Chapter 3

L-asparaginase production using *Aspergillus niger*

3.1 Introduction

The demand for the L-asparaginase (EC 3.5.1.1) enzyme will continue to grow several folds in forthcoming years due to the applications in both the food processing industries as well as a therapeutic drug (El-Naggar and El-Shweihy, 2020). To cater to the huge requirements, it is essential to develop an economically viable production process for the L-asparaginase enzyme. The substrates required for the microbial production of enzyme preparations account upto 30% of the total production cost (Ravindran and Jaiswal, 2016). To make the production process cost-effective, there is considerable interest in researchers to investigate cheaper and readily available agro-industrial substrates (Shakambari *et al.*, 2016). Agroindustrial and food processing by-products possess tremendous potential to act as inexpensive growth substrates due to their nutritional content. These agro-industrial wastes can be directly used as a source of nutrients for the microbial growth and production of different enzymes. Various agro-industrial substrates including bran of pulses, wheat bran, groundnut oil cake, coconut oil cake and many others has been explored and reported by researchers for L-asparaginase production (Mishra, 2006; Kumar *et al.*, 2011; Vala *et al.*, 2018; Ghosh *et al.*, 2013).

Niger (*Guizotia abyssinica*) seed alias nug seed is an oil crop cultivated in India, Ethiopia and other Asian countries (Solomon and Zewdu, 2009). In India, the crop is cultivated majorly for oil extraction that can be utilized for various diverse applications. After the oil extraction step, the leftover byproduct in the form of niger de-oiled cake was obtained in large quantities. The niger de-oiled cake is rich in protein (35-40%) and other organic and inorganic constituents (Bhagya and Sastry, 2005; Solomon and Zewdu, 2009). Moreover, it is free from any toxic factors and can be easily utilized for the growth of microorganisms (Gessesse 1997). Presently, the leftover de-oiled cake is used as animal feed and is available in huge quantities at a very low cost. Several literature studies reported that the utilization of the substrates having higher protein or higher nitrogen content (lower C/N content) have resulted in higher L-asparaginase production (Mishra, 2006; Kumar *et al.*, 2011). The present study reports the novel utilization of the inexpensive, nutrient-rich substrate in the form of niger de-oiled cake for the production of L-asparaginase enzyme with an aim to substantially lower the production cost. Moreover, the present study is the first to experimentally report using the CHNS analysis that the L-asparaginase production is positively influenced by the lower C/N content of the agro-substrate.

Optimization of bioprocesses to achieve the highest product formation can be performed using several methods. Identification of the crucial factors and their optimum levels is the principal function in any optimization study (Singh *et al.*, 2021). Recently, a novel methodology of machine learning using artificial neural networks (ANN) for non-linear multivariate modeling has been utilized to optimize process variables in several studies (Desai *et al.*, 2008; Haider *et al.*, 2008). The ANN approach offers a significant edge over the previously used response surface methodology (RSM) models as ANN can approximate all categorical types of non-linear functions. In contrast, RSM can only be used to solve quadratic functions (Santos *et al.*, 2017; Ekpenyong *et al.*, 2021). Given the light above, the present study holds two primary objectives: (i) judicious selection of low-cost agro-substrate for the cost-effective production of L-asparaginase, (ii) efficient modeling of the physical process parameters using the ANN approach to achieve optimum production of L-asparaginase. The rationale behind the present study of utilizing agro-substrates is their enormous availability and inexpensive source of nutrients that can be utilized to lower the production cost of the valuable therapeutic enzyme.

Experimental

3.2 Materials and methods

3.2.1 Substrates and Microorganism

Different agro-substrates viz., rice husk, wheat bran, niger de-oiled cake, maize bran, linseed de-oiled cake and pea pod husk were procured from the local markets in Varanasi and nearby areas. The substrates were then dried, grounded in a grinder and sieved to obtain homogenous biomass of 1.2 – 1.4 mm size for the enzyme production.

The L-asparaginase producing fungus, *Aspergillus niger* used in the present study was a local isolate obtained from the IIT (BHU), India and previously reported for production of L-asparaginase using bran of pulses (Mishra, 2006). The fungal culture was maintained on Czapek Dox's medium with the following composition (g/l) in distilled water: L-asparagine, 10; glucose, 2; KH_2PO_4, 1.5; KCl, 0.52; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.2; $CuNO_3.3H_2O$, 0.2 and agar, 20; pH 6.2 (Gulati *et al.*, 1997).

3.2.2 Screening for Amidohydrolases activities using Plate Assay

Initial screening for the enzyme activities of various amidohydrolases (asparaginase, glutaminase) on *Aspergillus niger* was performed using the modified Czapek Dox medium with the following composition (g/l): L-asparagine or L- glutamine, 10; Glucose, 2; KH_2PO_4, 1.5; KCl, 0.52; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.2; $CuNO_3.3H_2O$, 0.2; agar, 20 and bromothymol blue dye as pH indicator. Bromothymol blue dye (0.007%) was added into the above-said modified Czapek Dox medium containing L-asparagine and L-glutamine. Bromothymol blue was chosen due to its advantages of producing sharp contrast and a clear region of hydrolysis (from yellow at acidic pH to blue color at alkaline pH) (Mahajan *et al.*, 2013).

3.2.3 Preparation of Substrates

Ten grams of selected agro-substrates were taken in separate Erlenmeyer flasks and moistened with 0.01 M phosphate buffer (pH 6.0). The contents were thoroughly mixed and sterilized by autoclaving at 121 °C, 15 psi for 15 minutes unless otherwise stated. Separate experiments were also performed by autoclaving at different time periods to determine the effects of autoclaving time on the suitable substrate.

3.2.4 Elemental composition analysis

The chemical nature of the substrate directly influences the enzyme production during the fermentation. The selected agro-substrates utilized in the study were analyzed for the determination of the chemical composition (CHNS composition) using the CHNS Elemental Analyzer (Model Euro EA 3000, EUROVECTOR S.P.A, Milano Italy).

3.2.5 Fermentation and extraction

Sterilized solid substrate media in Roux bottles (SSF) were inoculated with 2 ml of spore suspension (1 x 10^7 cfu/ml) and incubated at 30 °C under stationary conditions for 120 hours. The extraction of the crude enzyme was carried out by mixing weighed quantities of the moldy substrate with phosphate buffer (1:5). The contents were stirred and then filtered through Whatman filter paper 1. The filtrate obtained was then centrifuged and the clear supernatant was utilized for enzyme assay.

The production experiments were also carried out under submerged conditions (SmF) in which Erlenmeyer flasks containing ten grams of solid substrates in 50 ml of sterile medium were incubated at 160 rpm in a shaking incubator for 120 hours. The outcomes obtained were compared with the synthetic medium proposed by Gulati *et al.* (1997) containing glucose and L-asparagine. Experiments were performed in triplicates and the estimation of enzymatic activity for the L-asparaginase enzyme was calculated with mean values ± standard deviations. **Figure 3.1** demonstrates the methodology utilized for L-asparaginase production.

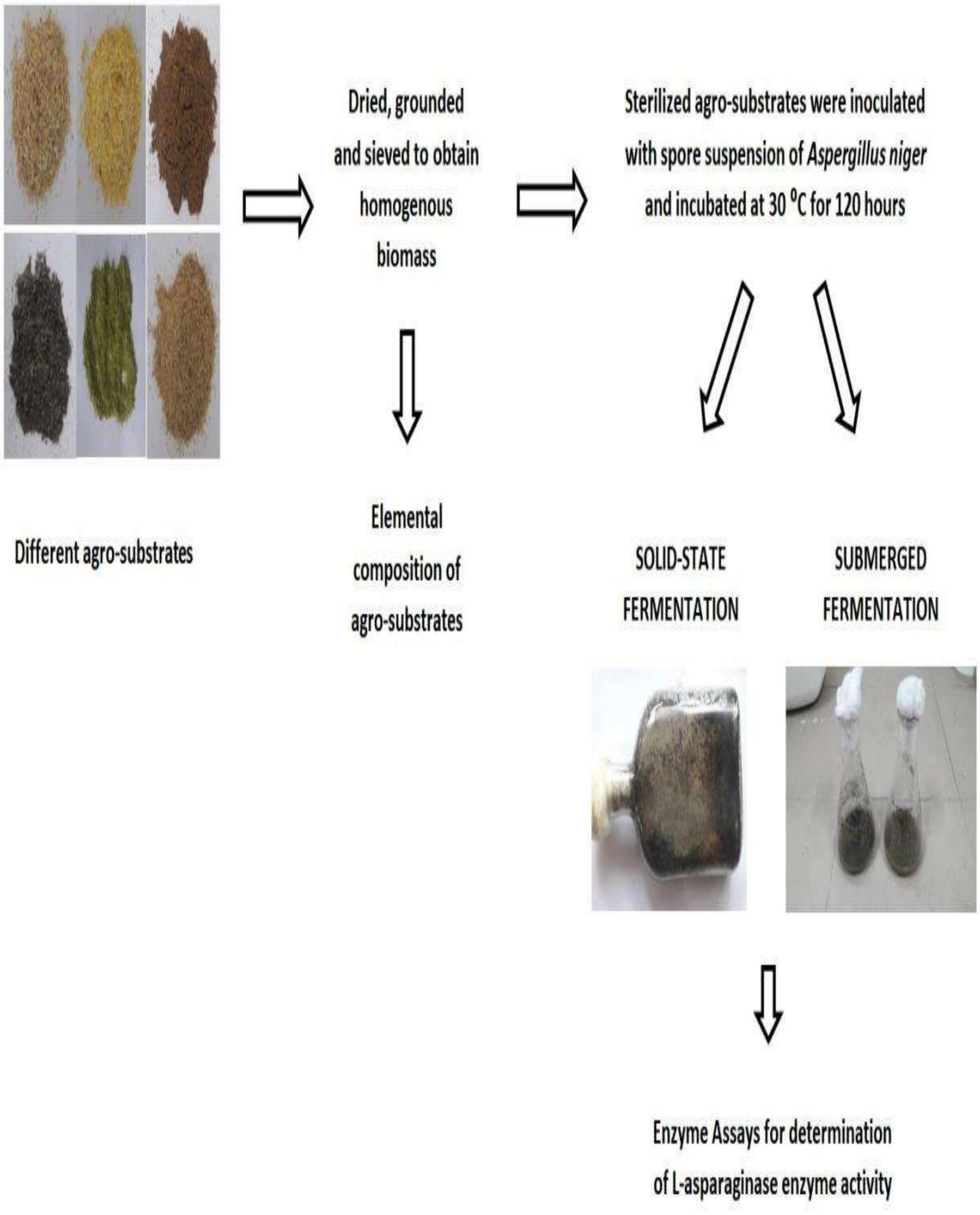

Figure 3.1: Diagrammatic representation of the methodology used for L-asparaginase production.

3.2.6 Assay for the enzymatic activity of L-asparaginase

Quantitative enzyme assays were performed using the β-aspartyl hydroxamate (AHA) method (Drainas *et al.*, 1977; Magri *et al.*, 2018). 4 ml of reaction mixture containing 10 mM L-asparagine as a substrate was mixed with 100 mM hydroxyl ammonium sulphate, 20 mM phosphate buffer, deionized water and cell-free supernatant as a crude enzyme source. The reaction mixture was incubated for 30 minutes at 37 ^{0}C and stopped using a mixture of 5% $FeCl_3$ / 2.5% TCA solution as stopping reagent. The brown coloured reaction product, ferric AHA formed due to the reaction between the AHA and $FeCl_3$ was quantified colorimetrically at 500 nm. A standard curve was prepared by multiple dilutions of standard AHA stock solution (Standard AHA was procured from Sigma Aldrich, USA). The outcomes of experiments were expressed as international unit (IU) of enzyme activity per gram of dry substrate (IU/gds). One IU of L-asparaginase enzyme activity is defined as the amount of particular enzyme needed to form one μmole of β-aspartyl hydroxamate/minute.

3.2.7 Process parameters optimization using RSM and ANN approach

The process variables utilized for the optimization studies were autoclaving time (X_1), moisture content (X_2), temperature (X_3) and pH (X_4), as shown in **Table 3.1**. The RSM and ANN analyses were performed using the Minitab® 18.0 and Matlab R2017a software programs respectively.

Table 3.1: Process parameters selected for SSF of niger de-oiled cake for L-asparaginase production.

Parameters	Levels		
	-1	0	+1
Autoclaving time (min)	20	30	40
Moisture content (%)	50	60	70
Temperature (^{0}C)	25	30	35
pH	5.0	6.0	7.0

The modeling was initiated with the Box-Behnken design (BBD) of experiments consisting of 27 experimental runs to predict the ranges of the process variables and their interactive effects on the asparaginase production. The variables were evaluated at different coded levels -1, 0, +1 in the experimental design, as shown in **Table 3.2**. The experimental runs were utilized to construct a second-order quadratic model for L-asparaginase production in which the interrelation between the outcome and the variables was expressed using the following regression equation

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j$$

Where y is the response variable, β_0 is the constant, n indicates the number of factors, β_i is the linear coefficient, β_{ii} is the quadratic coefficient of input factor X_i and β_{ij} is the interaction coefficient of input factor X_i and X_j.

Table 3.2: Box-Behnken experimental design matrix indicating four factors with their coded and experimental values and the response in terms of experimental and predicted values of L-asparaginase production (The experimental values obtained were mean values of triplicates ± standard deviation).

Run	Physical process parameters and levels				L- asparaginase enzyme Activity (IU/gds)		
	(X_1) (min)	(X_2) (%)	(X_3) (°C)	(X_4)	Experimental Values	RSM Predicted Values	ANN predicted values
1	-1 (20)	-1(50)	0 (30)	0 (6.0)	22.06 ± 1.32	21.178	22.063
2	1 (40)	-1 (50)	0 (30)	0 (6.0)	24.28 ± 1.23	23.523	24.330
3	-1 (20)	1 (70)	0 (30)	0 (6.0)	25.63 ± 1.44	25.316	25.635
4	1 (40)	1 (70)	0 (30)	0 (6.0)	25.31 ± 1.27	25.121	25.312
5	0 (30)	0 (60)	-1 (25)	-1 (5.0)	27.42 ± 1.68	26.088	27.407
6	0 (30)	0 (60)	1 (35)	-1 (5.0)	24.69 ± 1.45	24.348	24.609
7	0 (30)	0 (60)	-1 (25)	1 (7.0)	28.24 ± 2.01	27.511	28.407
8	0 (30)	0 (60)	1 (35)	1 (7.0)	26.82 ± 1.74	27.081	27.797
9	-1 (20)	0 (60)	0 (30)	-1 (5.0)	22.45 ± 1.18	22.848	22.452
10	1 (40)	0 (60)	0 (30)	-1 (5.0)	24.34 ± 1.65	25.263	24.343
11	-1 (20)	0 (60)	0 (30)	1 (7.0)	26.68 ± 1.53	26.266	26.680
12	1 (40)	0 (60)	0 (30)	1 (7.0)	25.89 ± 1.36	26.001	25.893
13	0 (30)	-1 (50)	-1 (25)	0 (6.0)	22.61 ± 0.98	23.388	22.624
14	0 (30)	1 (70)	-1 (25)	0 (6.0)	27.51 ± 1.85	27.591	27.528
15	0 (30)	-1 (50)	1 (35)	0 (6.0)	23.21 ± 1.46	23.638	23.211
16	0 (30)	1 (70)	1 (35)	0 (6.0)	25.44 ± 1.71	25.171	25.440
17	-1 (20)	0 (60)	-1 (25)	0 (6.0)	22.63 ± 1.38	23.555	22.621
18	1 (40)	0 (60)	-1 (25)	0 (6.0)	26.31 ± 1.87	26.585	25.927
19	-1 (20)	0 (60)	1 (35)	0 (6.0)	24.14 ± 1.32	24.425	24.185
20	1 (40)	0 (60)	1 (35)	0 (6.0)	23.91 ± 1.44	23.545	23.844
21	0 (30)	-1 (50)	0 (30)	-1 (5.0)	24.62 ± 1.63	24.731	24.619
22	0 (30)	1 (70)	0 (30)	-1 (5.0)	23.98 ± 1.29	24.220	23.980
23	0 (30)	-1 (50)	0 (30)	1 (7.0)	23.11 ± 1.26	23.430	23.110
24	0 (30)	1 (70)	0 (30)	1 (7.0)	29.23 ± 2.06	29.678	28.419
25	0 (30)	0 (60)	0 (30)	0 (6.0)	34.44 ± 1.74	34.480	34.479
26	0 (30)	0 (60)	0 (30)	0 (6.0)	34.23 ± 1.98	34.480	34.479
27	0 (30)	0 (60)	0 (30)	0 (6.0)	34.77 ± 1.94	34.480	34.479

In addition, a predictive artificial neural network (ANN) model with feed-forward architecture was created, trained and evaluated by utilizing the BBD experimental set. A neural network consisting of an input layer with four independent variables, a hidden layer of 6 neurons and an output layer having enzyme activity as the response was used in the study. Each input in the input layer was assigned a suitable weight (*W*) and bias (*b*). The sum of the weighted inputs and the bias served as the input to the transfer functions. The hidden layer and the output layer utilized the tangent sigmoidal (*tansig*) and the linear (*purelin*) transfer functions respectively for efficient results. The network randomly divided the data into three sets: training set, validation test and test set in the ratio of 70:15:15. The training of neural network was performed using three different training algorithms and the results were compared with the aim to predict the optimum enzyme activity (IU/gds) under the given experimental conditions. The three training algorithms utilized in the current study were Levenberg-Marquardt (LM) algorithm, scaled conjugate gradient (SCG) algorithm and BFGS quasi-Newton algorithm (BFGS). The output generated in the form of predictive bioprocess model using ANN was evaluated for the low value of mean squared error (MSE) (expressed as equation 2), low value of root mean equared error (RMSE) (expressed as equation 3), and high values of correlation coefficient (R) and regression coefficient (R^2).

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(\acute{y}_i - y_i)^2 \tag{2}$$

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(\acute{y}_i - y_i)^2} \tag{3}$$

Where $\acute{y}_i$ and y_i are the experimental and predicted values respectively.

3.2.8 Authentication of the experimentally designed model

The optimized levels of process variables obtained from both the BBD and ANN based optimizers were validated and compared by performing experimental runs in triplicates.

3.3 Results and Discussion

3.3.1 Qualitative screening for asparaginase and glutaminase activities

The microorganism *Aspergillus niger* was qualitatively screened for the amidohydrolases, asparaginase and glutaminase enzymes. Based on the plate assays, the microorganism shows a sharp zone of hydrolysis (appearance of blue color due to the release of ammonium ions) on a medium containing 1% (w/v) L- asparagine as shown in (**Figure 3.2a**). On the glutaminase assay plate, no zone of hydrolysis was observed as indicated by the absence of release of ammonia (no change in color) as shown in (**Figure 3.2b**). Thus, indicating that the major enzyme responsible for the hydrolysis activity was L-asparaginase.

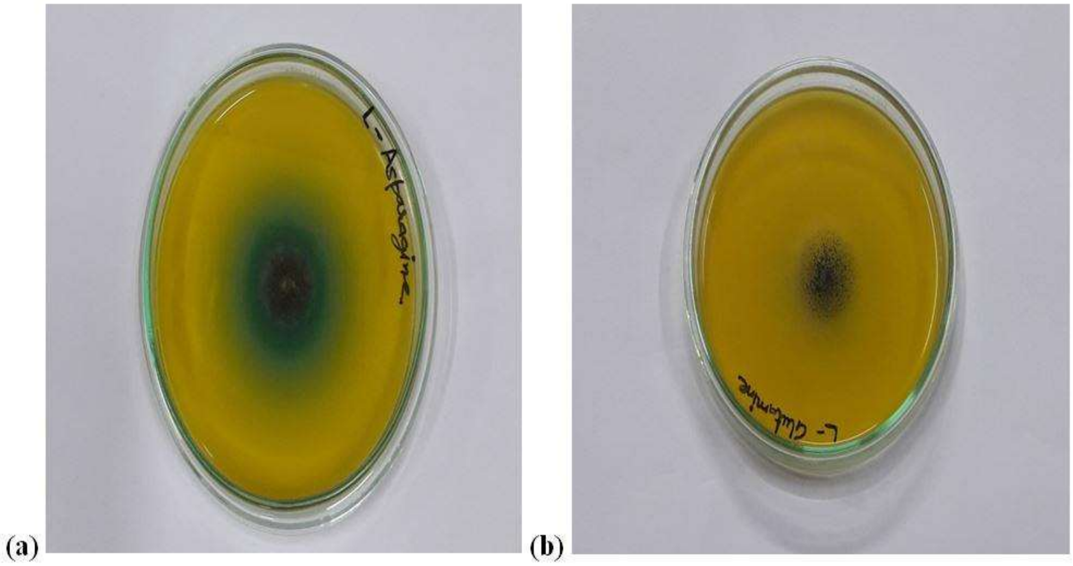

Figure 3.2: Plate assay for screening of L-asparaginase utilizing diverse nitrogen sources. (a) Plate assay showing growth and asparaginase production on the plate containing L-

asparagine. (b) Plate assay indicating the absence of glutaminase production on the plate containing glutamine (Sharma and Mishra, 2022).

3.3.2 L- asparaginase production parameters

The judicious selection of substrates, appropriate microorganism and the optimum levels of physical process parameters are the desired prerequisite for a successful fermentation process (Pandey *et al.*, 2000; Lizardi-Jiménez and Hernández-Martínez, 2017). Initially, the production of L-asparaginase was carried out on several agro-substrates at 30°C with initial moisture (%) of 70%, autoclaving time of 15 minutes, 5.5 pH for 6 days in solid state fermentation (SSF) and also under submerged conditions in the submerged fermentation (SmF). Among the various agro-substrates tested in SSF, maximum production of enzyme (25.42 ± 1.65 IU/gds) was obtained using niger de-oiled cake followed by maize bran (12.03 ± 0.9 IU/gds) and pea pod husk (10.17 ± 1.1 IU/gds). Wheat bran and linseed de-oiled cake resulted in enzyme production of 9.13 ± 0.82 IU/gds and 7.94 ± 0.45 IU/gds respectively. The lowest enzyme production was obtained using rice husk (1.94 ± 0.23 IU/gds) as substrate. The variations observed in enzyme activities distinctly demonstrate the importance of substrate chosen for enzyme production (**Figure 3.3**).

Under submerged conditions (SmF process) in the present study, lower L-asparaginase enzyme titres were observed on all the above tested agro-industrial substrates. Moreover, on utilizing synthetic medium, the L-asparaginase production using *Aspergillus niger* was still low with an enzyme activity of 0.11 ± 0.02 IU/mL. Similar observations were reported by Gulati *et al.* (1997) in which an enzyme activity of 0.07 IU/mL was obtained. Therefore, further part of the study was carried out by focussing on the solid-state fermentation methodology for the L-asparaginase production using agro-industrial wastes. Many previous studies on the different enzyme production processes have reported higher productivities in SSF than Smf processes (Diaz-Godinez *et al.*, 2001; Elinbaum *et al.*, 2002).

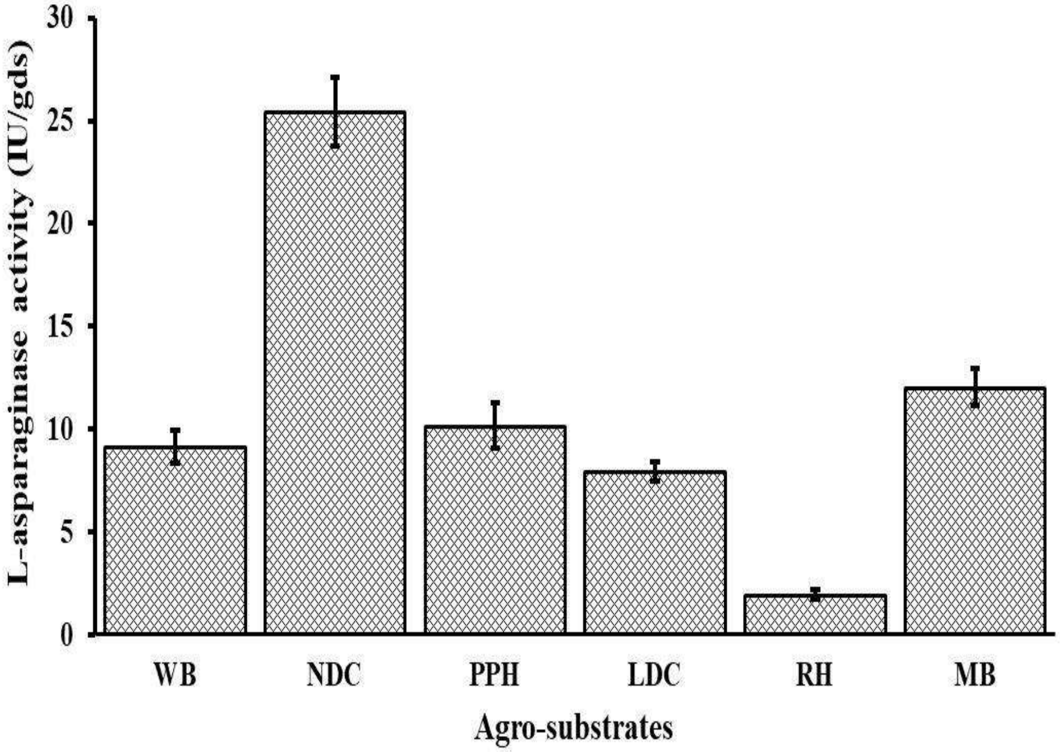

Figure 3.3: Effect of agro-substrates on L-asparaginase production. WB: wheat bran, NDC: niger de-oiled cake, PPH: pea pod husk, LDC: linseed de-oiled cake, RH: rice husk, MB: maize bran (Sharma and Mishra, 2022).

Moreover, it has been reported in several studies that the substrates having high protein content or higher nitrogen content (lower C/N content) have a positive impact on L-asparaginase production (Mishra, 2006; Kumar *et al.*, 2011). The primary factor responsible for the high asparaginase activity from niger de-oiled cake is the high protein content ranging from 35-40%. The niger de-oiled cake is also a low cost, excellent substrate for microbial growth and therefore possesses immense potential in bioprocess industries (Gessesse, 1997; Solomon and Zewdu, 2009).

3.3.3 Elemental composition analysis of the agro-substrates

In view of the light, the chemical composition of the various substrates was also investigated by performing the CHNS analysis on elemental analyser. The significant variation in enzyme activity seen using different substrates was attributed to the different chemical compositions in terms of C, H, N and S content (**Table 3.3**). The agro-substrates possessing high nitrogen (%) and lower C/N content served as better substrates for L-asparaginase production. Similar observations were seen in our previous study in which bran of leguminous pulses were utilized for the production of L-asparaginase enzyme (Mishra, 2006).

Table 3.3: Chemical composition (CHNS analysis) of various agro-industrial wastes used in the study.

Agro-substrate	Nitrogen (%)	Carbon (%)	Hydrogen (%)
Niger de-oiled cake	8.949	52.234	5.241
Maize bran	6.131	43.036	6.066
Wheat bran	4.648	43.144	5.070
Linseed de-oiled cake	6.265	45.994	5.370
Rice husk	1.419	42.240	5.762
Pea pod husk	5.605	39.675	6.080

3.3.4 Process modeling using statistical (RSM) and machine learning (ANN) models

After the selection of the most suitable substrate in the form of niger de-oiled cake, the influence of the process parameters viz. autoclaving time, moisture content, temperature and pH were investigated and compared using the RSM and ANN modeling with the goal to achieve the increased L-asparaginase production.

For the RSM study, the Box Behnken design was utilized to construct a second-order quadratic model for L-asparaginase production. The enzyme activity obtained during the experiments varied from 22.06 ± 1.32 IU/gds to 34.77 ± 1.94 IU/gds. Application of multiple regression analysis on the experimental outcomes resulted in the formation of a second-order polynomial equation that explains the production of L-asparaginase enzyme using niger meal as substrate.

$$Y_{activity} = -502.2 + 4.758\ X_1 + 5.885\ X_2 + 11.43\ X_3 + 36.87\ X_4 - 0.05558\ X_1^2 - 0.05138\ X_2^2 - 0.1758\ X_3^2 - 3.828\ X_4^2 - 0.00635\ X_1{*}\ X_2 - 0.01955\ X_1{*}\ X_3 - 0.0670\ X_1{*}\ X_4 - 0.01335\ X_2{*}X_3 + 0.1690\ X_2{*}\ X_4 + 0.0655\ X_3{*}\ X_4 \qquad (2)$$

where X_1 is autoclaving time (min), X_2 is moisture content (%), X_3 is temperature (°C) and X_4 is pH.

Table 3.4: ANOVA studies for the quadratic model.

Source	DF	Adj. SS	Adj. MS	F Value	P-value
Model	14	318.307	22.736	35.42	<0.0001
(X_1) : Autoclaving time (min)	1	3.467	3.467	5.40	0.038
(X_2) : Moisture content (%)	1	24.682	24.682	38.45	<0.0001
(X_3) : Temperature (°C)	1	3.532	3.532	5.50	0.037
(X_4) : pH	1	12.958	12.958	20.19	0.001
(X_1^2)	1	164.724	164.724	256.63	<0.0001
(X_2^2)	1	140.768	140.768	219.31	<0.0001
(X_3^2)	1	103.019	103.019	160.50	<0.0001
(X_4^2)	1	78.132	78.132	121.73	<0.0001
($X_1.X_2$)	1	1.613	1.613	2.51	0.139
($X_1.X_3$)	1	3.822	3.822	5.95	0.031
($X_1.X_4$)	1	1.796	1.796	2.80	0.120
($X_2.X_3$)	1	1.782	1.782	2.78	0.122
($X_2.X_4$)	1	11.424	11.424	17.80	0.001
($X_3.X_4$)	1	0.429	0.429	0.67	0.430
Error	12	7.702	0.642	-	-
Lack of Fit	10	7.554	0.755	10.19	0.093
Pure error	2	0.148	0.074	-	-
Total	26	326.009			

* DF - Degree of freedom, Adj.SS- Adjusted sum of squares, Adj. MS- Adjusted mean of squares.

The results obtained from the experimental design depicted that the moisture content (*P*-value < 0.001) was the most critical factor in influencing the SSF production process. The model terms (linear and quadratic) of variables were found to be highly significant as indicated by their *P* values in **Table 3.4**. Fisher F-test value of 35.42 signifies that the model was highly significant. The lack-of-fit (LOF) *P*-value of the model was found to be 0.093 (insignificant) which signified that the regression model adequately explains the functional interdependence between the experimental factors and the response values. The RSM model depicted R^2 value of 97.64%, adjusted R^2 of 94.88%, and predicted R^2 value of 86.55% respectively. The surface plots were prepared and evaluated to ascertain the optimal levels of physical process parameters affecting the L-asparaginase production using niger de-oiled cake as an SSF substrate (**Figure 3.4**).

The ANN modeling initiated with ANN architecture consisting of 4 inputs (above-mentioned process parameters) and enzyme activity (IU/gds) as output and was utilized to generate the predictive bioprocess model (**Figure 3.5**). The training of the network was performed using three feedforward back propagation algorithms (LM, SCG and BFGS). The LM algorithm or damped least square method is a widely used training algorithm for functions that involves sum of squared errors and is implemented using trainlm function in neural networks (Suryawanshi *et al.*, 2019). The SCG algorithm is an advanced method of supervised learning that avoids cumbersome line search approach and is implemented using the trainscg function in neural network (Suryawanshi *et al.*, 2019). Similarly, BFGS quasi-newton algorithm is a widely used method for faster convergence of the neural networks and is implemented using the trainbfg function (Robitaille *et al.*, 1996).

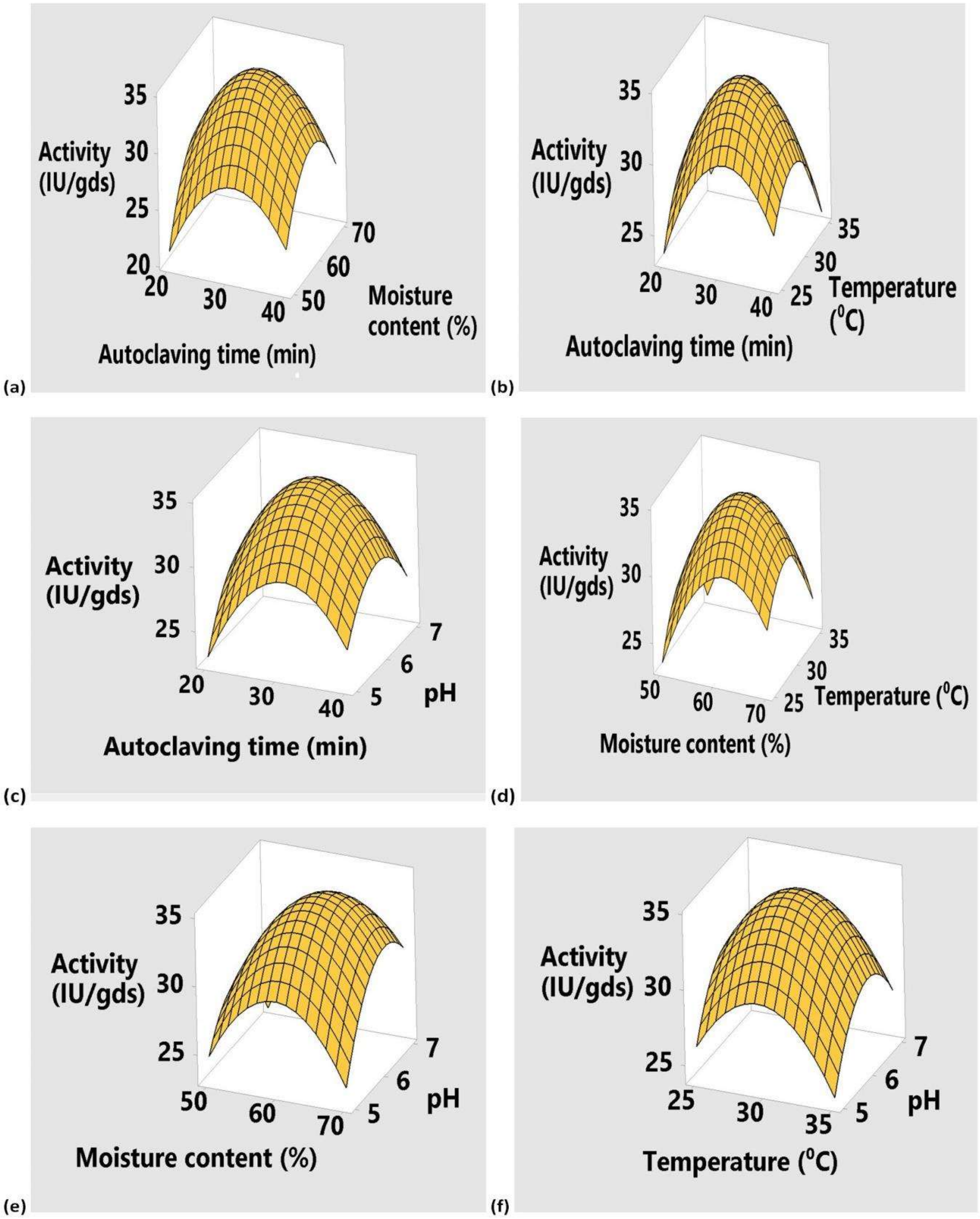

Figure 3.4: Response surface plots of the quadratic model depicting the interactive effects between the process parameters. Plots showing interaction between (a) autoclaving time (min) and moisture content (%) (b) autoclaving time (min) and temperature (⁰C) (c)

autoclaving time (min) and pH (d) moisture content (%) and temperature (ºC) (e) moisture content (%) and pH (f) temperature (ºC) and pH (Sharma and Mishra, 2022).

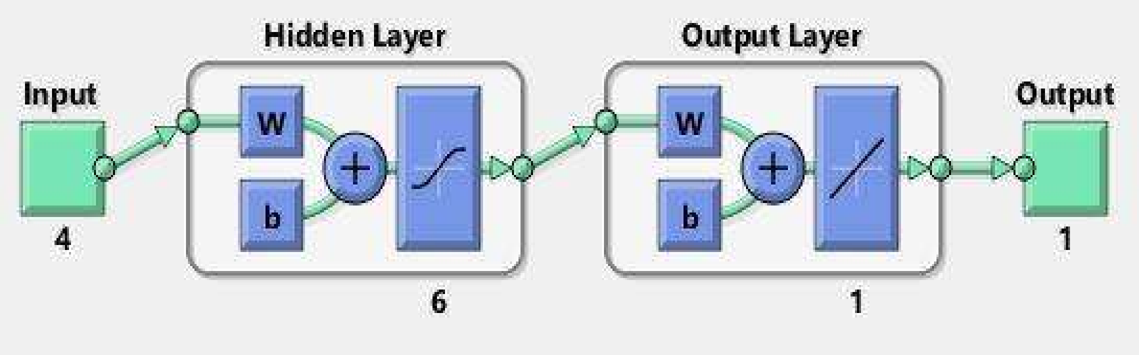

Figure 3.5: Neural network topology consisting of 4 inputs, 6 hidden neurons, 1 output (4-6-1) (Sharma and Mishra, 2022).

The regression plots obtained using the tested training algorithms were compared to select the best training algorithm. The regression plot obtained using the LM training algorithm depicted an overall R-value of 0.997 for all the data sets (**Figure 3.6a**). The regression plot acquired using the SCG algorithm represented an overall R¯value of 0.98 as shown in **Figure 3.7a**. The overall R value obtained using the BFGS training algorithm was 0.990, but the R-value for the test data set was 0.934 as shown in **Figure 3.8a**. In comparison, it can be seen that LM algorithm performed better than the SCG and BFGS training algorithms by attaining high R values for all the training, test and validation data sets. Moreover, it can be seen from the performance plot of LM algorithm (**Figure 3.6b**), that the mean square error for all the three data sets (training, validation and test data sets) was sufficiently low, in comparison to the mean square error for all the three data sets in the performance plots of SCG (**Figure 3.7b**) and BFGS algorithms (**Figure 3.8b**). In another study on process optimization using ANN, researchers have compared three different training algorithms and found the best optimization results using Levenberg-Marquardt algorithm (Suryawanshi *et al.*, 2019).

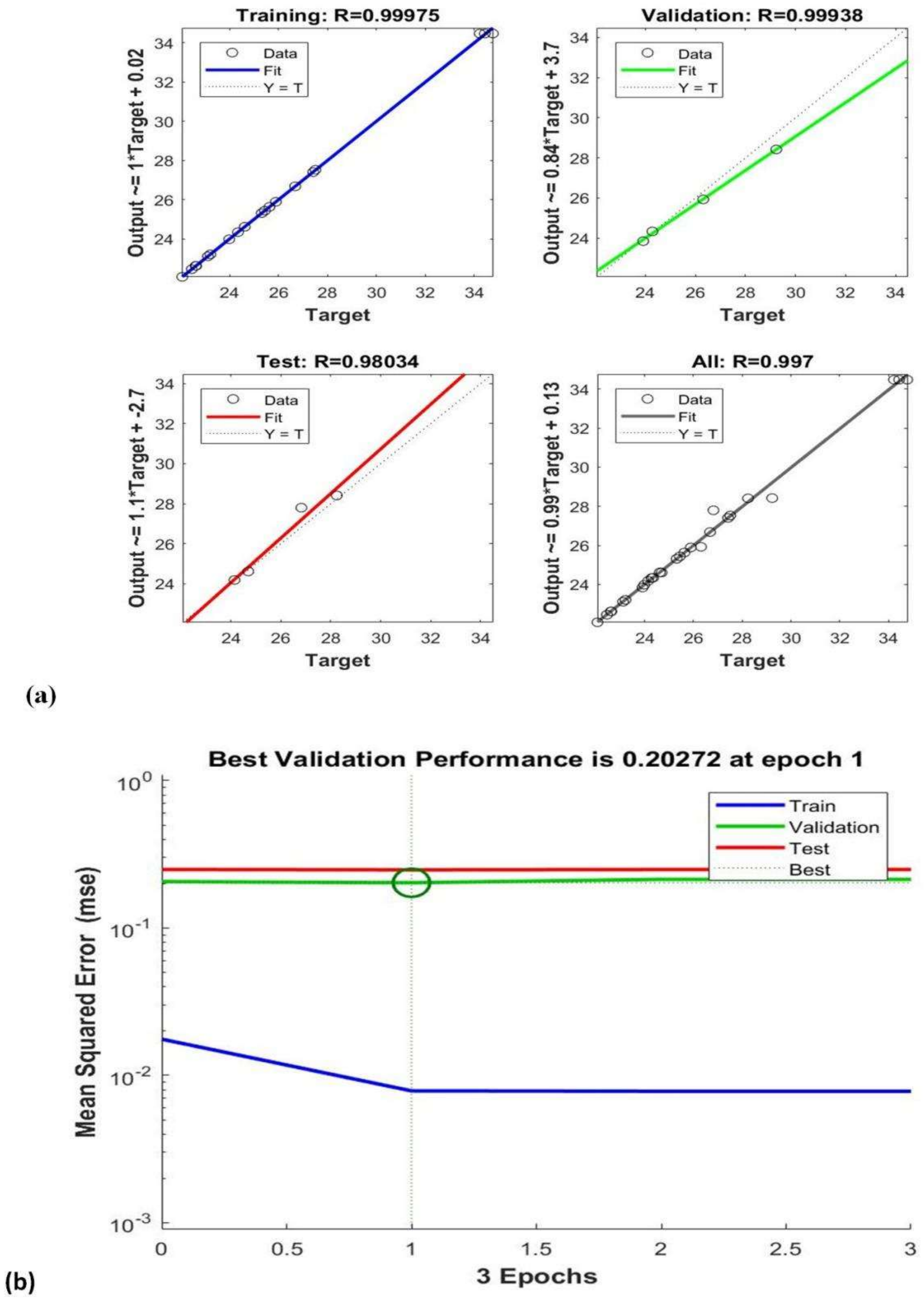

Figure 3.6: Outputs of a neural network trained with Levenberg-Marquardt (LM) training algorithm (a) Regression plot depicting R values for the training, validation, and test data sets. (b) Performance plot showing mean square error of training, validation and test data sets (Sharma and Mishra, 2022).

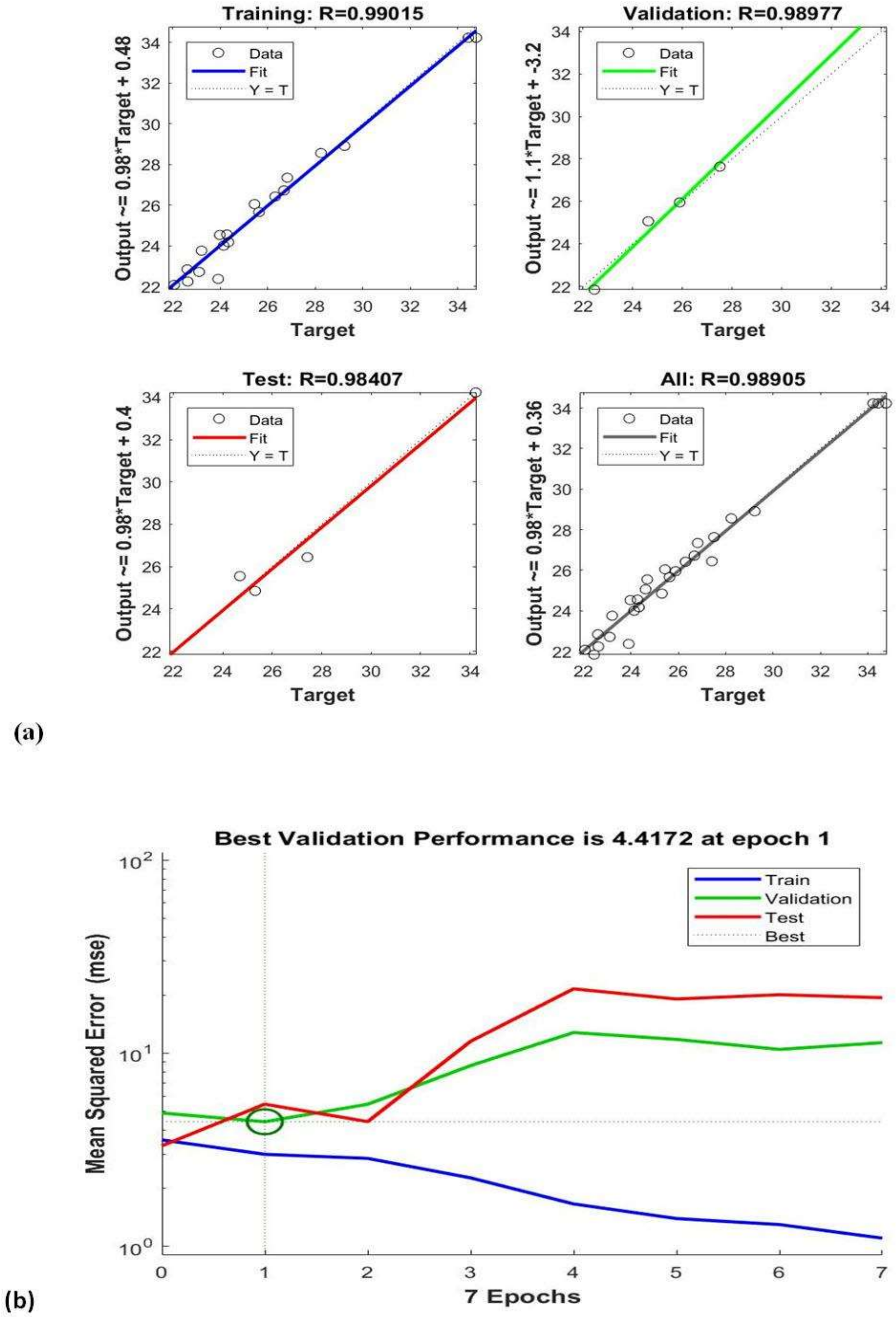

Figure 3.7: Outputs of a neural network trained with scaled conjugate gradient (SCG) training algorithm (a) Regression plot depicting R values for the training, validation, and test data sets. (b) Performance plot depicting mean square error of training, validation and test data sets (Sharma and Mishra, 2022).

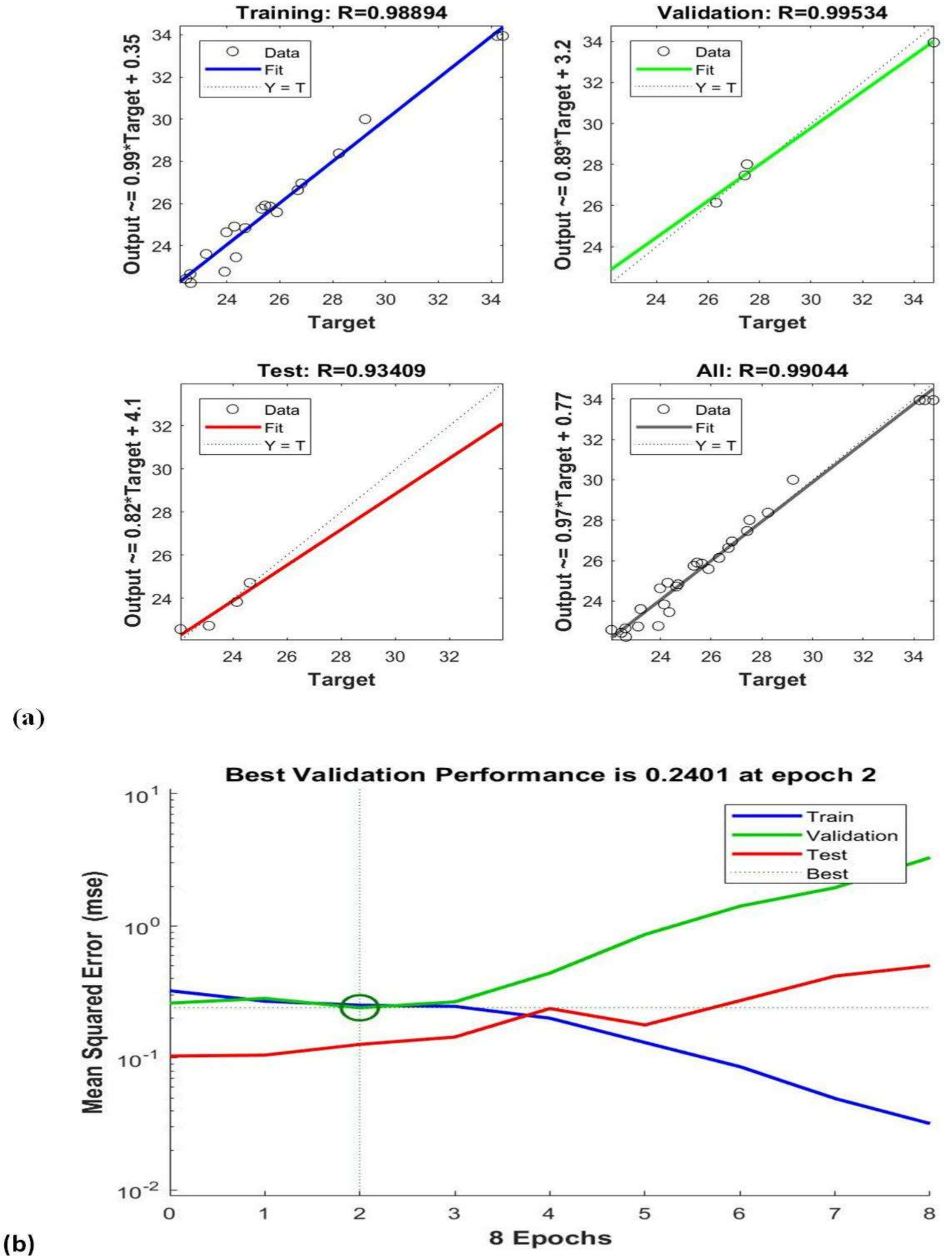

Figure 3.8: Outputs of a neural network trained with BFGS quasi-Newton (BFGS) training algorithm (a) Regression plot depicting R values for the training, validation, and test data sets. (b) Performance plot depicting mean square error of training, validation and test data sets (Sharma and Mishra, 2022).

The predicted results obtained from ANN using LM training algorithm are denoted in **Table 3.2**. The optimum network weights and biases used for the 4-6-1 topology of constructed ANN are denoted in **Table 3.5**. The predictive outcomes obtained using ANN (LM algorithm) demonstrated the mean squared error of 0.072 and root means squared error of 0.268 in the generated model. The results obtained using RSM showed higher deviations than the experimental data sets compared to ANN by depicting 0.285 value of mean squared error and 0.534 value of root means squared error.

Table 3.5: Optimum weights and biases for implementation of 4-6-1 ANN topology.

Input (4)- Hidden layer (6)					Hidden layer (6)-Output(1)	
Weights				Bias (b1)	Weights	Bias (b2)
0.4441	-0.0345	-0.8729	1.7857	-2.4943	0.4176	-1.1047
-3.6173	-0.7394	-1.3116	-2.3693	3.3023	0.8782	
-0.4252	4.7952	1.6269	1.8452	1.564	0.8134	
1.6979	-0.0140	2.517	-1.4302	1.7772	0.7567	
-1.4646	0.5752	2.1975	-3.7435	-2.156	-0.9082	
-0.0480	1.7804	0.5930	2.0864	2.3526	-0.8206	

The ANN model also revealed better results than the RSM model in terms of regression coefficient (R^2). The ANN and RSM models depicted regression coefficients of 0.994 and 0.976 respectively. Many research studies have previously expressed the superiority of the artificial neural network (ANN) models over the traditional RSM strategy in the optimization of fermentation process parameters (Nelofer *et al.*, 2012; Venkateswarulu *et al.*, 2017; Mukherjee *et al.*, 2019).

3.3.5 Effects of process parameters on L-asparaginase production in SSF

The effects of the process parameters on the enzyme production can be evaluated through the outcomes obtained during the experimental runs. It can be seen that the variations in the autoclaving time of the substrate lead to significant differences in the L-asparaginase enzyme production. Maximum enzyme activity was observed at 30 minutes of autoclaving (**Table 3.2**). Low enzyme production was observed both at 20 minutes and 40 minutes of autoclaving at 121 ^{o}C. It can be inferred from the outcomes that the autoclaving of the solid substrate for shorter time period than the optimum leads to incomplete breakdown of nutrients, whereas autoclaving for more extended periods than the optimum triggers lowered nutrient availability.

Variations in the moisture content (%) lead to significant differences in the L-asparaginase production. The crucial importance of the moisture levels on the enzyme production was evident by its p-value <0.0001. The moisture content of 62% was found to be optimal and a further increase in moisture (%) resulted in lesser enzyme production. High moisture levels tend to have a negative impact on enzyme production due to the reduction in the porosity of the substrate, structural changes in the substrate particles and lower gas exchange. Also, low moisture content than the optimum results in the reduction of the substrate swelling and nutrient solubility (Boratyński *et al.*, 2018).

pH is a significant factor influencing any microbial bioprocess. From the experimental results, it can be seen that a pH of 6.2 was found optimum for microbial L-asparaginase production using niger de-oiled cake as substrate. Although little loss of enzyme activity was observed at neutral pH, but significant amount of enzyme activity is retained. In another study on SSF, optimum pH of 7.0 was reported using *Fusarium culmorum* on soybean meal as substrate (Meghavarnam *et al.*, 2017). Similarly, the optimum incubation temperature was found to be 30°C in our study of SSF. In another study on the L-asparaginase from *Aspergillus niger*, the enzyme was quite stable in the range from 20-40 °C (Vala *et al.*, 2018). Studies carried out by other researchers on L-asparaginase reported 25°C as optimum using passion fruit peel flour as SSF substrate (da Cunha *et al.*, 2018). The significant enzyme activity observed at near physiological pH and temperature makes the L-asparaginase enzyme from *Aspergillus niger* an attractive candidate for pharmaceutical sector.

3.3.6 Validation of experimentally designed models

Experiments were conducted at optimum levels of process parameters provided by the response optimizer tool of RSM in Minitab and the ANN model (trained with LM algorithm) in Matlab for the maximization of the L-asparaginase production. The response optimizer of RSM and the ANN model predicted the enzyme activity of 34.71 IU/gds and 34.63 IU/gds respectively at the optimized level of process parameters: autoclaving time (X_1) of 30.3 min, moisture content (X_2) of 62%, temperature (X_3) of 30°C and 6.2 pH (X_4). The experimental enzyme activity of 34.65 ± 2.18 IU/gds was observed which is very close to ANN predicted activity in 96 hours of fermentation time with a productivity of 0.36 IU/gds/h.

A 1.36 fold improvement in enzyme activity was observed with optimized parameters when compared to the initial (unoptimized) conditions for production: autoclaving time of 15 min, moisture content of 70%, temperature of 30°C and pH 5.5 that resulted in enzyme activity of 25.42 ± 1.65 IU/gds using niger de-oiled cake. Various studies have been conducted by researchers in which the production of L-asparaginase was explored using different agro-industrial wastes. In one study, researchers have reported the maximum L-asparaginase production of 3.74 IU using wheat bran as substrate from *Cladosporium sp (*Kumar *et al.*, 2011). Another study reported the L-asparaginase production of 5.86 IU/gds and 7.21 IU/gds using coconut oil cake and soybean meal respectively (Ghosh *et al.*, 2013; Meghavarnam *et* *al.*, 2017). Shakambari *et al.*, (2017) reported the L-asparaginase activity of 1.40 ± 0.04 IU/ml by utilizing agricultural wastes in the form of onion and garlic peels. Apart from our study using the fungus *Aspergillus niger*, the production of L-asparaginase enzyme has also been explored in various *Aspergillus* sp. under both submerged and solid-state fermentation conditions. Costa-Silva *et al.*, (2019) reported the L-asparaginase production using *Aspergillus terreus* CCT 7693 and obtained a specific activity of 13.81 IU/g under submerged fermentation conditions. In another study, L-asparaginase enzyme activity of 20.58 IU/gds was reported using *Aspergillus tubingensis* IBBL1 when grown on a tri-substrate mixture consisting of cottonseed cake, wheat bran and red gram husk (Doriya *et al.*, 2018).

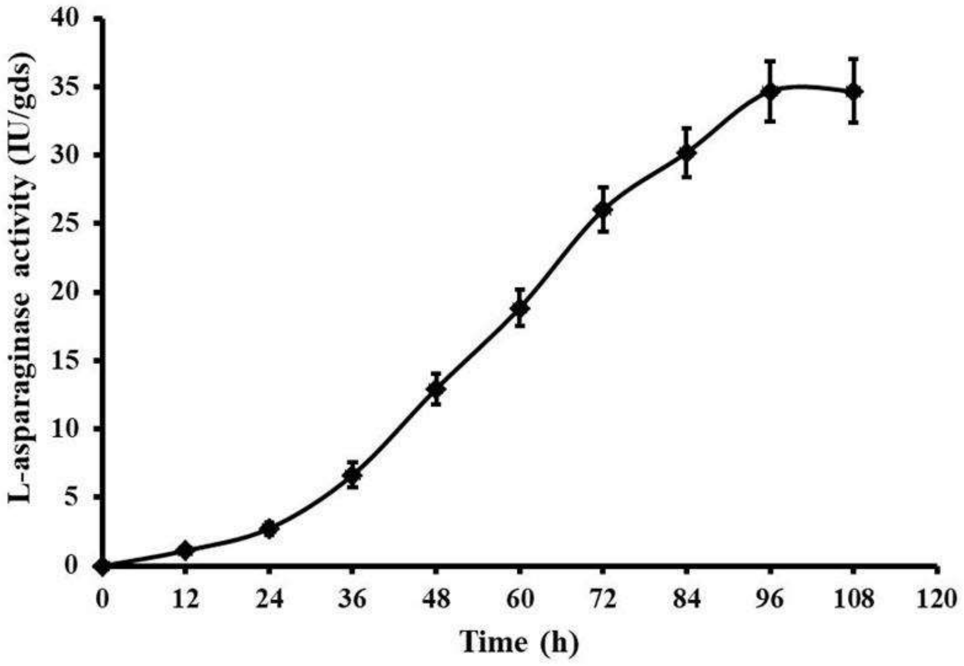

Figure 3.9: Production time profile of L-asparaginase enzyme using niger de-oiled cake as substrate at temperature of 30⁰C and 6.2 pH (Sharma and Mishra, 2022). Difference significant: $p < 0.05$ (analysis performed using two-way ANOVA).

Table 3.6: Effect of fermentation time on L-asparaginase production.

Fermentation time (h)	Enzyme activity (IU/gds)[a]
0	0
12	1.12 ± 0.2
24	2.74 ± 0.46
36	6.65 ± 0.78
48	12.94 ± 1.13
60	18.85 ± 1.3
72	26.02 ± 1.6
84	30.21 ± 1.8
96	34.65 ± 2.18
108	34. 63 ± 2.24

[a] Difference significant: $p< 0.05$ (analysis performed using two-way ANOVA)

Both the ANN and RSM models showed good predictions but the ANN model performed better than RSM model due to the close prediction with obtained experimental values, low root means square error (RMSE) and high value of regression coefficient. **Table 3.6** shows the effect of fermentation time on L-asparaginase production using niger de-oiled cake as substrate and the results were depicted as means ± standard deviations. Two way analysis of variance (ANOVA) was performed to assess the significant differences between the means for each fermentation time. Significant differences were observed as p value<0.05 was obtained. The production kinetics profile in **Figure 3.8** demonstrates that the fermentation period of 96 h was optimal for the maximum production of L-asparaginase enzyme. The niger de-oiled cake (agricultural waste biomass) can be efficiently utilized as a sole source of nutrients to produce the L-asparaginase enzyme at an economical cost.

In terms of protein content, the niger de-oiled cake used in our study is better than other reported agro-industrial substrates like bran of different pulses (19-22%), coconut oil cake (25-26%) and wheat bran (9.6-18.6%) (Mishra, 2006; Sadh *et al.*, 2018; Onipe *et al.*, 2015). In terms of cost economics with other agro-industrial residues, the price of niger de-oiled cake (US $ 200-230 per ton) is comparable to wheat bran (US $ 210-225 per ton) but cheaper than coconut oil cake (US $ 310-340 per ton) and different bran of pulses (US $ 250-260 per ton) that has been reported for L-asparaginase production. Moreover, the cost of niger de-oiled cake is much cheaper than each of the constituents of semi-synthetic medium (glucose, L-asparagine and a complex nitrogen source) used for L-asparaginase production.

3.4 Conclusion

In the present study, a readily available and inexpensive agroindustrial byproduct was valorized into value added product through microbial route. A waste of the food processing industry in the form of niger (*Guizotia abyssinica*) de-oiled cake was utilized for growth and L-asparaginase production. The agro-substrate niger de-oiled cake on elemental analysis exhibited lower C/N content that resulted in the higher production of L-asparaginase enzyme. Moreover, the substrate can be utilized as a sole source (without the addition of supplements) for the L-asparaginase production. Enzyme activity of 34.65 ± 2.18 IU/gds was achieved in optimization studies using machine-learning based ANN technique. In comparison to the RSM, the ANN approach performed better in predictive capabilities even with limited number of experiments. Considering the extremely low cost of niger de-oiled cake in comparison to the costly semi-synthetic medium requirements, enormous amounts, easy availability and inherent nutritional constituents, utilization of niger (*Guizotia abyssinica*) de-oiled cake for the production of L- asparaginase offers a promising and reliable process to cater to the demands of L-asparaginase application in pharmaceutical and food industries.

SSF offered higher product concentration and simple working controls. Moreover, the agro-substrates in SSF simulated the natural conditions required for the filamentous fungal growth and L-asparaginase production. Although better yields of L-asparaginase production was observed in solid-state fermentation than submerged conditions on the agro-wastes using the fungus *Aspergillus niger*, one drawback in the form of decreased enzyme activity was observed on scale up (> 1 Kg) of the solid state fermentation at high solid loading. The major reason might be an increase in the temperature with the increase in the bed thickness at high solid loadings during the SSF process.

With the success obtained in the SSF process for L-asparaginase production using agro-wastes in comparison to the submerged process, further study in chapter 4 focussed on finding a low cost soluble substrates that can be efficiently utilized for the L-asparaginase production in submerged fermentation processes using a safe and new bacterial source for L-asparaginase production.