L-ASPARAGINASE ENZYME PRODUCTION FROM MARINE FUNGUS *BEAUVERIA BASSIANA* – STATISTICAL OPTIMIZATION

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ABSTRACT

Statistical optimization was done to enhance the production of L-asparaginase by submerged fermentation from the marine fungus *Beauveria bassiana,* which was isolated from sediments and corals of sea. Optimization of different process parameters for the production of L-asparaginase and validation using response surface methodology involving central composite design (CCD) was performed with four variables, carbon source, nitrogen source which are essentially important for the growth of the microorganism and other conditions like pH and inoculum level, which include dextrose at 0.43% w/V, L-asparagine 1.1%w/V, pH 7.5 and an inoculum level of 10.2% w/V, respectively. Extracellular production of L-asparaginase by *B. bassiana* and its potential for L-asparaginase production is reported in the present study.

Keywords: Marine *Beauveria bassiana*, L-asparaginase, central composite design, optimization

INTRODUCTION

L-Asparaginase is ubiquitous in nature and produced by fungi, bacteria, yeasts and actinomycetes. Currently, L-asparaginase isolated from *Escherichia coli* and Erwinia sp. is used in the treatment of acute lymphoblastic leukaemia (ALL). Hypersensitivity and inactivation of the immune system are drawbacks for L-asparaginase of bacterial origin[1-2] and that from fungi have better patient compatibility[3] possessing an antitumor property with a non-toxic effect were found be an alternative choice[4]. Marine derived fungi have been reported as a source for novel metabolites and have been explored as L-asparaginase producers in the past[5-6] and several other fungi were isolated from marine environments and endophytes seaweed and also from tropical soil[7].

Asparagine is a nutritional requirement for both normal and tumor cells and is converted to aspartic acid and ammonia. This reaction is catalysed by the enzyme L-asparaginase (L-ASP). The viability of abnormal cells is impacted by reduced levels of asparagine, as opposed to normal cells, which possess the enzyme asparagine synthetase that can produce asparagine from aspartic acid. In contrast, cancer and tumor cells exhibit low levels of this enzyme.

MATERIALS AND METHODS

Microorganism

Beauveria bassiana isolated from sea sediments and corals was used in the present study[8], which was done in A.U. College of Pharmaceutical Sciences, Visakhapatnam. Yeast extract malt extract (YEME) agar medium was used for the maintenance of isolates was at 28 ^{o}C for a period of one week and the slants that were sporulated were preserved at 2-8 ^{0}C in the refrigerator and subcultured monthly and to perform taxonomic studies[8].

Production of enzyme by submerged fermentation (SmF)

SmF was carried out in asparagine dextrose salts (ADS) broth, containing L-asparagine, dextrose, K_2HPO_4 and $MgSO_4.7H_2O$ with concentration of 1.0% w/V, 0.2% w/V, 0.05% w/V and 0.01% w/V, respectively, at pH 7.4 and 28^{0}C, 125 rpm for 8 days for the isolate and the enzymatic activity was performed according to method of Imada et al.[9]

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Scanning electron microscopy

A 2.5% glutaraldehyde solution in a 0.1M phosphate buffer at pH 7.2 was used to treat vials containing microorganism cells. The treatment was carried out for 24 h at a temperature of 4^0C. Over 4 h, a 2% solution of aqueous osmium tetroxide was utilized for post-fixation purposes. Subsequently, dehydration was carried out using alcohol, and critical point drying was performed to facilitate microscopic analysis. The specimens were affixed using dual-sided carbon conductivity adhesive tape and coated with a thin layer of gold via an automated sputter coater (Model: JOEL-JFC-1600). The samples were then examined at the desired level of magnification using a scanning electron microscope (Model: JOEL-JSM 5600). The work mentioned above was conducted by the RUSKA Laboratory, affiliated with the College of Veterinary Sciences at SVVU in Rajendranagar, Hyderabad, India.

Statistical optimization of L-asparaginase production from marine *B. bassiana*

For statistical optimization, Design Expert software of Version 12.0 (Stat-Ease, Minneapolis, USA) was used for studying individual and combined interactive effects of influencing variables on the enzyme production[10].

Central composite design

Thirty experimental runs were carried out by considering four process variables and single response i.e enzyme activity in IU mL^{-1}. Regression, graphical analysis of the data and for the estimation of the coefficients of the regression equation software was used as given by the equation 1. The generalized second order polynomial model used was;

$$Y = \beta o + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} x_i x_j \quad \text{.....(1)}$$

where Y is the predicted response, βo the intercept term, β_i the linear effect, β_{ii} is the squared effect and β_{ij} is the interaction effect of the variables $x_i x_i^2$ and $x_i x_j$ respectively

Purification and characterization of L-asparaginase

The purification step of fermented broth[8] was carried out at cool temperature[11].

Protein precipitation

This step was done using ammonium sulphate[8] (80% saturation), 8.5 pH for 24 h in a cold room, centrifuged at 5000 rpm at cool temperature for 15 minutes and further purification was done with precipitate obtained.

Dialysis

The precipitate obtained in the above step was dissolved in Tris buffer 1 M pH 8.0, until the dialysate was tested negative for ammonia presence. Dialysis membrane-100 procured from Hi-Media was used in the study and the samples were estimated for protein and assay of enzyme after dialysis.

Filtration of gel

Sephadex LH-20 column, which was pre-equilibrated with a 0.01 M TRIS buffer at pH 8.5, was applied to the dialyzed sample. Fractions having shown enzyme activity were pooled and lyophilized.

Determination of molecular weight of L-ASP

SDS-PAGE was performed to the purified fraction for molecular weight determination of the enzyme using molecular markers that are standard. Each sample (50 µL) was loaded into alternate wells and a mixture of different molecular weight markers was used.

RESULTS AND DISCUSSION

Taxonomic studies

Rapid growth rate having 1 to 3 cm colony diameter was observed. Cottony colony texture having white on the front side and yellowish on the reverse side was observed, as shown in Fig. 2. Hyphael conidiogenous cells were extravagant at the base and were typically flask – shaped and terminated in a thin zigzagging filament forming dense clusters appearing as small crumbly balls. Sympodial geniculate growth was observed as shown in Fig. 1. Hyaline conidia, one – celled, globose to ellipsoid with diameter range 2-3 µm was seen.

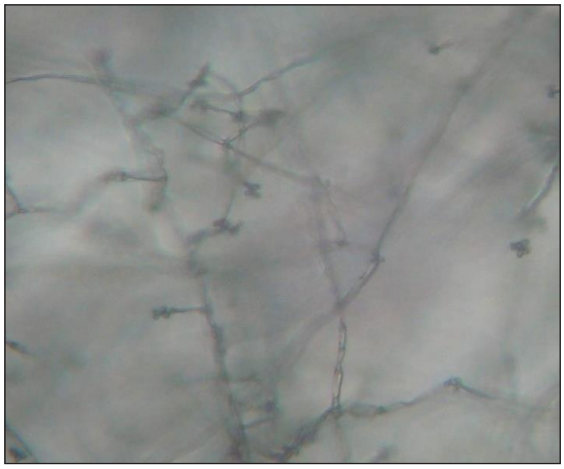
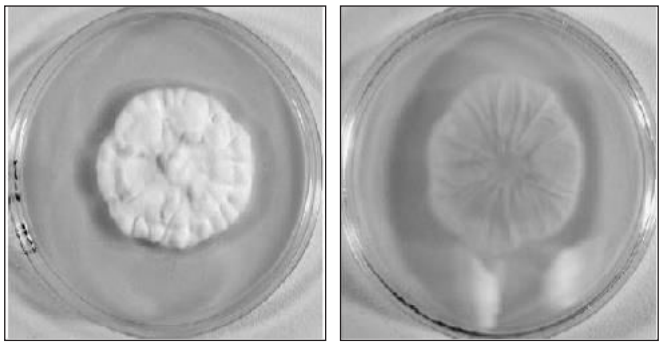


Fig. 1: Microscopic morphology of *B. bassiana* under 400x magnification

Rapid growth rate of *B. bassiana* was observed. The colonies achieve a diameter of 1 to 3 cm following incubation at 25 ^{0}C for about a week on Sabouraud's dextrose agar (SDA). The texture was cottony and the surface was white to yellowish white as shown in Fig. 2.



A. Front side **B. Reverse side**

Fig. 2: White, cotton textured colony of *B. bassiana* on Sabouraud's dextrose agar

The isolate was sent to IMTECH, Chandigarh, India and based on microscopy and macroscopy, the culture was identified and confirmed as *Beauveria bassiana* and was deposited in IMTECH with accession number MTCC 10368.

Scanning electron microscopy

Dense clusters of conidiogenous cells appearing as tiny crumbly balls in the aerial hyphae were observed. The size of the spore was about 2.61-2.92 µm. The scanning electron photograph of *B. bassiana* was shown in Fig. 3.

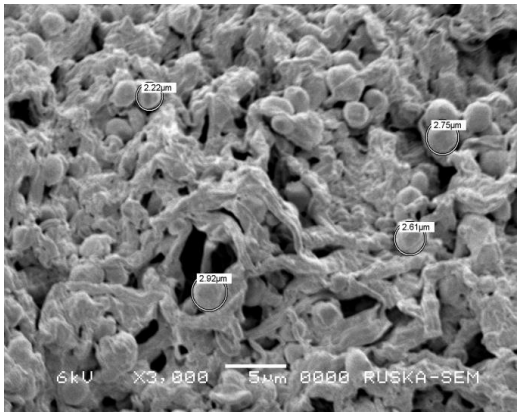


Fig. 3: SEM photograph of *B. bassiana*

Statistical analysis

To analyze the experimental data ANOVA and generate contour plots software was employed. The combination of different optimized parameters, which gave maximum enzyme yield, was tested experimentally for model verification.

In order to optimize L-asparaginase production by *B. bassiana* in pre-defined media, the combined effect of process variables (dextrose, L-asparagine, pH, inoculum level) were assessed using central composite design (Table I). The composition of the experimental design and the responses obtained for each combination of the quantitative variables which were under study were shown in Table II. L-asparaginase activity varied over a range (from 10.44 to 19.65 IU mL^{-1}) depending on the selected variable grouping. Statistics is best fitted inferring good agreement with the experimental data with the model by the determination of R^2 as shown in Table III and Table IV. In this case, the value of the determination coefficient (R^2 =0.9684) indicated that the model could explain 96.84% of the variability in the response. The value of the adjusted determination coefficient (Adj R^2 =0.93) was also very high indicating the significance of the model[12, 13]. The model F-value of 2.60 with a low chance value (P_{model} F >= 0.001) demonstrated a very high significance for the regression model[12- 13].

Table I: Experimental range and levels of variables

Variables	Experiment range and levels				
	-2	-1	0	+1	+2
Dextrose D(% w/V)	0.2	0.3	0.4	0.5	0.6
L-asparagine A (% w/V)	0.8	0.9	1.0	1.1	1.2
pH P	6.5	7.0	7.5	8.0	8.5
Inoculum level I (% V/V)	5	7.5	10	12.5	15

The following is a regression equation (Eq. 2) with enzyme yields and test variables in coded units.

Y=19.31 - 2.16 * D -1.15 * A - 1.39 * P - 4.10 * I + 0.83 * D * D + 2.08 * A*A + 0.52 * P * P + 0.09 * I * I + 0.56* D* A + 0.25 * D * P - 1.02 * D * I - 0.67 * A * P + 0.90 * A *I + 0.98 * P * I. ---------- (2)

The significance of each coefficient of the equation was determined by t- test and p- values and the larger the extent of t- value and smaller the p-value, the more significant is the consequent coefficient in Table III. In the pareto chart it was observed that square terms of inoculum level and pH, interaction terms of dextrose with L-asparagine and pH were not significant, while all other remaining terms were significant (Fig. 4). It was observed that all variables have higher effect at their quadratic terms

particularly inoculum which indicate that each variable had its own significance and they were controlling the fermentation in their own way. These results indicated that the process parameters played a vital role in the production of L-asparaginase by *B. bassiana*.

Contour plots of the response surface, with all other variables held constant at zero, are valuable for comprehending the main effects and interactions of two factors. These plots can be readily generated through a systematic approach to the model, wherein the values of one factor are plotted against a range of values of the second factor (ranging from -2 to +2) while adhering to a specified Y value constraint. The yield values were predicted from contour plots (Fig. 5 to Fig. 10) and found that the optimum concentration of dextrose was 0.43% w/V, L-asparagine 1.1% w/V, optimum pH 7.5 and an optimum inoculum level with 10.2% V/V.

Table II: CCD consisting of 30 experiments

Runs	D	A	P	I	Coefficients assessed by	L-asparaginase activity (IU mL^{-1})	
						Observed response	Predicted response
1	-1	-1	-1	-1	Full factorial design	13.73	13.62
2	1	-1	-1	-1		13.30	12.85
3	-1	1	-1	-1		14.24	13.58
4	1	1	-1	-1		15.04	14.77
5	-1	-1	1	-1		15.13	14.92
6	1	-1	1	-1		15.93	15.96
7	-1	1	1	-1		13.80	13.53
8	1	1	1	-1		17.12	16.53
9	-1	-1	-1	1		14.73	14.67
10	1	-1	-1	1		12.09	11.84
11	-1	1	-1	1		15.68	15.14
12	1	1	-1	1		14.73	14.28
13	-1	-1	1	1		17.33	17.09
14	1	-1	1	1		16.08	16.08
15	-1	1	1	1		16.42	16.21
16	1	1	1	1		17.57	17.16
17	-2	0	0	0	Star points (8 points)	13.46	14.13
18	2	0	0	0		15.31	15.81
19	0	-2	0	0		14.10	14.90
20	0	2	0	0		18.72	19.08
21	0	0	-2	0		15.93	15.98
22	0	0	2	0		15.93	17.03
23	0	0	0	-2		10.44	11.00
24	0	0	0	2		10.57	11.18
25	0	0	0	0	Central points (7 points)	19.50	19.31
26	0	0	0	0		19.27	19.31
27	0	0	0	0		18.73	19.31
28	0	0	0	0		19.12	19.31
29	0	0	0	0		19.64	19.31
30	0	0	0	0		19.57	19.31

Table III: Estimated regression coefficients for yield of L-asparaginase by *B. bassiana*

	Effect	Std. error	t-value	p-value	Coefficient
Mean	19.31	0.25	74.97	0.00	19.31
D	-2.16	0.24	-9.00	0.00	-1.08
D*D	0.83	0.25	3.25	0.005	0.42
A	-1.15	0.24	-4.80	0.0002	-0.57
A*A	2.08	0.25	8.11	0.000001	1.04
P	-1.39	0.24	-5.80	0.00003	-0.69
P*P	0.52	0.25	2.04	0.005	0.26
I	-4.10	0.24	-17.05	0.00000	-2.05
I*I	0.09	0.25	0.35	0.7	0.04
D*A	0.56	0.31	1.78	0.009	0.28
D*P	0.25	0.31	0.81	0.4	0.12
D*I	-1.02	0.31	-3.24	0.005	-0.51
A*P	-0.67	0.31	-2.14	0.04	-0.33
A*I	0.90	0.31	2.87	0.01	0.45
P*I	0.98	0.31	3.11	0.007	0.49
R^2=0.96					
R^2 (adj)=0.93					

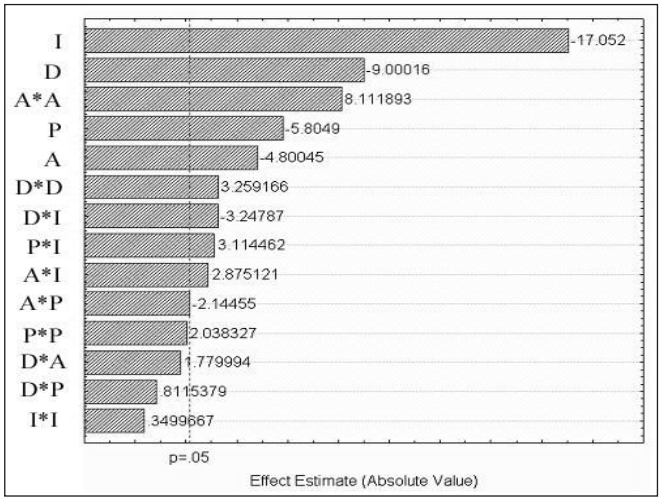


Fig. 4: Pareto chart of standardized effects of *B. bassiana*

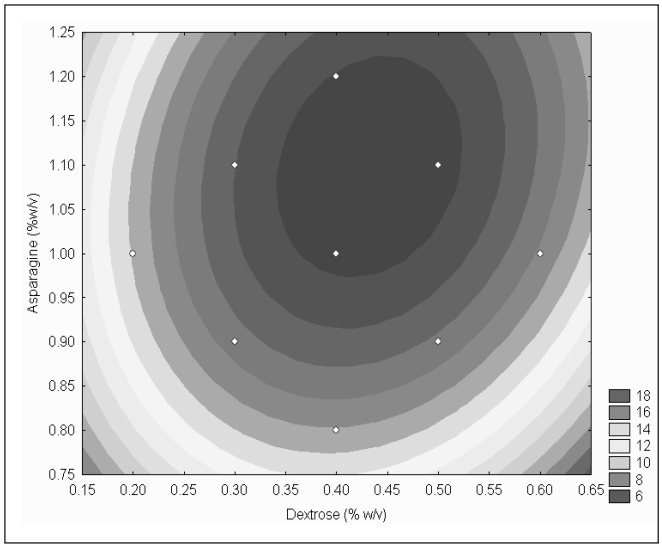


Fig. 5: Contour plot showing the effect of dextrose and asparagine on L-asparaginase production

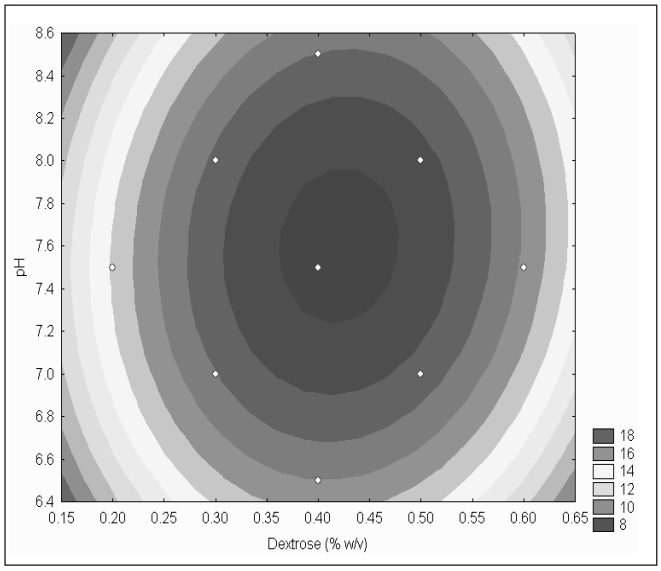


Fig. 6: Contour plot showing the effect of dextrose and pH on L-asparaginase production

Table IV: Purification of L-asparaginase from *B. bassiana*

Purification Steps	Total activity (IU)	Total protein(mg)	Specific activity (IU mg^{-1})	Purification fold	Yield (%)
Fermented broth	90.46	5.2	17.39	----	100
Ammonium sulphate precipitation	56.68	0.8	70.85	4.07	62.60
Dialysis	49.96	0.09	555.11	31.92	55.20
Sephadex LH-20 chromatography	45.85	0.04	1146.2	65.91	50.68

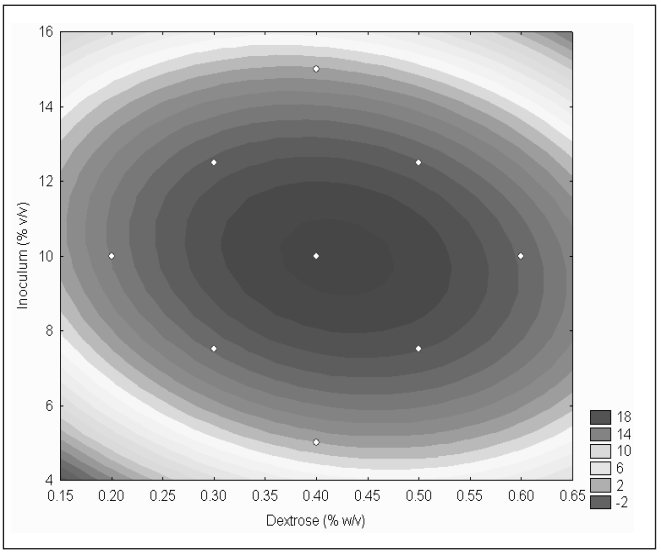


Fig. 7: Contour plot showing the effect of dextrose and inoculum level on enzyme production

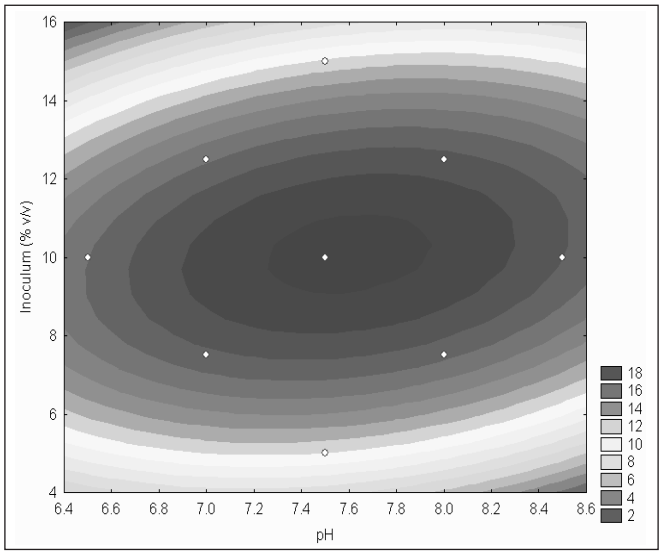


Fig. 9: Contour plot showing the effect of pH and inoculum level on enzyme production

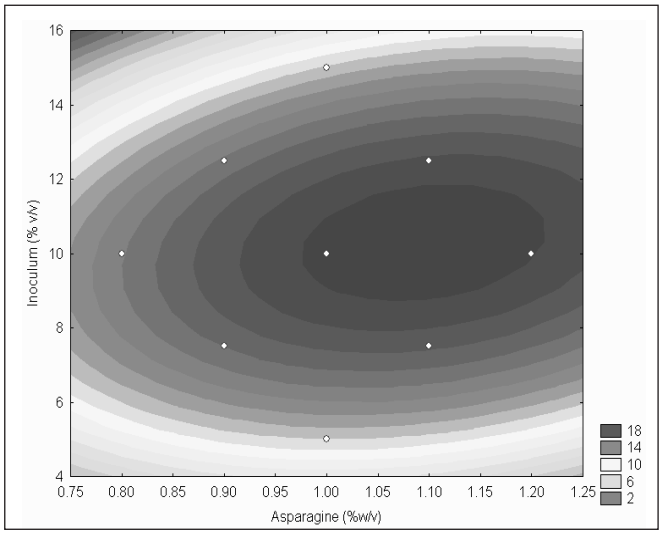


Fig. 8: Contour plot of showing the effect of asparagine and inoculum level on enzyme production

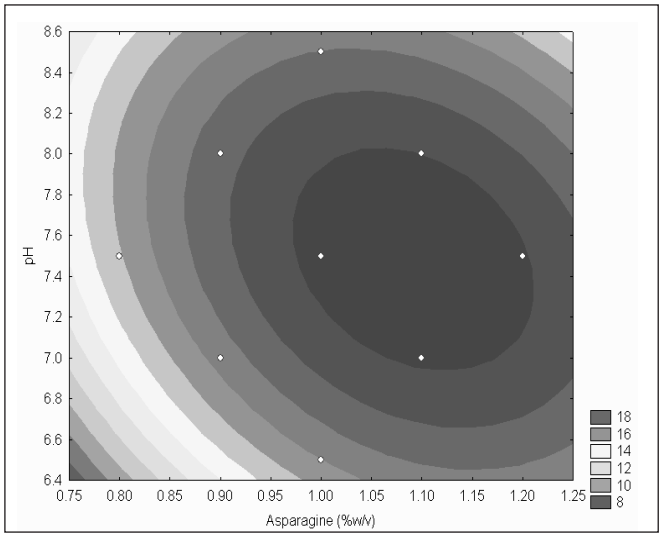


Fig. 10: Contour plot showing the effect of asparagine and pH on L-asparaginase production

Validation of the model

The best possible conditions conventional for the production of L-asparaginase from *B. bassiana* were dextrose, 0.43% w/V; L-asparagine, 1.1% w/V; pH 7.5 and inoculum level of 10.2% V/V. The maximum response to produce enzyme was 19.64 IU mL^{-1} whereas the predicted value was 19.31 IU mL^{-1} indicating a strong agreement between them. To prove that model a significant, the experimental values were found to be very close to the predicted values.

Purification of enzyme from *B. bassiana*

The addition of ammonium sulphate resulted in protein precipitation. Following dialysis and Sephadex LH-20 column chromatography, the enzyme's specific activity increased to 555.11 and 1146.2 IU mg^{-1}, respectively. The Sephadex LH-20 purification step resulted in a 65.91 purity of the enzyme, with a 50.68% recovery rate. Table IV provides a summary of the purification procedures employed for L-asparaginase.

Molecular weight determination of L-Asparaginase

SDS-PAGE of the purified L-asparaginase has shown the presence of a single band related with L-asparaginase enzyme, having a molecular weight of 68 kDa as shown in Fig. 11.

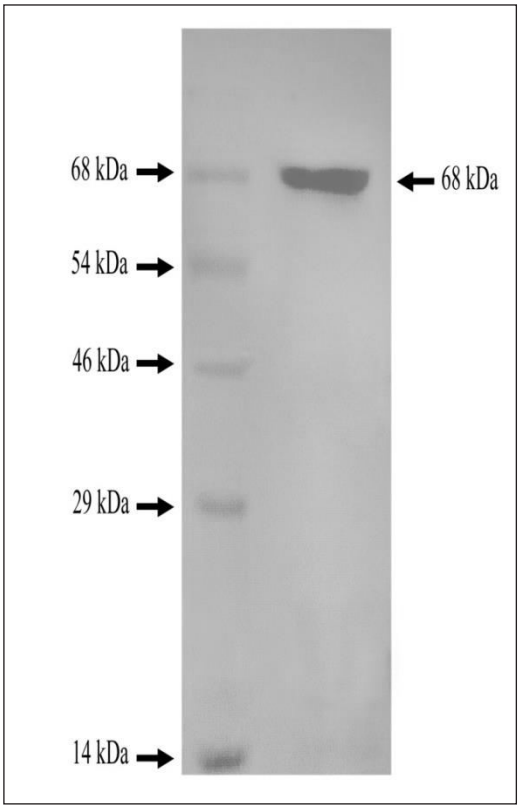


Fig. 11: SDS-PAGE of L-asparaginase obtained from *B. bassiana*

CONCLUSION

Using a conventional method with central composite design (CCD) proved to be a dependable and efficacious approach in optimizing bioprocess variables for enzyme production by *B. bassiana*. This methodology facilitated the identification of statistically significant factors and the determination of optimal concentrations of these factors in the fermentation medium. Contour plots are a valuable tool for effectively visualizing various factors' primary effects and interactions. The Sephadex LH-20 purification step increased the purity of L-asparaginase up to 65.91, with a recovery rate of 50.68%. The findings presented in this study suggest that implementing a central composite design (CCD) may prove advantageous in enhancing the enzymatic activity of *B. bassiana*. Concurrently, the purified enzyme was subjected to SDS-PAGE analysis, which revealed the existence of a solitary band linked to the L-asparaginase enzyme, exhibiting a molecular weight of 68 kDa.

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