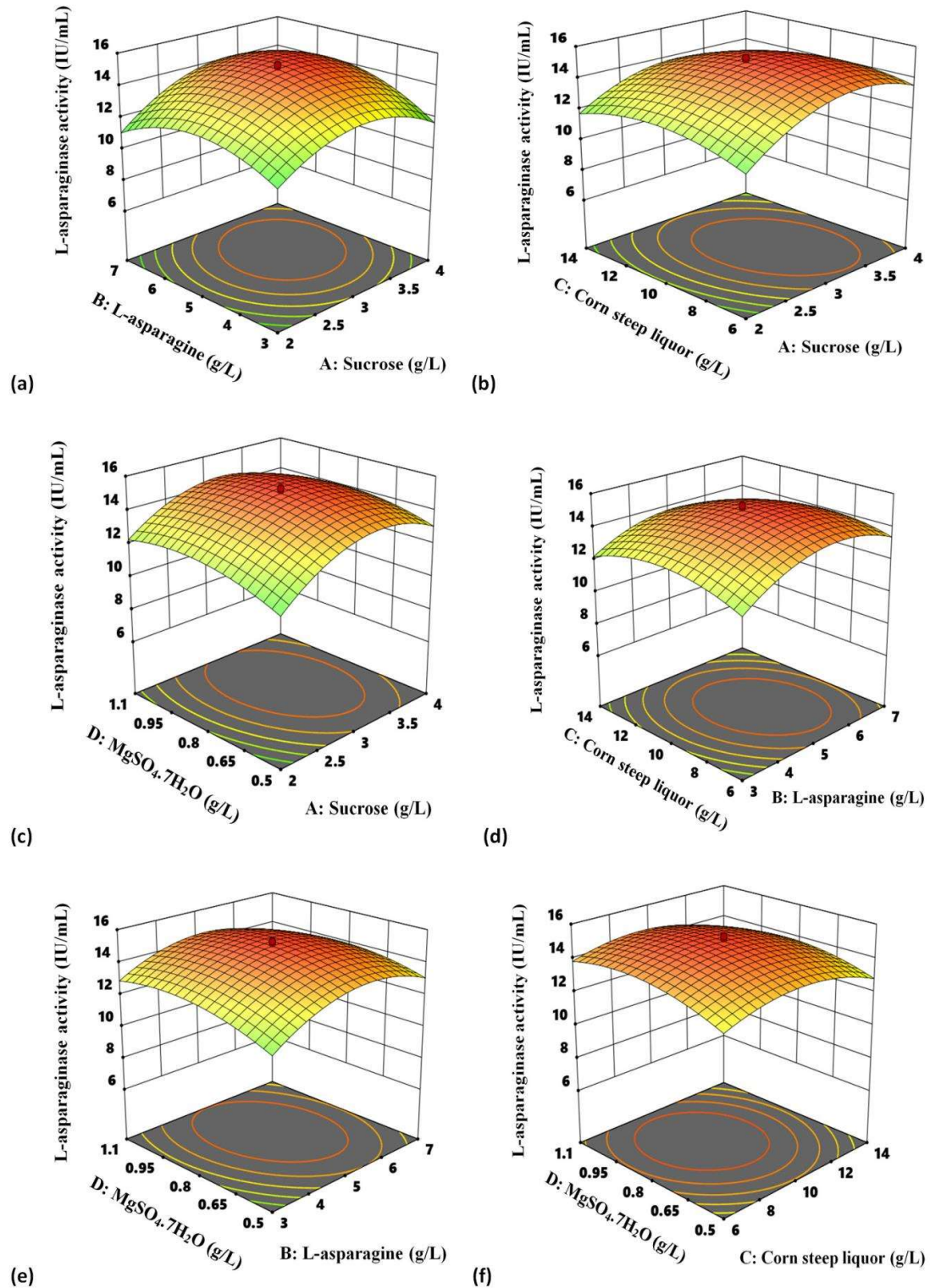
X<sub>1</sub> – Sucrose, X<sub>2</sub> – L-asparagine, X<sub>3</sub> – corn steep liquor, X<sub>4</sub> – MgSO<sub>4</sub>.7H<sub>2</sub>O.

The model terms were found to be significant due to very low  $p$ -values ( $P < 0.05$ ) (**Table 4.4**). The significant interaction effects among variables ( $p < 0.05$ ) were shown by sucrose with L-asparagine ( $p < 0.001$ ), sucrose with corn steep liquor ( $p < 0.048$ ), L-asparagine with corn steep liquor ( $p < 0.017$ ) and L-asparagine with  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $p < 0.030$ ). The outcomes of the RSM designs can be efficiently demonstrated using the surface and contour plots (Zarei *et al.*, 2010; Priyadharshini *et al.*, 2016; Bagewadi *et al.*, 2018). The three-dimensional (3D) surface plots were displayed to examine the association between the optimal levels of the medium constituents and the obtained L-asparaginase activity (**Figure 4.3**). The two dimensional contour plots were also constructed for estimating the range of the enzyme activities on selecting the combination of medium components (**Figure 4.4**).

**Table 4.4:** ANOVA studies for the quadratic model.

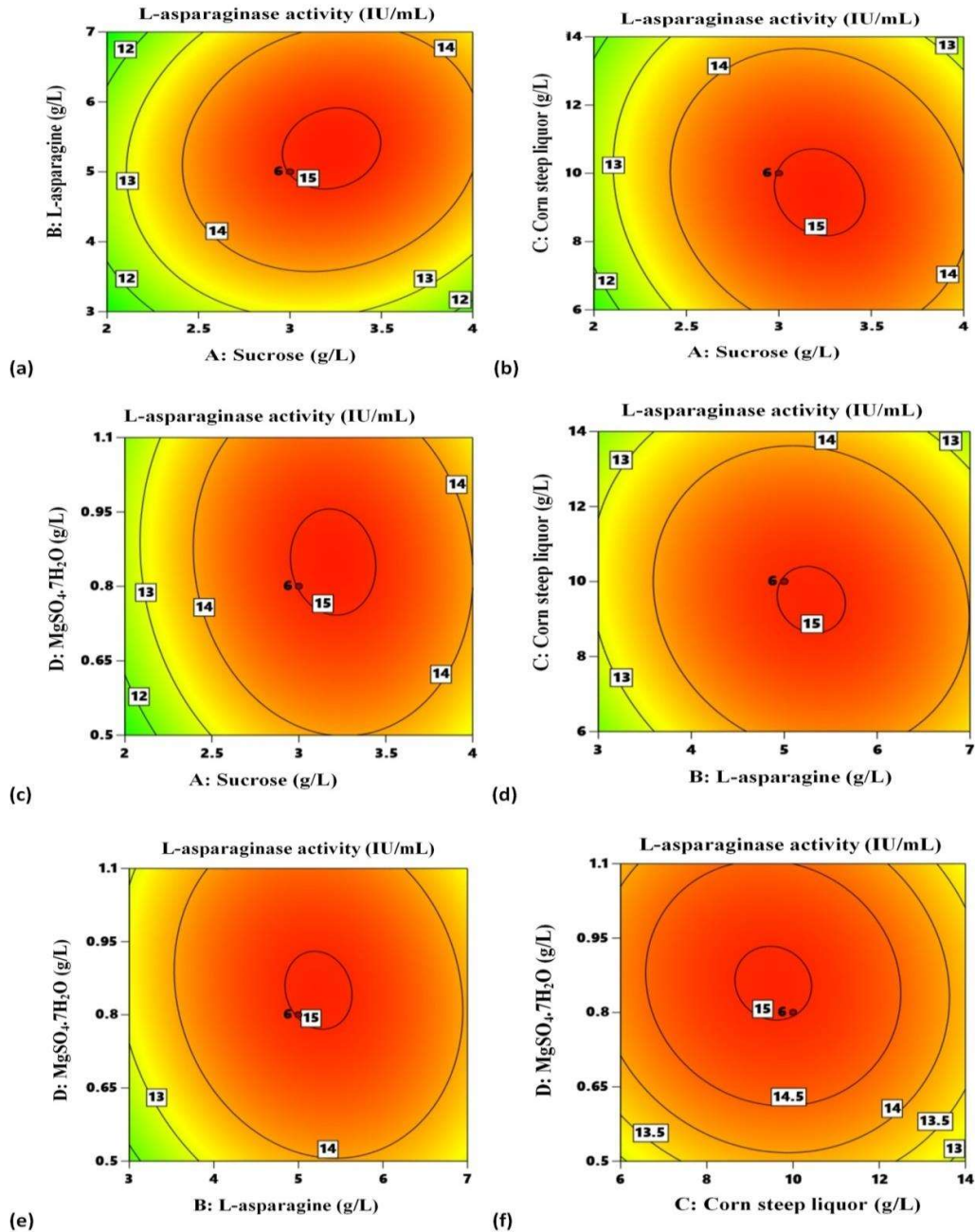
Source	df	SS	MS	F Value	P-value
Model	14	164.61	11.76	62.53	<0.0001
(X <sub>1</sub> ): Sucrose (% w/v)	1	12.08	12.08	64.24	<0.0001
(X <sub>2</sub> ): L-asparagine (% w/v)	1	3.72	3.72	19.80	0.0005
(X <sub>3</sub> ): Corn steep liquor (% w/v)	1	0.983	0.983	5.23	0.0372
(X <sub>4</sub> ): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1	2.21	2.21	11.75	0.0037
(X <sub>1</sub> <sup>2</sup> )	1	80.85	80.85	429.97	<0.0001
(X <sub>2</sub> <sup>2</sup> )	1	59.45	59.45	316.17	<0.0001
(X <sub>3</sub> <sup>2</sup> )	1	28.07	28.07	149.28	<0.0001
(X <sub>4</sub> <sup>2</sup> )	1	18.16	18.16	96.56	<0.0001
(X <sub>1</sub> .X <sub>2</sub> )	1	3.04	3.04	16.16	0.0011
(X <sub>1</sub> .X <sub>3</sub> )	1	2.06	2.06	10.94	0.0048
(X <sub>1</sub> .X <sub>4</sub> )	1	0.5837	0.5837	3.10	0.0984
(X <sub>2</sub> .X <sub>3</sub> )	1	1.35	1.35	7.21	0.0170
(X <sub>2</sub> .X <sub>4</sub> )	1	1.07	1.07	5.70	0.0306
(X <sub>3</sub> .X <sub>4</sub> )	1	0.360	0.360	1.91	0.1867
Residual	15	2.82	0.1880	-	-
Lack of Fit	10	2.36	0.2357	2.54	0.1572
Pure error	5	0.4632	0.0926	-	-
Total	29	167.43			

df - Degree of freedom, SS- sum of squares, MS- mean squares.



**Figure 4.3:** Surface plots depicting the optimal range of medium components and their interactions required for maximizing the L-asparaginase production. Plots showing

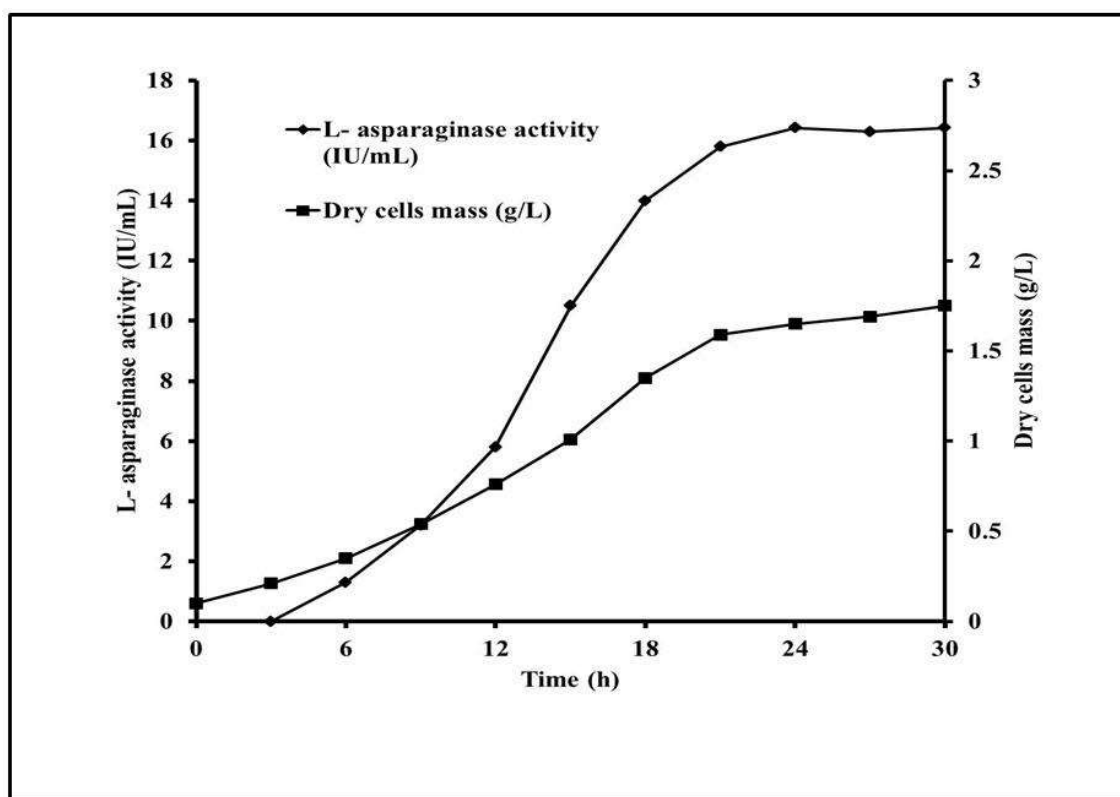
interaction between (a) Sucrose and L-asparagine; (b) Sucrose and corn steep liquor; (c) Sucrose and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; (d) L-asparagine and corn steep liquor; (e) L-asparagine and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; and (f) Corn steep liquor and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Sharma and Mishra, 2023).



**Figure 4.4:** Contour plots depicting the optimal range of medium components and their interactions required for maximizing the L-asparaginase production (Sharma and Mishra, 2023).

#### 4.3.4 Scale-up and validation of the experimental model

The production studies in 3.7 L stirred bioreactor resulted in  $16.42 \pm 0.17$  IU/mL of L-asparaginase enzyme activity at an initial pH of 7 and temperature of 37 °C (Figure 4.5). Cell biomass was also enhanced from 1.57 g/L to 1.75 g/L. The production of L-asparaginase is usually followed by an increase in the pH of the culture medium. A change in pH from 7.0 to 8.8 was observed that indicated the interaction of L-asparaginase with L-asparagine (substrate), leading to the release of ammonia.



**Figure 4.5:** Growth and production profile of L-asparaginase production by *Bacillus indicus* MTCC 4374 using the optimized medium in a laboratory scale bioreactor at an initial pH of 7, temperature of 37 °C, and agitation speed of 200 rpm (Sharma and Mishra, 2023).

#### 4.4 Conclusion

The high costs and the multifarious side-effects associated with the L-asparaginase formulations have sparked the interest to explore new L-asparaginase preparations that possess the desired properties. The present study reports a new and safe L-asparaginase preparation from *Bacillus indicus* MTCC 4374 that causes potent cytotoxicity and apoptosis in leukemia cells. Chapter 4 describes the bench scale L-asparaginase production process in which cheaper substrates, viz. corn steep liquor and sucrose were utilized in comparison to the yeast extract, peptones and glucose. The production process was optimized using the response surface methodology (RSM) approach. Initially, Plackett Burman design (PBD) was utilized for the screening of the significant medium constituents in which four out of eight medium components viz. sucrose, L-Asparagine, corn steep liquor and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  were found to have a significant impact on the L-asparaginase yield. The concentrations of these constituents were then optimized according to the central composite experimental design in which  $15.34 \pm 0.24$  IU/mL of L-asparaginase activity was achieved using 3 g/L of sucrose, 5 g/L of L-asparagine, 10 g/L of corn steep liquor, and 0.8 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . The shake flask optimization studies were then validated in 3.7 L stirred bioreactor which resulted in  $16.42 \pm 0.17$  IU/mL (7 % enhancement) of L-asparaginase enzyme activity in 24 h at an initial pH of 7 and temperature of 37 °C. The outcomes obtained using cheaper substrates can be highly useful for the industrial production of the L-asparaginase enzyme at low-cost.