

Article

Comparative Evaluation of RSM and ANN Models on Prediction of Cellulase Production by *Bacillus paralicheniformis* Using *Plumeria alba* in Submerged Fermentation

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Abstract

This study reports cellulase production by *Bacillus paralicheniformis* using *Plumeria alba* leaf powder under submerged fermentation with a focus on systematic bioprocess optimization. Physical parameters were first optimized using a one-factor-at-a-time (OFAT) approach, followed by optimization of yeast extract, MgSO₄ and (NH₄)₂SO₄ via a central composite design (CCD) and response surface methodology (RSM). An artificial neural network (ANN) with a 5:3:1 network trained by the Levenberg–Marquardt algorithm further improved prediction of carboxymethylcellulase (CMCase) and filter paper cellulase (FPase) activities. This study is the first to exploit *Plumeria alba* leaf powder as an untapped, low-cost lignocellulosic substrate for cellulase production by *B. paralicheniformis* and uniquely benchmarks RSM against ANN-based modeling to identify superior predictive frameworks for bioprocess optimization. Under optimized conditions (24 h, 4% *w/v* substrate, 1% *v/v* inoculum), the maximum FPase and CMCase activities reached 60.53 IU/mL/min and 332.10 IU/mL/min respectively. Partial characterization showed optimum FPase and CMCase activities at 50 °C and 70 °C, respectively, at pH 7.5. Enzymes also showed activation by NaCl and some select solvents while tolerating a broad range of metal ions. The enzymatic hydrolysis of *P. alba* biomass released 59.42 mg/mL total reducing sugars after 8hr, confirming efficient saccharification from a low-cost feedstock. The ANN model ($R^2 = 97.59\%$ for CMCase; 85.95% for FPase) outperformed RSM ($R^2 = 85.95\%$ and 78.25% , respectively), while radial basis function optimization reached 99.99%. These findings highlight *B. paralicheniformis* cellulase as a promising biocatalyst for biorefinery applications and demonstrate the value of integrating RSM and ANN for process optimization.

Keywords: *Bacillus paralicheniformis*; *Plumeria alba*; Central Composite Design (CCD); submerged fermentation; Artificial Neural Network (ANN)



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1. Introduction

Lignocellulosic biomass has received increasing attention as renewable, low-cost substrate to produce cellulolytic enzymes, including endoglucanase [1,2]. Lignocellulosic materials are primarily composed of three structural polymers: cellulose, hemicelluloses

and lignin [3]. Cellulose is the most abundant organic polymer, accounting for 30% to 50% of all organic materials [4,5]. Its high energy content and availability make it a cost-effective and sustainable resource for bioconversion applications [6,7].

Soil vegetation is largely composed of cellulosic materials, making cellulose decomposition fundamental to the biological carbon cycle [2]. Enzymes are preferred for this purpose due to their high substrate specificity, catalytic efficiency and minimal impact compared with their chemical alternatives [8,9]. Cellulose can be hydrolyzed to glucose by the synergistic action of a cellulase enzyme system comprising endoglucanase (carboxymethyl-cellulase), exoglucanase (cellobiohydrolase), and β -glucosidase [10,11]. Endoglucanase starts hydrolysis by randomly cleaving internal β -1,4-glucosidic bonds within amorphous regions of the cellulose chain, generating new reducing and non-reducing chain ends and reducing the degree of polymerization [12–14]. Subsequently, exoglucanase acts on these free chain ends, releasing cellobiose as the primary product. Finally, β -glucosidase cleaves cellobiose into fermentable glucose monomers, relieving product inhibition of the upstream enzymes [13].

Microbial cellulases are advantageous for industrial applications due to their low production cost, biodegradability and environmental safety [15]. Cellulase-producing bacteria have been isolated from diverse environments, such as soil, compost, decaying plant matter and organic-rich substrates, enabling the selection of strains with high cellulolytic potential [16,17]. Among cellulolytic microorganisms, organisms of the genus *Bacillus* are particularly known for their rapid growth, biosafety, and capacity to secrete thermostable and alkali-stable extracellular cellulases under extreme environmental conditions [18–26]. Notable cellulase-producing species include *Bacillus cellulosilyticus* [27], *Bacillus subtilis*, and *Bacillus sphaericus* [18]. Other genera with documented cellulolytic activity include *Clostridium*, *Pseudomonas*, and *Cellulomonas* [28,29]; *Actinomycetes*, *Streptomyces*, and *Erwinia* [30]; *Thermomonospora* and *Microbispora* [31]; and *Acetovibrio*, *Paenibacillus*, and *Bacteroides* [10,11].

Cellulase can be produced via two major fermentation strategies: submerged fermentation (SmF) and solid-state fermentation (SSF) [32,33]. Both approaches have been extensively investigated using low-cost agro-industrial substrates [34,35]. SmF is generally preferred for bacterial fermentation because bacteria require some water content for growth, whereas SSF, which is conducted in the absence of free water, is more suited to filamentous fungi [36]. SmF also offers distinct process advantages, including ease of sterilization, precise parameter control, and simpler downstream processing compared with SSF [37,38]. Consequently, cellulase production from *Bacillus* species and related bacteria, including *Pseudomonas aeruginosa* [39], *Bacillus* sp. LFC15 [40], *B. subtilis* [41], *B. thuringiensis* [42], *B. subtilis* BS06 [43], and *Bacillus megaterium* BM05 [44], has been predominantly studied under SmF conditions.

Maximizing cellulase yield requires systematic optimization of fermentation and medium parameters [45]. Optimization strategies include one-variable-at-a-time (OVAT) approaches and statistical designs such as central composite design, full factorial design [9], and response surface methodology (RSM) [46–48]. Although OVAT is straightforward, it is time-consuming and fails to capture interactions among variables [49]. RSM overcomes this limitation by enabling simultaneous optimization of multiple parameters through a reduced number of experimental runs while also modeling the interactions between process variables [50–52]. RSM is a well-established statistical and mathematical tool used to evaluate the relationship between independent variables and a response function. More recently, artificial neural networks (ANNs) have been employed alongside RSM to improve predictive accuracy in nonlinear bioprocess systems, given their ability to model complex input–output relationships without assuming a predetermined functional form [53,54].

Cellulases have many industrial applications, including in textile processing, food and beverage production, brewing, and paper and pulp industries, where they have replaced several chemically intensive treatments [55]. Given this versatility and the growing demand for cost-effective enzyme production, the present study was conducted to evaluate cellulase production by *B. paralicheniformis* using *Plumeria alba* as a lignocellulosic substrate under submerged fermentation conditions. *P. alba* is a fast-growing tropical tree widely distributed across South and Southeast Asia, the Caribbean, and Sub-Saharan Africa, where its leaves accumulate as underexploited botanical waste. Unlike conventional lignocellulosic substrates such as sugarcane bagasse or rice straw, *P. alba* leaf biomass remains largely unutilized industrially, offering a non-competitive, low-cost feedstock option [56]. Its leaves are rich in cellulose and associated cell wall polymers that serve as both carbon source and inducer for cellulase biosynthesis in cellulolytic microorganisms [57]. Process parameters were optimized using both RSM and ANN approaches, and the resulting enzyme was further characterized.

2. Materials and Methods

2.1. Microorganism

Bacillus paralicheniformis was obtained from the microbial culture collection of the Department of Biotechnology, University of Sargodha, Sargodha, Pakistan. The strain was previously isolated from soil and was maintained on nutrient agar slants at 4 °C until further use in cellulase studies.

2.2. Preparation of Inoculum

Inoculum was prepared by transferring a loopful of a 24-h old strain of *B. paralicheniformis* culture into nutrient broth, followed by incubation at 35 °C and shaking at 120 rpm for 24 h. The resulting cell suspension (optical density = 600) was used directly as the inoculum source in further fermentation studies.

2.3. Substrate Preparation

Leaves of *Plumeria alba* were obtained from University of Sargodha, Sargodha, Pakistan. The leaves were washed thoroughly to remove surface debris and then oven-dried at 70 °C until constant weight was achieved. The dried biomass was ground into fine powder, sieved to a uniform particle size and stored in air-tight containers at room temperature until use.

2.4. Fermentation Technique

Fermentation was carried out in a 250 mL Erlenmeyer flask containing 15 mL of medium supplemented with 4% (*w/v*) of *P. alba* leaf powder as the substrate. Flasks were autoclaved at 121 °C and at 15 psi for 15 min. After cooling, flasks were inoculated under aseptic conditions with 1% (*v/v*) of 24-h old vegetative culture of *B. paralicheniformis* and incubated at 35 °C and 120 rpm in a shaking incubator. After 24 h, the fermented broth was filtered through muslin cloth and centrifuged at 10,000 × *g* for 10 min at 4 °C to remove cell debris. The clarified supernatant was collected as the crude enzyme extract for subsequent assays.

2.5. Enzyme Activity Assay

CMCase (Carboxymethyl cellulase) and FPase (filter paper cellulase) were estimated as previously described [58]. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under standard assay conditions.

2.6. Optimization of Fermentation Parameters

2.6.1. One-Factor-at-a-Time (OFAT) Approach

Inoculum size and substrate concentration were initially screened using the one-factor-at-a-time (OFAT) strategy, where one variable is varied while all others are held constant [59]. Substrate concentration varied from 0.5% to 5% (*w/v*), and inoculum size ranged from 0.5% to 3% (*v/v*). Optimum values identified by the OFAT approach were subsequently used as baseline conditions for central composite design (CCD) optimization of nutritional parameters.

2.6.2. Central Composite Design (CCD) and Response Surface Methodology (RSM)

A CCD under RSM was designed to optimize three nutritional variables: yeast extract (A), MgSO_4 (B) and $(\text{NH}_4)_2\text{SO}_4$ (C). Each variable was tested at five coded levels (−2, −1, 0, +1, and +2), as shown in Table 1. The relationship between coded and actual values is given by Equation (1).

$$x_i = \frac{X_i - X_o}{\Delta X_i} \quad (1)$$

where x_i is the coded value, X_i is the actual value of the variable, X_o is the actual value at the center point, and ΔX_i is the step change. Experimental data were fitted to the second-order polynomial model (Equation (2)) using STATISTICA version 13.5 (TIBCO Software Inc., Palo Alto, CA, USA) software.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (2)$$

where Y is the predicted response (enzyme activity); β_0 is the intercept; β_1 , β_2 , and β_3 are linear coefficients; β_{11} , β_{22} , and β_{33} are quadratic coefficients; and β_{12} , β_{13} , and β_{23} are interaction coefficients.

Table 1. Coded and actual levels of three independent variables used in CCD.

Independent Variable	Label	−2	−1	0	+1	+2
Yeast extract	A	−0.17045	0.670448	0	0.25	0.50
MgSO_4	B	−0.06818	0.268179	0	0.10	0.20
$(\text{NH}_4)_2\text{SO}_4$	C	−0.17045	0.670448	0	0.25	0.50

2.7. Artificial Neural Network (ANN)

An ANN model was designed using the Neural Network Toolbox in MATLAB R2015a (The MathWorks Inc., Natick, MA, USA), to predict and optimize CMCase and FPase production. The network architecture consisted of three input variables (yeast extract, MgSO_4 , and $(\text{NH}_4)_2\text{SO}_4$), one hidden layer, and one output layer (enzyme activity), corresponding to a (3– n –1) topology. The optimal number of neurons in the hidden layer was determined iteratively by training multiple networks and selecting the configuration that minimized root mean square error (RMSE). The Levenberg–Marquardt backpropagation algorithm (trainlm) was used for training. The dataset was partitioned into training (70%), validation (15%), and test (15%) subsets. Model performance was evaluated using the coefficient of determination (R^2) and RMSE. The ANN architecture is shown in Figure 1. In which Blue nodes show the input variables, purple nodes show the hidden layers (five neurons in each hidden layer), and orange node represent the output layer.

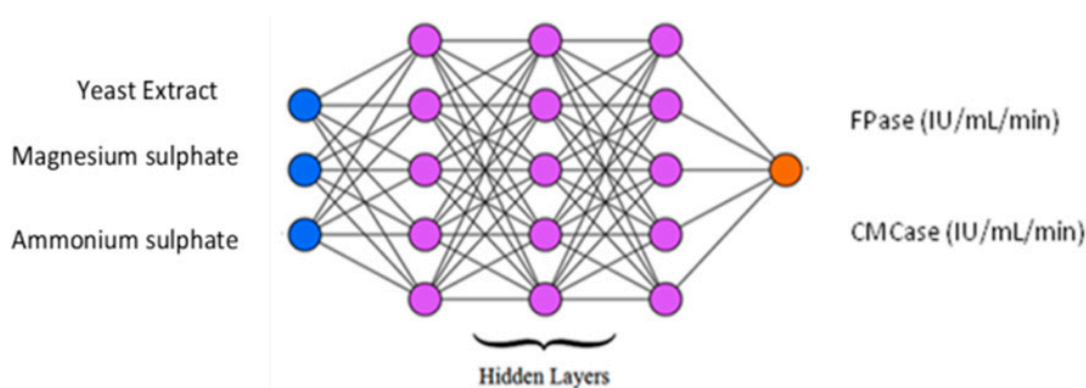


Figure 1. ANN network architecture.

2.8. Enzyme Characterization

2.8.1. Effect of pH on Cellulase Activity

The effect of pH on cellulase activity was determined by incubating crude enzyme in appropriate buffers across a pH range of 4.0–11.0 (at 0.5-unit intervals) at 50 °C for 30 min. Residual activity was measured using the DNS (3,5-dinitrosalicylic acid) colorimetric method [59].

2.8.2. Effect of Temperature on Cellulase Activity and Thermostability

The effect of temperature on activity was assessed by incubating CMCase with 0.5 mL of 1% (*w/v*) carboxymethyl cellulose (CMC) prepared in 0.05 M sodium citrate buffer (pH 5.0), and FPase with 0.5 mL of 0.05 M sodium citrate buffer (pH 5.0), across a temperature range of 30–90 °C. Thermostability was evaluated by pre-incubating the enzyme at the same temperature range for a defined period, after which residual activity was measured under standard assay conditions.

2.8.3. Effect of Metals Ions on Cellulase Activity

The influence of metal ions (FeSO₄, AlCl₃, CaCl₂, MgSO₄, MnSO₄, NaCl, and CuSO₄) on cellulase activity was assessed at concentrations of 1, 3, and 5 mM. CMCase and FPase activities were measured using the standard DNS assay [59].

2.8.4. Effect of Surfactants and Organic Solvents on Cellulase Activity

The effects of surfactants (SDS and Triton X-100) and organic solvents (hexane, propanol, butanol, and ethanol) on enzyme activity were evaluated using the standard DNS assay [59].

2.8.5. Saccharification

Saccharification was performed in 500 mL Erlenmeyer flasks containing 100 mL of crude enzyme and 4% (*w/v*) *P. alba* leaf powder substrate, incubated at 50 °C for varying time intervals. Following hydrolysis, the reaction mixture was filtered through muslin cloth and centrifuged at 10,000× *g* for 10 min. The percentage saccharification was calculated using Equation (3) [43].

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugars released (mg/mL)} \times 0.9}{\text{Substrate used (mg/mL)}} \times 100 \quad (3)$$

3. Results and Discussion

3.1. Effect of Inoculum Size on Cellulase Production

Inoculum size was optimized by evaluating concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (*v/v*). Maximum CMCase (13.09 IU/mL/min) and FPase (31.19 IU/mL/min) activities were achieved at inoculum sizes of 1.0% and 0.5%, respectively, while minimum enzyme production was observed at 2.5% (Figure 2). Enzyme yield declined progressively beyond the optimal inoculum concentration, likely due to the rapid depletion of nutrients and the accumulation of metabolic by-products under high-cell-density conditions, which impose substrate limitation and catabolite repression [60]. These results are consistent with previous reports of maximum cellulase production at 1% inoculum size in *B. amyloliquefaciens* and *Bacillus megaterium* under submerged fermentation conditions. Optimal inoculum sizes of 2% [61–63] and 5% have also been reported for other *Bacillus* strains, indicating that the optimal cell density for cellulase production is strain- and medium-dependent [19].

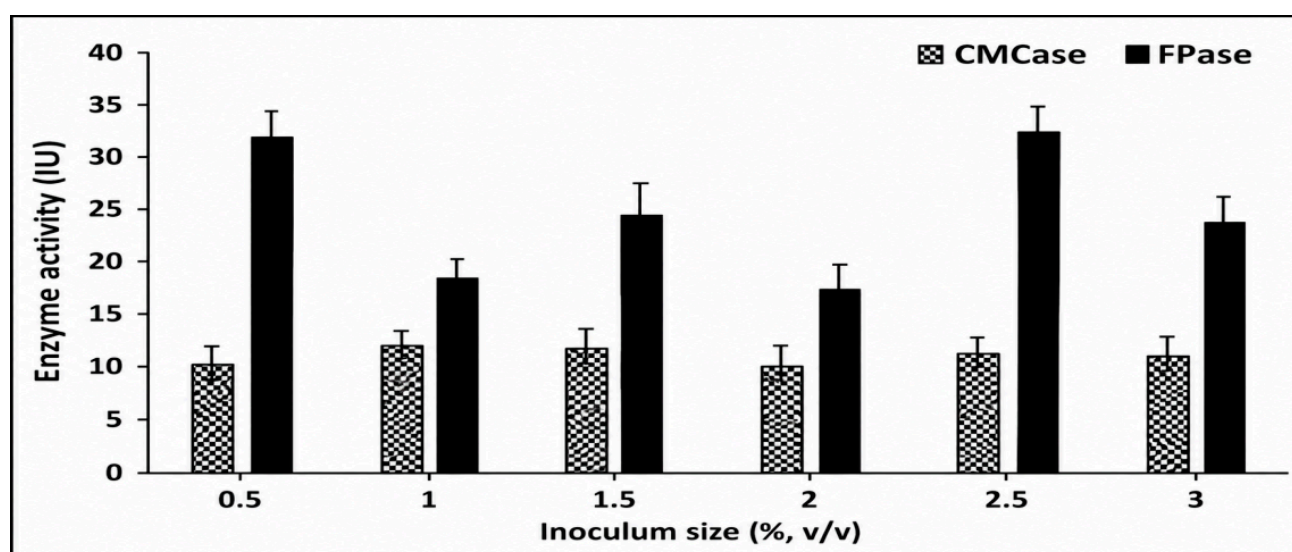


Figure 2. Effect of inoculum size on CMCase and FPase activities produced by *Bacillus paralicheniformis* using *Plumeria alba* leaf powder as substrate under submerged fermentation conditions.

3.2. Effect of Substrate Concentration on Cellulase Production

The effect of *P. alba* leaf powder concentration on cellulase production was evaluated across a range of 0.5–5.0% (*w/v*). Maximum CMCase (18.62 IU/mL/min) and FPase (46.61 IU/mL/min) activities were achieved at 4% (*w/v*) substrate, while minimum activities of 3.72 IU/mL/min (CMCase) and 11.44 IU/mL/min (FPase) were recorded at 0.5% substrate (Figure 3). Enzyme production declined at concentrations exceeding 4%, likely due to substrate inhibition of microbial growth and reduced mass transfer efficiency at elevated solid loadings [44]. These findings are consistent with previous reports of optimal cellulase production at 4% wastepaper substrate by *Cellulomonas* sp. TSU03 [64] and at 3% substrate by a newly isolated *Cellulomonas* sp. Lower optimal substrate concentrations have also been reported, including 2% wheat straw for *Bacillus megaterium* BM05 (yielding 0.716 ± 0.04 U/mL) [44], 2% rice hull [65] and 1% fruit pulp [2] for *B. amyloliquefaciens*, 1–2% wheat straw [66] for *Aeromonas* sp., and 0.5% cellobiose for *Bacillus* strain CTP-09, collectively indicating that optimal substrate concentration is substrate- and strain-specific [67].

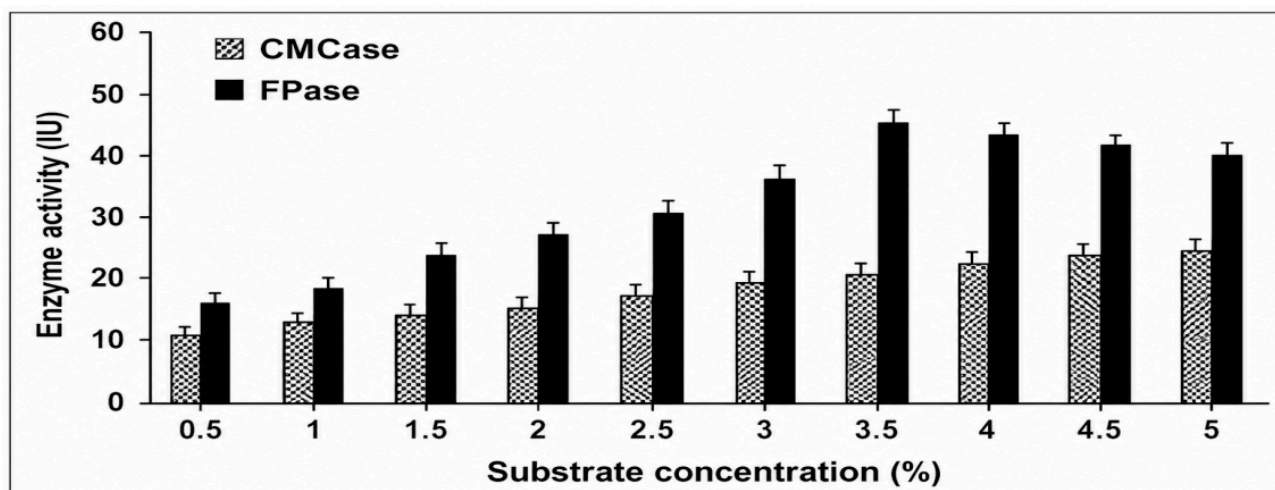


Figure 3. Effect of *P. alba* leaf powder concentration on CMCase and FPase activities produced by *Bacillus paralicheniformis* under submerged fermentation conditions.

3.3. CCD for CMCase and FPase Production by *Bacillus paralicheniformis* Under SMF

A CCD under RSM was employed to investigate the effects of three nutritional variables—yeast extract (A), MgSO_4 (B), and $(\text{NH}_4)_2\text{SO}_4$ (C)—on CMCase and FPase production. A total of 20 experimental runs were conducted, and the observed and predicted enzyme activities for each run are presented in Table S1. CMCase activity ranged from 41.03 to 60.53 IU/mL/min across all runs; Run 19 yielded the maximum CMCase activity (60.53 IU/mL/min), with a residual difference of 1.84 IU/mL/min between observed and predicted values. FPase activity ranged from 100.40 to 332.10 IU/mL/min; Run 6 yielded the maximum FPase activity (332.10 IU/mL/min), with a residual difference of 72.12 IU/mL/min, reflecting the sensitivity of FPase production to medium composition. The relationships between the independent variables and CMCase and FPase responses are described by the following second-order polynomial regression equations (Equations (4) and (5)):

$$\text{CMCase IU/mL} = 10.70 + 94.4A + 225.3B + 108.9C - 12.4A^2 - 204B^2 - 59.2C^2 - 295.3AB - 127.0AC - 344.4BC \quad (4)$$

$$\text{FPase IU/mL} = 143.6 + 529A + 568B + 233C - 585A^2 + 1233B^2 - 124C^2 - 1458AB - 270AC - 2418BC \quad (5)$$

3.4. Analysis of Variance (ANOVA) for CCD Models

The statistical significance of both regression models was assessed by ANOVA. The CMCase and FPase models were significant at $p = 0.003$ and $p = 0.021$, respectively (Table 2), confirming that the models adequately describe the relationship between the independent variables and enzyme production. The coefficient of determination (R^2) was 85.95% and 78.25% for CMCase and FPase, respectively, indicating that the models accounted for most of the variability in the observed responses. The adjusted R^2 values of 73.31% (CMCase) and 58.67% (FPase) further confirm model reliability, with the lower adjusted R^2 for FPase suggesting greater unexplained variance, possibly attributable to uncontrolled environmental factors or additional interaction terms not included in the model. All linear terms (A, B, and C), quadratic terms (A^2 , B^2 , and C^2), and interaction terms ($A \times B$, $A \times C$, and $B \times C$) were statistically significant, indicating that both individual variable effects and their pairwise interactions are important determinants of cellulase production.

Table 2. Analysis of variance (ANOVA) for CMCase and FPase production through *B. paralicheniformis* in SmF.

	Source	DF	Adj. SS	Adj. MS	F-Value	p-Value
CMCase (IU/mL/min)	Model	9	2617.21	290.801	6.80	0.003
	Linear	3	845.42	281.808	6.59	0.010
	Yeast	1	616.23	616.229	14.41	0.004
	MgSO ₄	1	82.50	82.499	1.93	0.195
	(NH ₄) ₂ SO ₄	1	146.70	146.695	3.43	0.094
	Square	3	238.95	79.651	1.86	0.200
	A × A	1	8.70	8.698	0.20	0.662
	B × B	1	60.15	60.149	1.41	0.263
	C × C	1	197.10	197.101	4.61	0.057
	Two-way interaction	3	1532.83	510.945	11.94	0.001
	A × B	1	435.90	435.903	10.19	0.010
	A × C	1	504.01	504.014	11.78	0.006
	B × C	1	592.92	592.917	13.86	0.004
	Error	10	427.78	42.778		
	Lack of fit	5	427.59	85.519	2233.30	0.000
	Pure error	5	0.19	0.038		
	Total	19	3044.99			
FPase (IU/mL/min)	Source	DF	Adj. SS	Adj. MS	F-value	p-value
	Model	9	85,656	9517.3	4.00	0.021
	Linear	3	20,066	6688.6	2.81	0.094
	Yeast	1	437	436.7	0.18	0.678
	MgSO ₄	1	3264	3263.7	1.37	0.269
	(NH ₄) ₂ SO ₄	1	16,365	16,365.4	6.87	0.026
	Square	3	23,447	7815.8	3.28	0.067
	A × A	1	19,288	19,287.7	8.10	0.017
	B × B	1	2189	2189.4	0.92	0.360
	C × C	1	865	864.9	0.36	0.560
	Two-way interaction	3	42,143	14,047.6	5.90	0.014
	A × B	1	10,622	10,622.5	4.46	0.061
	A × C	1	2278	2278.4	0.96	0.351
	B × C	1	29,242	29,242.0	12.28	0.006
	Error	10	23,815	2381.5		
	Lack of fit	5	15,849	3169.9	1.99	0.234
	Pure error	5	7966	1593.1		
	Total	19	109,471			

3.5. Interaction Effects of Process Variables: Response Surface and Contour Plot Analysis

Response surface and contour plots were generated to visualize the pairwise interaction effects of the three independent variables on CMCase and FPase production. Observed and predicted value plots of CMCase (IU) and FPase (IU) shown in Figure 4. Clustering of blue circles around the 45° line predicts the agreement strength between observed and predicted values, circles closed to line show greater predictive performance between observed and predicted values as compared to points which are far away from line which indicate weaker predictions. For each contour plot, two variables were varied simultaneously, while the third was held at its central (zero-coded) level. The interactions examined were yeast extract (A) × MgSO₄ (B), yeast extract (A) × (NH₄)₂SO₄ (C), and MgSO₄ (B) × (NH₄)₂SO₄ (C). These plots enabled identification of the optimal variable combinations for maximum cellulase production, as shown in Figure 5.

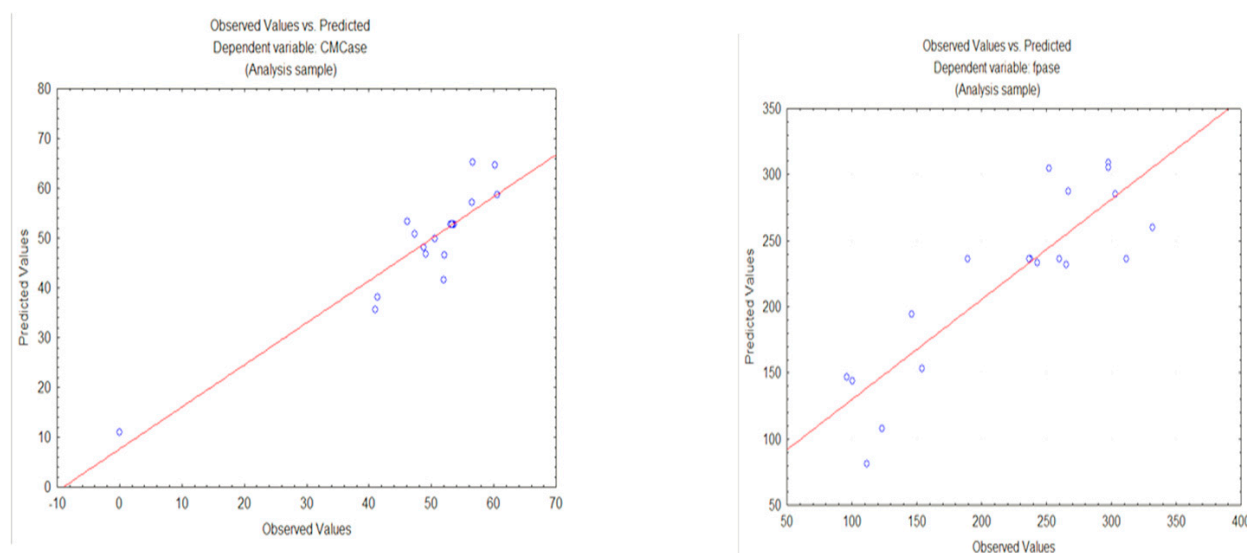


Figure 4. Observed values vs. predicted values of CMCase and FPase activities.

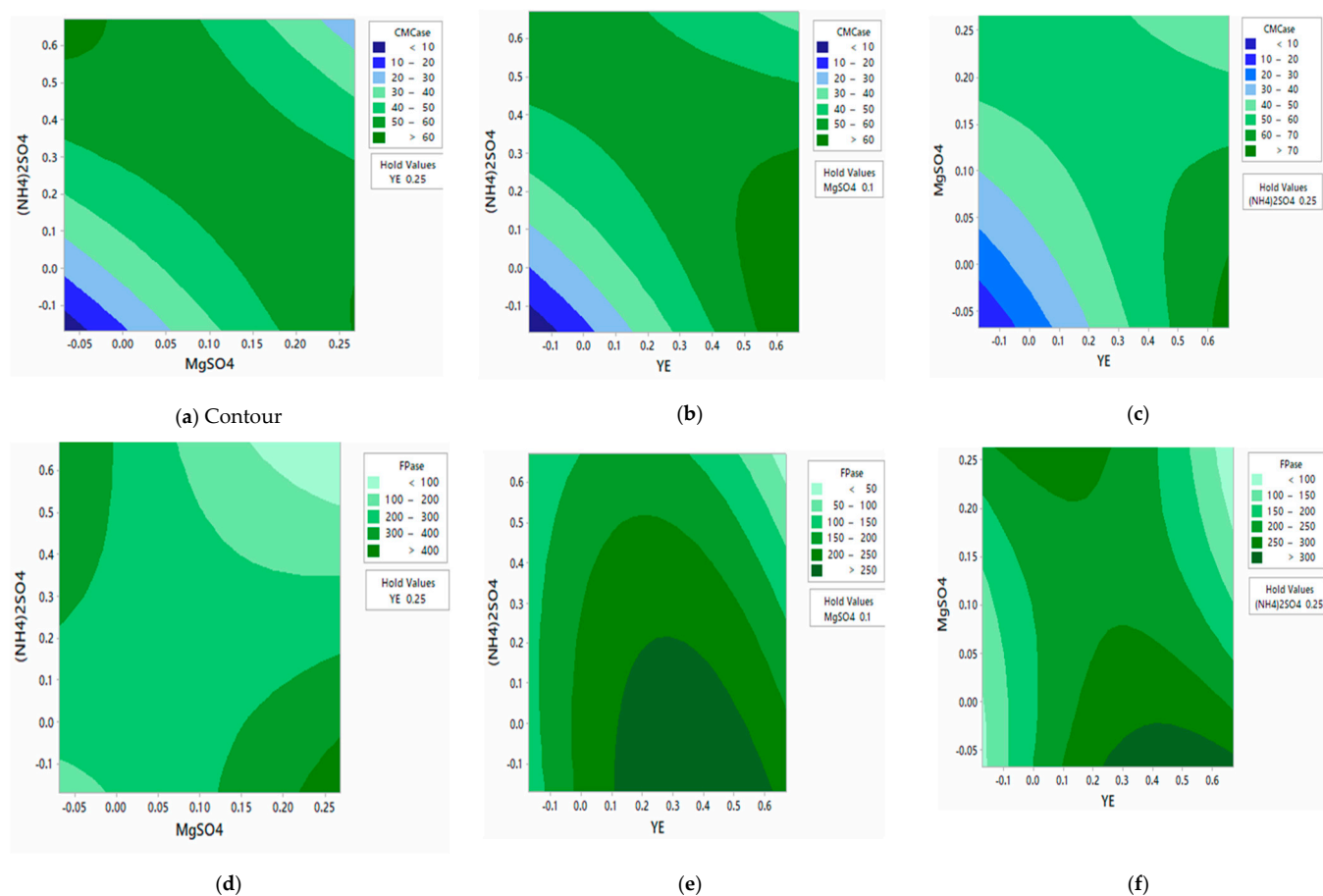


Figure 5. Response surface contour plots explaining interrelating effects of (a) MgSO_4 vs. $(\text{NH}_4)_2\text{SO}_4$, (b) yeast extract vs. $(\text{NH}_4)_2\text{SO}_4$ and (c) MgSO_4 vs. yeast extract on CMCase and (d) MgSO_4 vs. $(\text{NH}_4)_2\text{SO}_4$, (e) yeast extract vs. $(\text{NH}_4)_2\text{SO}_4$ and (f) MgSO_4 vs. yeast extract on FPase production by *Bacillus paralicheniformis* by using *Plumeria alba* in submerged fermentation. The plot was made by keeping one variable constant.

3.6. Characterization of Cellulases

3.6.1. Effect of pH on Activity of Cellulases

The effect of pH on cellulase activity was studied across a range of pH 4.0–11.0 using appropriate buffer systems. Maximum CMCase activity (19.99 IU/mL/min) and FPase activity (39.07 U/mL/min) were both observed at pH 7.5, while minimum CMCase (9.587 IU/mL/min) and FPase (14.98 IU/mL/min) were recorded at pH 11.0 (Figure 6). The enzyme retained activity across pH 7.0–11.0, with activity declining progressively beyond the optimum, consistent with denaturation of the active site at extreme pH values. The observed neutral-to-alkaline optimum is characteristic of *Bacillus*-derived cellulases and aligns with reports of optimal CMCase activity at pH 7.5 in *Bacillus licheniformis* and endoglucanase stability across pH 6.5–9.5 [17]. Similar neutral optima have been reported [68] for cellulases from *B. subtilis* M-11 (stable at pH 7.0–9.5) [69] and *B. subtilis* subsp. *subtilis* A-54 (optimum at pH 6.5 and stable at pH 6.5–8.0) [70], as well as for *B. megaterium* (peak activity at pH 6.5 and stable at pH 6.0–8.0) [44]. Lower pH optima have also been reported within the genus, including pH 5.0 for *B. mycoides* AR20-61 [71], pH 5.0 for *B. subtilis* subsp. JJBS300 CMCase, pH 5.0–6.5 for various *Bacillus* strains [72], and pH 9.2 for extracellular cellulase from *B. subtilis*, collectively demonstrating that optimal pH for *Bacillus* cellulases is strain-specific and spans a broad range from acidic to alkaline conditions [73].

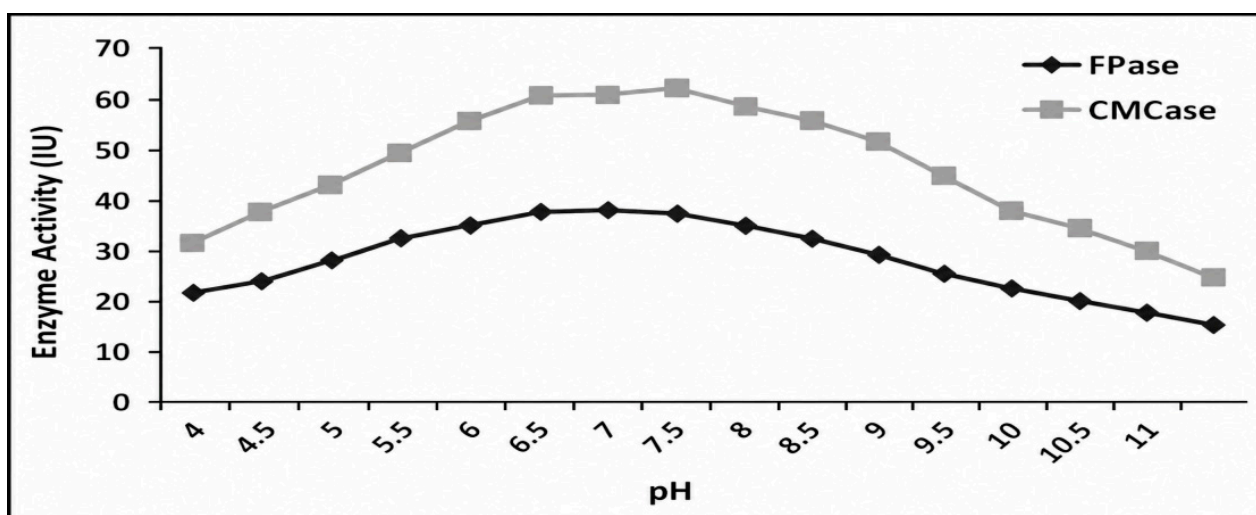


Figure 6. Effect of pH on CMCase and FPase activities by *Bacillus paralicheniformis*.

3.6.2. Effect of Temperature on Cellulase Activity and Thermostability

Maximum CMCase activity (19.58 IU/mL/min) and FPase activity (49.75 IU/mL/min) were recorded at 70 °C and 50 °C, while maximum thermostability of CMCase (15.88 IU/mL/min) and FPase (39.86 IU/mL/min) were estimated at 30 °C and 60 °C respectively (Figure 7). The difference in optimum activity and stability (70 °C vs. 30 °C) suggests that the enzyme achieves a peak catalytic turnover at high temperatures but prolonged exposure above 30 °C leads to progressive thermal denaturation. Moderate temperature optima have also been reported [68] for *B. subtilis* M-11 (stable at 25–60 °C), *Bacillus licheniformis* (optimum at 50 °C) [73], *Bacillus* sp. (optimum at 50 °C), and *B. subtilis* (stable at 20–45 °C) [74], while *Bacillus* strain CTP-09 exhibited exceptional thermostability at 80 °C [75]. Collectively, these indicate that thermal optima among *Bacillus* cellulases vary considerably by strain and enzyme type. This thermophilic activity of CMCase is consistent with reports of *Bacillus*-derived cellulases at elevated temperatures such as *Bacillus* sp.

strain G4 (maximum activity at 60 °C and stable below 50 °C) and *Bacillus licheniformis* AU01 (optimum activity at 50 °C and retaining 80% activity at 80 °C) [76].

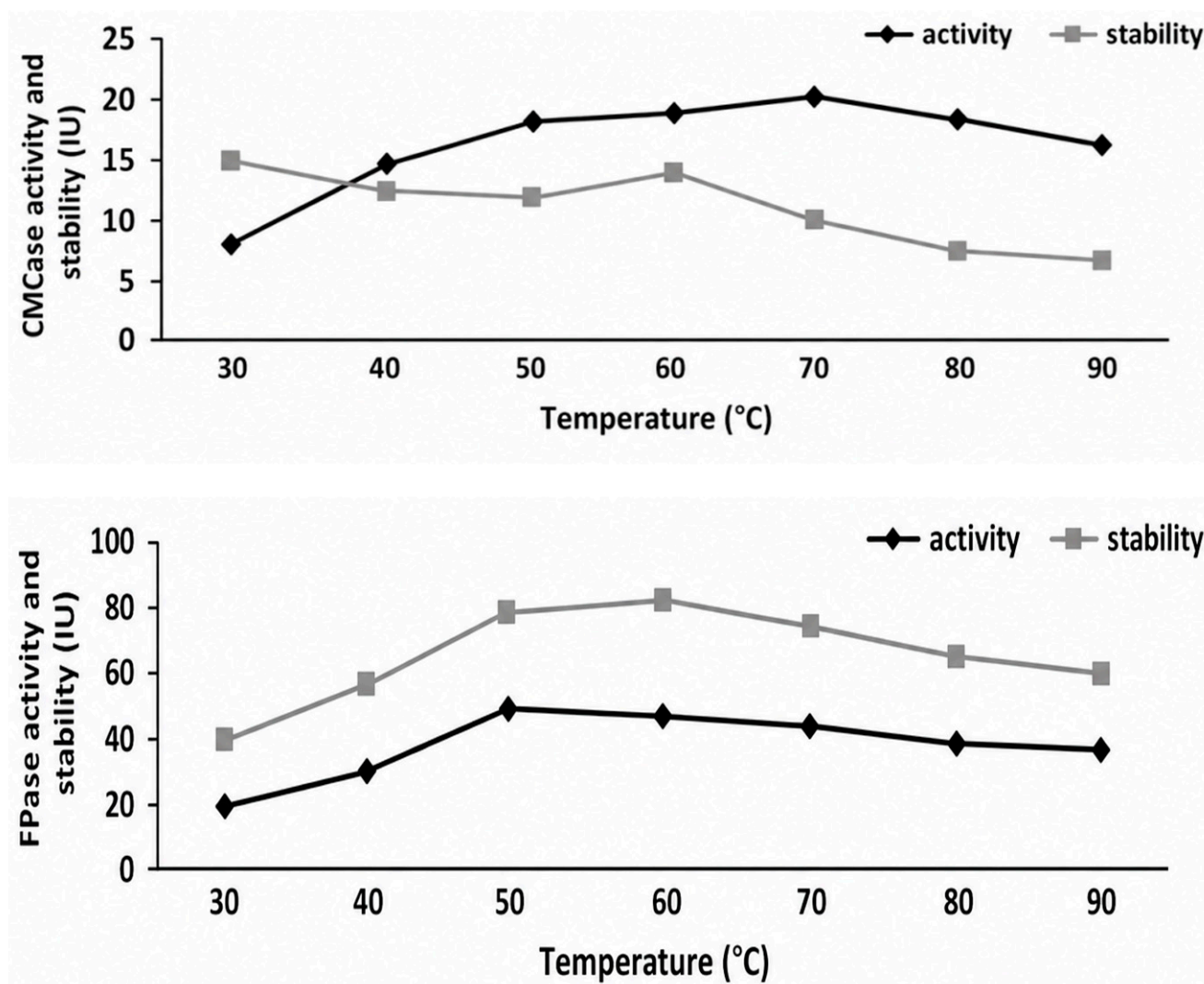


Figure 7. Effect of temperature on CMCase and FPase activity and thermostability in *Bacillus paralicheniformis*.

3.6.3. Effect of Solvents and Surfactants on Cellulase Activity

The effect of organic solvents (ethanol, propanol, butanol, and hexane) and surfactants (SDS and Triton X-100) on CMCase and FPase activity were determined at 0.5% concentration (Table 3). Ethanol had the highest relative activity, with CMCase and FPase reaching 235.26% and 169.56% of control, respectively. This indicates that the enzyme is ethanol-tolerant. Butanol and SDS produced the lowest CMCase and FPase activities, respectively. Similar reports indicate that SDS inhibits cellulases from *B. subtilis* YJ1, *Bacillus* sp. strain G4 [77], and *Bacillus licheniformis* [78]. This is likely due to SDS-induced disruption of the enzyme's hydrophobic core and active site architecture. Activation by Triton X-100 has been reported for cellulase from *Bacillus* sp. AC-1, though in the present study, Triton X-100 yielded intermediate activity (CMCase: 91.45%; FPase: 149.54%), suggesting surfactant effects are enzyme- and concentration-dependent [79].

Table 3. Effect of solvents and surfactants on relative activity (%) of CMCase and FPase by *Bacillus paralicheniformis*.

Reagent	CMCase (%)	FPase (%)
Blank (Control)	100	100
SDS	98	40.23
Triton-X	91.45	149.54
Hexane	166.66	121.82
Propanol	192.15	146.88
Butanol	89.15	81.23
Ethanol	235.26	169.56

3.6.4. Effect of Metal Ions on Cellulase Activity

The effects of seven metal ion supplements at concentrations of 1, 3 and 5% on CMCase and FPase activities are presented in Table 4. Maximum CMCase (185%) and FPase (273.3%) were obtained with 1% and 5% NaCl respectively. This shows that Na⁺ is a good activator of both enzyme components, which is consistent with reports of NaCl-mediated cellulase activation in *Bacillus pumilus* EWBCM1 [62] and *B. subtilis* subsp. JJBS300 [72]. Minimum CMCase (53.7%) and FPase (58.7%) activities were recorded with 1% MnSO₄ and 3% CaCl₂ respectively. FeSO₄, AlCl₃, MgSO₄, and CuSO₄ showed concentration-dependent activating effects on both CMCase and FPase. This is generally consistent with reports of Fe³⁺ and Mg²⁺ activation and Co²⁺/Mn²⁺ activation in *B. subtilis* YJ1 [77], while inhibitory effects of ZnCl₂ on *B. licheniformis* cellulase and Cu²⁺ on *Bacillus* sp. have been attributed to coordination with catalytic residues at the enzyme active site [80,81].

Table 4. Effect of different metal ion concentrations on relative activity (%) of CMCase and FPase from *Bacillus paralicheniformis*.

Metal Ion	CMCase 1% w/v	FPase 1% w/v	CMCase 3% w/v	FPase 3% w/v	CMCase 5% w/v	FPase 5% w/v
Blank	100	100	100	100	100	100
FeSO ₄	130.5	170.6	165.4	187.9	177.5	214.9
AlCl ₃	171.6	169.6	130.8	208.2	127.9	236.3
CaCl ₂	77.7	150.1	135.5	58.7	142.6	247.3
MgSO ₄	110.9	179.9	118	187.3	110.52	262.6
MnSO ₄	53.7	134.3	75.8	65.8	83.03	101
NaCl	185	130.2	148.9	172.7	175.9	273.3
CuSO ₄	89.7	167.7	124.8	156.3	147	195.7

3.7. Saccharification of *Plumeria alba* by Crude Cellulase

The saccharification potential of crude cellulase was evaluated using *P. alba* leaf powder as the substrate. The maximum saccharification (19.81%) was achieved after 4 h, while maximum total reducing sugar release (59.42 mg/mL) was observed at 8 h of incubation at 50 °C (Figure 8). The efficiency of saccharification decreased after 24 h, likely due to product inhibition of cellulase activity from accumulated glucose and cellobiose at extended incubation times. The noted decrease in reducing sugar concentration may be attributed to microbial uptake of released sugars for biomass formation, maintenance, growth, enzyme production, and other metabolic activities. These values agree with previously reported saccharification yields from *Bacillus* cellulases, including 11.45% from *B. subtilis* BS06 [43] and 54.39% from *B. subtilis* K-18 [48]. In terms of total sugar release, reported values range from 12.71 mg/mL (*B. subtilis* K-18, 24 h) [14], 0.74 mg/mL (pretreated substrate, *B. subtilis*, 53 h) [82], and 38.0 ± 0.23 mg/mL (*B. paralicheniformis*, 8 h) [83] to 31.42 mg/mL (*B. cereus*, 6 h at 50 °C) [52], indicating that the present enzyme's saccharification performance is

competitive within the *Bacillus* genus, though variability across studies reflects differences in substrate type, pretreatment, and assay conditions.

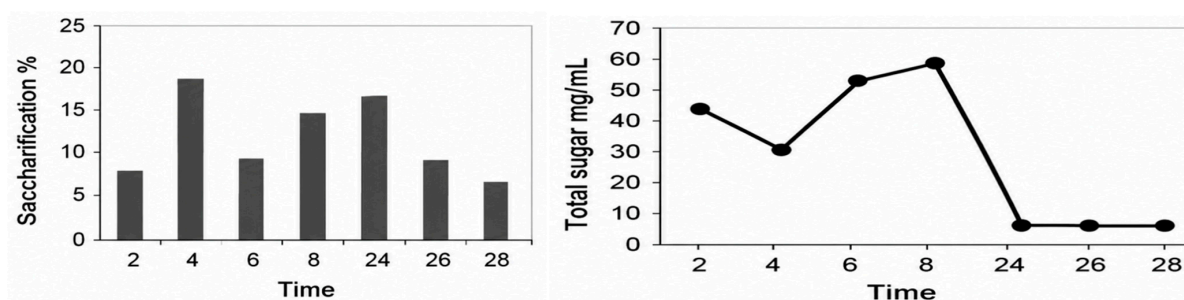


Figure 8. Percentage saccharification and total reducing sugar yield (mg/mL) of *Plumeria alba* hydrolyzed by crude cellulase from *Bacillus paralicheniformis* at defined time intervals.

3.8. Artificial Neural Network (ANN) Modeling

An Artificial Neural Network (ANN) model was developed using experimental data collected from the RSM-CCD optimization process to predict CMCase and FPase production [84]. The model also tried to capture nonlinear relationships among the fermentation variables which may not be fully described by the second-order polynomial RSM model. For CMCase (Figure S2), regression plots, error histograms, and training state plots confirmed strong agreement between observed and predicted values across all data subsets. The R^2 value of 0.9524 and RMSE of 2.6928 IU/mL indicate that the ANN model accurately describes the relationship between the nutritional variables and CMCase production (Table 5). Key training performance metrics included a final MSE of 4.62×10^{-6} , the best validation MSE of 1.68×10^{-2} , the best test MSE of 3.50×10^{-2} , and convergence at epoch 9 of 12 total epochs using the Levenberg–Marquardt (*trainlm*) algorithm [85,86].

Table 5. Comparative predictive performance of MLP, RBF, and GRNN models on CMCase and FPase production.

Models	R^2	RMSE	MAE
MLP	0.18102	0.18447	0.13172
RBF	0.99994	0.0016163	0.000683
GRNN	0.20867	0.18133	0.098592

For FPase (Figure S3), the ANN model yielded an R^2 of 0.7033 and an RMSE of 40.30 IU/mL, reflecting greater variability in FPase prediction, consistent with the lower adjusted R^2 observed for FPase in the RSM model (Section 3.4). The training MSE was 7.02×10^{-2} , the best validation MSE was 3.42×10^{-1} , the best test MSE was 1.07×10^{-1} , and the model converged at epoch 2 of eight total epochs. The lower predictive accuracy for FPase suggests that additional variables or a larger training dataset may be required to fully capture the complexity of FPase production dynamics. Observed vs. predicted values for both enzymes are presented in Figure 9, and best validation performance plots are shown in Figure S4. The regression plots for CMCase (Figure S2a) and FPase (Figure S3a) demonstrate excellent predictive performance across all three data partitions. The 70% of CCD data allocated for training effectively captured the nonlinear relationships governing enzyme production, as reflected in the high training R values for both responses. Similarly, the 15% of data reserved for testing yielded well-optimized predictions, confirming that the trained model generalizes reliably to unseen data points. The low mean square error (MSE) values observed across the training, validation, and test phases collectively affirm the robustness and statistical acceptability of the ANN model [87]. The mild overfitting

observed in the validation performance plots is attributable to the relatively small size of the experimental dataset [88]. This is a common limitation when CCD-generated data are used as ANN inputs and does not meaningfully compromise model utility. The error histogram further supports model integrity, with most prediction errors concentrated near zero, indicating minimal systematic bias and high prediction accuracy in the model outputs. Overall, these diagnostic metrics confirm that the ANN model trained on CCD-derived data constitutes a reliable and well-fitted tool for predicting cellulase activity under the fermentation conditions examined in this study.

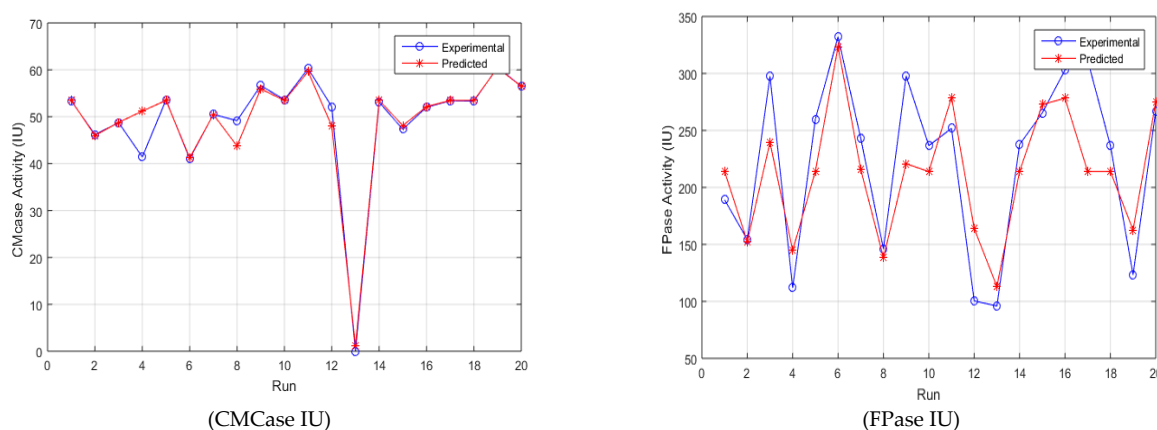


Figure 9. Observed vs. predicted values for CMCase and FPase activities generated by the ANN model.

Comparative Evaluation of ANN Architectures (RBF, MLP and GRNN)

Three ANN architectures, multilayer perceptron (MLP), radial basis function network (RBF), and generalized regression neural network (GRNN), were compared in their ability to predict CMCase and FPase production from the CCD dataset [89] (Figure 10; Table 5). The RBF model substantially outperformed both MLP and GRNN, achieving an R^2 of 0.99994, an RMSE of 0.00162, and an MAE of 0.000683, compared with R^2 values of 0.181 (MLP) and 0.209 (GRNN). The superior performance of RBF is consistent with its known advantage in approximating complex, localized nonlinear input–output relationships through Gaussian radial basis functions, making it particularly well suited for small, structured datasets such as those generated by a CCD [90]. Regression plots and error histograms for all three architectures are presented in Figure S5, further confirming the superior fit of the RBF model to the CCD experimental data.

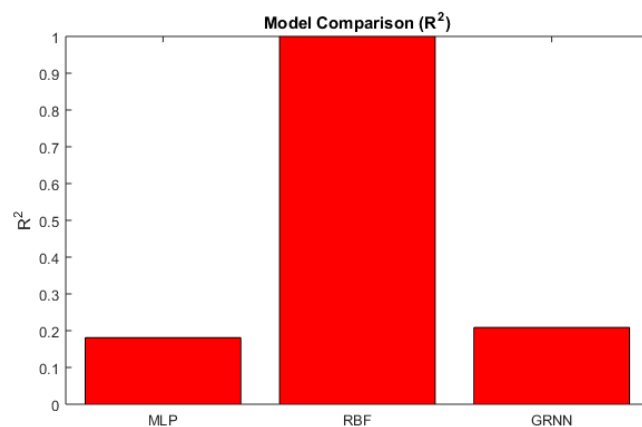


Figure 10. Comparison of R^2 values for MLP, RBF, and GRNN models for CMCase and FPase prediction.

4. Conclusions

Bacillus paralicheniformis showed strong cellulolytic activity when cultivated on *Plumeria alba* leaf powder as a sole carbon source under submerged fermentation conditions, yielding maximum CMCase and FPase activities under RSM-optimized nutritional parameters. A central composite design under RSM identified optimal combinations of yeast extract, MgSO_4 , and $(\text{NH}_4)_2\text{SO}_4$. ANN modeling, particularly the RBF architecture ($R^2 = 99.99\%$), outperforming RSM ($R^2 = 85.95\%$ and 78.25% for CMCase and FPase, respectively), and confirming the ANN's superior capacity to capture nonlinear variable interactions. Enzyme characterization revealed a neutral-to-alkaline pH optimum (7.5), broad thermostability, activation by ethanol and NaCl, and a saccharification efficiency of 19.81% with 59.42 mg/mL total reducing sugars released from *P. alba* substrate. This supports its potential for lignocellulosic biomass conversion. This study makes two novel contributions: the first documented use of *P. alba* leaf powder as a low-cost lignocellulosic substrate for *B. paralicheniformis* cellulase production and a rigorous comparative validation establishing the ANN as the preferred optimization framework. It also offers a scalable, cost-effective strategy for industrial cellulase production and biorefinery applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12070312/s1>, Figure S1. Desirability plots for predicted optimal levels of yeast extract (A), MgSO_4 (B), and $(\text{NH}_4)_2\text{SO}_4$ (C) maximizing CMCase (top) and FPase (bottom) activities produced by *Bacillus paralicheniformis* using *Plumeria alba* as substrate under submerged fermentation conditions; Figure S2: (a) ANN model performance on CMCase activity prediction: (a) regression plots showing the fit between observed and predicted values across training, validation, testing, and all data subsets; (b) error histogram; and (c) training state plot; Figure S3: ANN model performance on FPase activity prediction: (a) regression plots showing the fit between observed and predicted values across training, validation, testing, and all data subsets; (b) error histogram; and (c) training state plot; Figure S4: Plots of best validation performance for CMCase and FPase ANN models; Figure S5: Regression plots and error histograms for MLP, GRNN, and RBF network architectures; Table S1: CCD matrix with observed and predicted CMCase and FPase activities produced by *Bacillus paralicheniformis* using *Plumeria alba* as substrate under submerged fermentation conditions.

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Abbreviations

The following abbreviations are used in this manuscript:

RSM	Response Surface Methodology
OFAT	One Factor At a Time
CCD	Central Composite Design
ANN	Artificial Neural Network
CMCase	Carboxymethyl Cellulase

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