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RESEARCH ARTICLE

Maximizing enzyme production by standardizing process parameters through one factor at a time approach (OFAT) subjected to a statistical technique: Response surface methodology (RSM)

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Abstract

Enzymes play an important role in many industrial processes such as biofuel production, paper and pulp processing, food processing and waste management. Due to their multiple significant applications, there is growing need for maximizing their production. *Trichoderma* sp. is known for its potent enzyme-producing capabilities, making it an attractive candidate for enzyme production through solid-state fermentation (SSF). This study aimed to enhance enzyme production by employing a two-phase optimization strategy. The initial phase employed the one factor at a time (OFAT) approach to identify key process parameters (pH, temperature and incubation time) influencing enzyme activity. In the subsequent phase, response surface methodology (RSM) was used for further optimization, involving 20 experiments to assess the combined effects of multiple factors. OFAT analysis revealed that the concentration of substrate and incubation period were the most influential factors affecting enzyme production. In contrast, pH and temperature had a moderate yet still significant impact. RSM was used to fine-tune these parameters, resulting in optimized pH 5.5, 30 °C temperature and 7 days of incubation. Under these optimized conditions, enzyme production was approximately doubled compared to the baseline levels achieved without optimization. The findings are particularly relevant for industries such as biofuel, paper and pulp and waste management, where efficient enzymatic processes are essential for operational success and environmental sustainability. The methodology demonstrated in this study offers a practical and efficient framework for process optimization in enzyme production, potentially applicable to a broad range of microbial and enzymatic systems.

Keywords: biofuel; enzymes; optimization; RSM; solid state fermentation; sustainability

Introduction

Lignocellulose is the most abundant biopolymer in nature and its estimated global annual generation, i.e., 120×10^9 tons, is subjected to open burning which adversely affect the environment and human health (1). In recent years, biotechnological advancements have focused on utilizing renewable lignocellulosic resources for the production of bioenergy and value-added products (2). As a widely available and low-cost feedstock, lignocellulose is utilized across several industries (3).

Among woody biomass, pine needles are major recalcitrant lignocellulosic biomass rich in cellulose and hemicelluloses, along with lignin that cannot serve as fodder (4). The major challenge in these processes is complete hydrolysis of lignocellulosic material and the key step is its depolymerization of carbohydrates into sugars and further conversion into bioethanol. A clean and green biofuel, bioethanol, is of great importance as an alternative fuel to the current depletion of fossil fuels (5). The structural complexity and recalcitrance of lignin, cellulose and hemicellulose in plant cell walls necessitate effective pretreatment strategies (6).

Conventional pretreatment techniques, such as acid and alkali are used in various biorefineries that are not eco-friendly or economical (7). The production of bioethanol from lignocellulosic waste at the commercial level would require critical standardization of process parameters for the complete hydrolysis of biomass by

applying hydrolytic enzymes. The degradation in nature is caused by various potential bacterial and fungal species, but it occurs in a slow manner due to the lesser production of extracellular enzymes (8). Fungi are generally more effective than bacteria and archaea in degrading lignocellulosic biomass, especially under solid-state fermentation (SSF), which yields more robust enzymes compared to submerged fermentation (SmF).

Some of the most potential strains that produce bioproducts and enzymes under submerged fermentation are *Fusarium*, *Penicillium* and *Aspergillus* (9). Fungal hyphae can infiltrate deep within lignocellulosic biomass, generating extracellular hydrolytic enzymes that efficiently catalyze degradation processes. Many of these fungi secrete cellulolytic enzymes such as cellulase, xylanase and laccase, which collaborate synergistically to break down different biomass components (10). Laccase enzyme plays a crucial role in lignin breakdown, whereas xylanase and cellulase are primarily involved in hemicellulose and cellulose degradation, respectively.Traditional optimization techniques, such as the one-factor-at-a-time (OFAT) method, are often time-consuming and fail to account for interactions between variables. In contrast, Response Surface Methodology (RSM) is a powerful statistical approach that enables the efficient optimization of enzyme production by evaluating the combined effects of multiple parameters (11, 12).

RSM integrates experimental design, mathematical modelling and statistical analysis to identify optimal conditions for maximizing desired responses (13, 14). The optimization process focused on maximizing enzyme production while considering cost-effectiveness and environmental sustainability. The experimental design employed a central composite design (CCD) under RSM to evaluate the effects of various cultivation parameters, including pH, substrate concentration, temperature and incubation time (15). The main focus of this study was to maximize production of hydrolytic enzymes by optimizing all process parameters critically by applying a statistical approach (RSM) by subjection optimized conditions under one factor at a time approach which can be further enhance the rate of hydrolysis to release maximum of reducing sugars for bioconversion of cellulosic waste into bioethanol.

Materials and Methods

Biomass collection and its pretreatment

The pine needles were taken from coniferous forests of the Himalayas and chipped into fine particles of 2.0 mm mesh size by using a woodchipper/grinding machine. 100 g of biomass was taken and treated with microwave irradiation at 400 W for 2 min to enhance substrate accessibility.

Isolation of multiple enzymes producing fungus- *Trichoderma* sp.

To enrich lignocellulolytic fungi, 2 % (w/w) cellulose powder was mixed with 5 g of degraded, chopped wood samples and incubated at 28 ± 2 °C for 5 days.

1 g of cellulose-enriched wood sample was diluted in 9 mL of sterile water. The serial dilution method was performed for isolation by using Potato Dextrose Agar media, 28 ± 2 °C for 3 days and pure cultures were maintained. Qualitative screening was done by visualizing zones of hydrolysis for assessing their potential to produce hydrolytic enzymes. Identification of the fungus was done on the basis of phenotypic characterization by studying its cell morphology, mycelium shape and culture conditions (16).

Standardization of parameters through one factor at a one-factor-at-a-time technique (OFAT)

Process parameters were optimized using the One-Factor-at-a-Time (OFAT) approach. Temperature optimization was carried out at 20 °C, 25 °C, 30 °C, 35 °C, 40 °C and 45 °C. Different solid-to-moisture ratios (1:2, 1:3, 1:4, 1:5 and 1:6) were tested to determine the optimal moisture content for enzyme production. Incubation periods of 3, 5, 7, 10 and 12 days were analyzed at 30 °C under static conditions. For pH optimization, values ranging from 4.5 to 6.5 (4.5, 5.0, 5.5, 6.0 and 6.5) were evaluated to determine their effect on enzyme yield.

Optimization of process parameters through a statistical technique: Response surface methodology

Response surface methodology is a powerful statistical and mathematical technique in which a researcher can critically optimize the production parameters Such as pH, temperature, incubation time, carbon concentration and nitrogen concentration, affecting the whole process. This study employed RSM to optimize enzyme production from both untreated and pretreated pine needle biomass using a Central Composite Design (CCD). In the study, the input or independent variables were temperature, incubation time and pH and reducing sugars were the dependent variable. A CCD with three variables at five levels was used, resulting in 20 experimental runs, including six center points. The axial value (α) was set at 1.68 to ensure rotatability. The lower and higher levels of the independent variables used in the experiment are mentioned in Table 1. A quadratic model equation approximates the mathematical relationship of response laccase, cellulase, xylanase and reducing sugars. All the experiments were performed in triplicate and the response value mentioned is the average of the triplicates performed.

Table 1. Variables used in experimental design

Numeric	Name	Units	Low	High
A	Temperature	°C	20	30
B	Incubation time	Days	5	7
C	pH	-	5.0	6.0

Enzyme production and extraction

The selected *Trichoderma* sp. strain, capable of producing multiple hydrolytic enzymes, was first cultured on Potato Dextrose Agar (PDA) and incubated at 30 °C for 72 hr (17). As soon as the substantial growth (full plate) was observed, fungal culture was added separately to the 5 g of each flask containing untreated and pretreated pine needle biomass by scratching with 10 mL of autoclaved distilled water in the flask and incubated at 30 °C in static conditions. After incubation, enzymes were extracted by adding 50 mL of 0.05 M sodium citrate buffer (pH 5.5) to each flask containing the degraded biomass. The flasks were kept shaking at 120 rpm for 1 hr and separated using muslin cloth. The filtrate was centrifuged at 10000 rpm for 10 min at 4 °C and the resulting supernatant was collected for enzymatic activity assays (18).

Quantitative estimation of enzymes

Laccase

The laccase was estimated by following the standard method (19).

The reaction mixture typically includes the enzyme extract, buffer solution and substrate, incubated under optimal conditions. One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 µmol substrate/min under the assay conditions. This method provides a reliable quantitative measure of laccase activity at 420 nm in a spectrophotometer.

Cellulase

CMCase and FPase activities of the prepared reaction mixture were measured by applying method which involves incubating the enzyme extract with carboxymethyl cellulose (for CMCase) or filter paper strips (for FPase) in appropriate buffer, followed by quantification of released reducing sugars using the dinitrosalicylic acid (DNS) method (20). To quantify β-glucosidase enzyme standard method was used, which measures the release of p-nitrophenol or glucose from specific substrates under defined conditions (21).

Xylanase

To estimate xylanase activity, standard method was used, which involves the quantification of reducing sugars released from a xylan substrate using the dinitrosalicylic acid (DNS) reagent (22). The reaction mixture, containing the enzyme extract and xylan in suitable buffer, is incubated under optimal conditions and the absorbance is measured at 540 nm.

Reducing sugars

Reducing sugars were measured by applying method , in which the DNS reagent reacts with reducing sugars present in the sample to produce a coloured complex, the intensity of which is measured spectrophotometrically at 540 nm (22).

Protein estimation

A standard method was used to measure protein contents (23). In this assay, proteins react with copper ions under alkaline conditions, followed by a reaction with the Folin reagent, resulting in a blue-colored complex. The intensity of the colour, measured spectrophotometrically at 660 nm, is directly proportional to the protein concentration.

Statistical approach for critical optimization of parameters

In the present study, a software namely "Design Expert® version 7.0" i.e. a statistical software was applied for the analysis and interpretation of the data. This software was used to design experiments, optimize process parameters and for statistical analysis, particularly in the context of Response Surface Methodology (RSM). Here, the experimental designs we used is Central Composite Design (CCD) to evaluate the interactive effects of multiple variables on enzyme production. By employing this software, we analysed ANOVA, regression modeling and generated 3D surface graphs for identifying optimal operating conditions based on model predictions.

Generation and validation of model

A mathematical model was developed during the implementation of the Central Composite Design (CCD) under Response Surface Methodology (RSM) and its validity was assessed through confirmatory (checkpoint) experiments conducted under selected optimized conditions. A total of three independent validation experiments were performed to compare the experimentally obtained enzyme activity values with those predicted by the model. The prediction accuracy was evaluated by calculating the percentage error between the actual and predicted responses. A prediction error within ± 5 % was considered acceptable, indicating a good agreement between the experimental results and the model's predictions, thereby confirming the model's reliability.

Results

Isolation and phenotypic characterization of fungal strain *Trichoderma* sp.

The fungus was isolated from degraded wood samples by using Potato Dextrose Agar (PDA) and its pure culture was obtained. The strain was identified based on its morphological and physiological characteristics. The isolate was screened for its hydrolytic enzyme production potential through qualitative plate assays, where distinct zones of hydrolysis around the colonies were observed for laccase, cellulase and xylanase.

These zones of hydrolysis indicated that the strain is an efficient producer of these multiple hydrolytic enzymes.

One factor at a time technique (OFAT) for optimization of process parameters

Temperature

The effect of temperature on enzyme production was evaluated using both untreated and pretreated pine needle biomass. Enzyme activities were assessed at 25, 30, 35, 40 and 45 °C. Maximum enzyme activity was observed at 30 °C for both untreated and pretreated substrates. In untreated biomass, laccase, cellulase and xylanase activities were 2.70 U/g (0.07 U/mg), 24.60 U/g (0.69 U/mg) and 272.25 U/g (7.74 U/mg), respectively. Similarly, in pretreated biomass, the respective maximum enzyme activities were 2.70 U/g (0.07 U/mg), 23.25 U/g (0.69 U/mg) and 271.65 U/g (8.12 U/mg) (Fig. 1a).

The effect of substrate-to-moisture ratio on the production of laccase, cellulase and xylanase by *Trichoderma* sp. was evaluated using untreated and pretreated pine needle biomass. In untreated biomass, the highest activities were observed at a 1:4 ratio for laccase (3.60 U/g; 0.10 U/mg), cellulase (29.70 U/g; 0.87 U/mg) and xylanase (297.17 U/g; 8.73 U/mg). For pretreated biomass, optimal activities were laccase (3.60 U/g; 0.10 U/mg at 1:2), cellulase (29.40 U/g; 0.79 U/mg at 1:4) and xylanase (292.20 U/g; 7.91 U/mg at 1:4) (Fig. 1b). The impact of incubation period (3rd, 5th, 7th, 10th and 12th days) on enzyme and reducing sugar production was also assessed. In untreated biomass, peak laccase activity (4.20 U/g; 0.10 U/mg) was recorded on the 10th day, while in pretreated biomass, it reached 3.90 U/g with higher specific activity (0.20 U/mg) on the 7th day. Maximum cellulase activity was observed on the 7th day in both untreated (33.20 U/g) and pretreated (29.40 U/g) biomass. For xylanase, the highest activity in untreated biomass (298.45 U/g; 7.74 U/mg) occurred on the 10th day, while in pretreated biomass it peaked at 297.60 U/g on the 7th day (Fig. 1c).

pH

Effect of pH on enzymes activity from selected fungal isolates was evaluated using untreated as well as pretreated biomass. Fig. 2d shows the difference in multiple enzyme productions i.e. laccase, cellulase and xylanase at different pH levels i.e. 4.5, 5.0, 5.5, 6.0, 6.5 pH. Maximum laccase activity for *Trichoderma* sp. utilizing untreated biomass was observed i.e. 6.45 at 5.5 pH and 6.17 at 5.5 pH in case of pretreated biomass. Maximum cellulase activity for untreated biomass by *Trichoderma* sp. was observed 37.20 U/g and a specific activity of 2.48 U/mg at 5.0 pH and

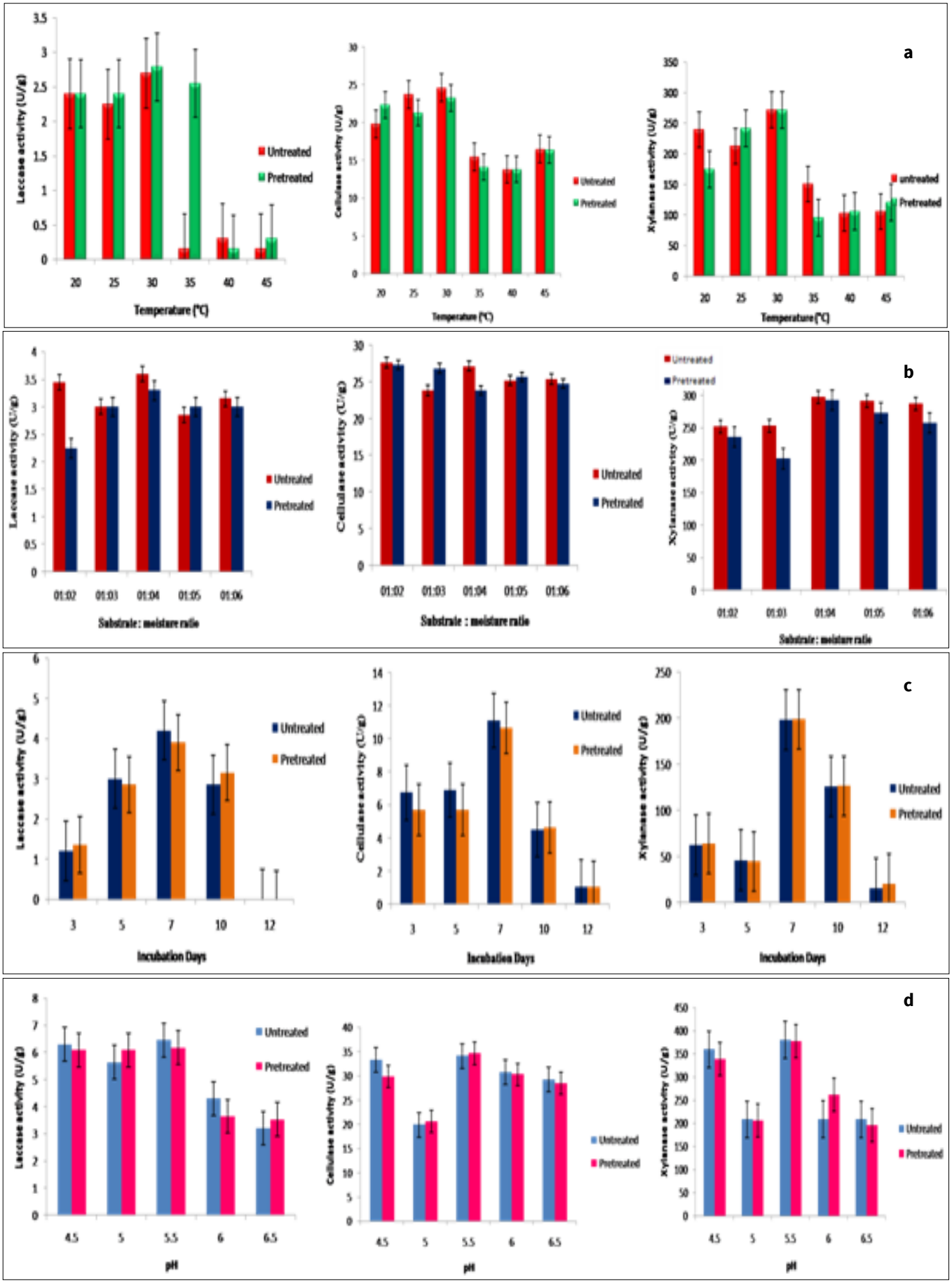

Fig. 1. a. Effect of temperature on laccase (i), cellulase (ii) and xylanase (iii) productions by *Trichoderma* sp. using untreated and pretreated pine needles biomass under SSF through OFAT; **b.** Effect of moisture content on laccase (i) cellulase (ii) and xylanase (iii) productions by *Trichoderma* sp. using untreated and pre-treated pine needles biomass under SSF through OFAT; **c.** Effect of incubation days on laccase (i) cellulase (ii) and xylanase (iii) productions by *Trichoderma* sp. using untreated and pre-treated pine needles biomass under SSF through OFAT; **d.** Effect of pH on laccase, cellulase and xylanase productions by *Trichoderma* sp. using untreated and pretreated pine needles biomass under SSF through OFAT.

maximum cellulase activity for pretreated biomass i.e., 37.50U/g at 5.5 pH. The effect of pH on xylanase production revealed maximum xylanase activity for untreated and pretreated biomass by *Trichoderma* sp., such as 380.00U/g with specific activity 7.96 U/mg at 5.0 pH and 376.99 U/g with specific activity 8.1 at 5.5 pH, respectively (Fig. 1d).

Response surface methodology (CCD) for optimization of enzyme and sugar production

The microwave pretreated pine needles biomass was used as the carbon source/ substrate for the enzyme production and compared with untreated biomass for efficient production of enzymes and reducing sugars. In the statistical approach, computers are programmed with data based on mathematical rules and statistical assumptions (24). Many optimization methods were applied to enhance the research experiments' efficiency, accuracy and performance (25). So, RSMs is also a statistical approach used in the present research to analyze the accuracy of the enzyme production parameters. The lignocellulolytic fungal strain *Trichoderma* sp. was taken for enzyme production under solid-state fermentation by varying temperature, incubation time and pH since diverse physico-chemical and biological factors influence the production of hydrolytic enzymes. This method required prior knowledge of significant culture conditions obtained from the previous One Variable at a Time (OVAT) approach experiment to achieve a more realistic model (26). Central Composite Design of RSM was applied for the standardization of independent variables for laccase, cellulase, xylanase and the dependent variable reducing sugar production from untreated and pretreated biomass. 20 experiments were runs in triplicates with different combinations of 3 factors for untreated and pretreated biomass. The dependent factors used to study factorial analysis were temperature, incubation period and pH. The whole experimental design with mentioned factors and their response values have been given in Tables 2 & 3 for untreated and pretreated pine needles biomass, respectively, which showed a considerable variation in the amount of laccase, cellulase xylanase as well as reducing sugars production depending upon the interaction among 3 independent variables.

By subjecting multiple regression models on the data obtained for *Trichoderma* sp. by untreated and pretreated pine needles biomass, a quadratic model was created for the response of laccase, cellulase, xylanase and reducing sugar activity. The significant terms of model were standardized by analysis of variance for untreated and pretreated biomass (ANOVA) in the present study ($p < 0.05$) and were recognized as A, B, C, A^2, B^2, C^2, AB, AC, BC for laccase, cellulase, xylanase and reducing sugar where temperature (A_1), incubation days (B_2) and pH (C_3) are dependent variables in all equations. By optimizing the above parameters by using RSM for untreated and pretreated pine needles biomass, maximum laccase (Table 4), cellulase (Table 5), xylanase (Table 6) and reducing sugar activity (Table 7) obtained was 6.90, 37.86, 398.00 U/g and 47.98 mg/g for untreated biomass and 6.70, 36.80, 392.10 U/g and 45.65 mg/g respectively for pretreated biomass at 30 °C, 7 days and pH 5.5.

Both the developed models were found strongly significant, as noticed by the model F-values and a low probability value ($P_{model}>F= 0.0001$). The coefficient of determination (R^2 value) for untreated biomass laccase, cellulase, xylanase and reducing sugar was calculated as 0.7163, 0.7085, 0.7110 and 0.7713, respectively, for *Trichoderma* sp. And coefficient determination (R^2) for pretreated pine needles biomass was found as 0.7596, 0.6662, 0.7851 and 0.7710, respectively, indicating a respective variability of untreated pine needles biomass was 71.63, 70.85, 71.10 and 77.13 % respectively and for pretreated pine needles biomass was 75.96, 66.62, 78.51 and 77.10 % respectively. A regression model with an R^2 value > 0.9 has a very high correlation.

The statistical significance of Eqn. 1 and 2 was checked by *F*-test and the analysis of variance (ANOVA) of the response surface quadratic model for *Trichoderma* sp. For laccase in Table 4 & 5.

Enzyme Activity 1 (Laccase untreated) = 5.58 - 0.76 × A + 0.21 × B - 0.22 × C - 0.70 × A2 - 0.48 × B2 - 0.76 × C2 + 0.13 × A × B + 0.094 × A × C - 0.21 × B × C **(Eqn. 1)**

Enzyme Activity 2 (Laccase pretreated) = 5.09 - 0.68 × A + 0.50 × B - 0.32 × C - 0.60 × A2 - 0.65 × B2 - 0.63 × C2 + 0.000 × A × B + 0.19 × A × C - 0.15 × B × C **(Eqn. 2)**

3D surface presentation under CCD for laccase production by *Trichoderma* sp. using untreated and pretreated pine needles biomass with interactions between different variables are represented in Fig. 2a(i-iii) & 2b(i-iii), where interaction between

Table 2. Optimization of process parameters for enhanced hydrolytic enzyme production using untreated pine needles biomass by response surface methodology for *Trichoderma* sp.

Std	Run	Incubation Days	Temperature	pH	Laccase (U/g)	Cellulase (U/g)	Xylanase (U/g)	Reducing Sugar (mg/g)
5	1.	5.00	25.00	6.00	3.90	27.60	124.05	31.5
15	2.	7.00	30.00	5.50	4.80	31.95	269.40	32.7
10	3.	10.36	30.00	5.50	5.50	31.95	201.00	29.45
12	4.	7.00	38.41	5.50	3.45	18.90	169.20	34.8
9	5.	3.64	30.00	5.50	5.81	18.75	129.45	18.45
19	6.	7.00	30.00	5.50	5.70	30.00	366.60	47.98
8	7.	9.00	35.00	6.00	2.40	15.15	169.60	38.60
4	8.	9.00	35.00	5.00	2.55	16.95	111.90	30.9
18	9.	7.00	30.00	5.50	5.10	27.30	331.50	36.78
6	10.	9.00	25.00	6.00	3.30	17.55	189.00	15.15
14	11.	7.00	30.00	6.34	3.75	26.40	374.20	24.8
7	12.	5.00	35.00	6.00	1.80	15.90	256.50	30
1	13.	5.00	25.00	5.00	3.60	16.95	335.40	29.98
3	14.	5.00	35.00	5.00	1.80	17.10	243.90	16.5
16	15.	7.00	30.00	5.50	5.25	37.86	398.00	29.55
20	16.	7.00	30.00	5.50	6.90	30.60	283.35	20.86
13	17.	7.00	30.00	4.66	4.95	29.25	300.60	29.7
17	18.	7.00	30.00	5.50	6.00	36.15	289.80	38.65
2	19.	9.00	25.00	5.00	4.50	20.10	300.90	14.55
11	20.	7.00	21.59	5.50	5.60	25.80	253.80	13.98

Table 3. Optimization of process parameters for enhanced hydrolytic enzyme production using microwave pretreated pine needles biomass by Response surface methodology for *Trichoderma* sp.

Std	Run	Incubation days	Temperature	pH	Laccase activity (U/g)	Cellulase (U/g)	Xylanase (U/g)	Reducing Sugar(mg/g)
5	1.	5.00	25.00	6.00	3.30	18.90	107.70	28.8
15	2.	7.00	30.00	5.50	4.50	28.35	253.80	29.95
10	3.	10.36	30.00	5.50	5.40	20.15	192.00	30.15
12	4.	7.00	38.41	5.50	3.15	16.35	151.80	29.89
9	5.	3.64	30.00	5.50	5.01	17.70	119.70	32.23
19	6.	7.00	30.00	5.50	5.10	28.65	228.60	45.65
8	7.	9.00	35.00	6.00	2.25	14.70	57.60	14.87
4	8.	9.00	35.00	5.00	2.10	15.30	104.70	19.96
18	9.	7.00	30.00	5.50	4.50	25.65	301.50	26.87
6	10.	9.00	25.00	6.00	2.85	16.20	177.00	32.54
14	11.	7.00	30.00	6.34	3.30	25.20	284.70	24.98
7	12.	5.00	35.00	6.00	1.50	14.85	243.00	29.87
1	13.	5.00	25.00	5.00	3.30	15.90	318.90	30.23
3	14.	5.00	35.00	5.00	1.95	16.65	227.70	18.78
16	15.	7.00	30.00	5.50	4.95	36.80	392.10	28.75
20	16.	7.00	30.00	5.50	5.55	30.00	253.35	16.63
13	17.	7.00	30.00	4.66	4.65	27.90	301.50	12.98
17	18.	7.00	30.00	5.50	6.70	27.85	283.80	26.32
2	19.	9.00	25.00	5.00	4.65	18.60	299.70	14.12
11	20.	7.00	21.59	5.50	4.95	25.05	237.90	13.78

Table 4. ANOVA analysis for laccase production from pine needles biomass using response surface for central composite design

Source	Sum of squares		Df		Mean square		F value		p-value prob>F			
	Untreated	P	U	P	U	P	U	P	U	P	U	P
Model	25.47	25.75	9	9	2.83	2.86	2.81	3.51	0.0619	0.0316	not significant	Significant
A	7.87	6.37	1	1	7.87	6.37	7.8	7.82	0.019	0.0189		
B	0.59	3.35	1	1	0.59	3.35	0.58	4.11	0.4639	0.0702		
C	0.69	1.4	1	1	0.69	1.4	0.68	1.72	0.4278	0.2195		
A^2	7.05	5.24	1	1	7.05	5.24	6.99	6.43	0.0246	0.0296		
B^2	3.3	6.03	1	1	3.3	6.03	3.27	7.4	0.1006	0.0215		
C^2	8.35	5.71	1	1	8.35	5.71	8.28	7	0.7194	0.0245		
AB	0.14	0	1	1	0.14	0	0.14	0	0.7972	1		
AC	0.07	0.28	1	1	0.07	0.28	0.07	0.35	0.5743	0.5699		
BC	0.34	0.18	1	1	0.34	0.18	0.34	0.22		0.6485		
Residual	10.09	8.15	10	10	1.01	0.81			0.013		Significant	
Lock of fit	9.15	6.86	5	5	1.83	1.37	9.71	5.32		0.0453		Significant
Pure Error	0.94	1.29	5	5	0.19	0.26						
Cor Total	35.56	33.89	19	19								

*U: Untreated: P: Pre-treated

Table 5. ANOVA analysis for pine needles hydrolysis and enzyme production from *Trichoderma* sp.

Source	Sum of squares		df		Mean square		F		p-value prob>F			
	U	P	U	P	U	P	U	P	U	P	U	P
Model	690.07	495.2	9	9	76.67	55.03	2.7	2.22	0.0688	0.1153	not significant	not significant
A	60.33	37.84	1	1	60.33	37.84	2.12	1.53	0.1756	0.2451		
B	15.18	27.67	1	1	15.18	27.67	0.53	1.12	0.4814	0.3158		
C	6.90E-03	2.94	1	1	6.90E-03	2.94	2.429-004	0.12	9.88E-01	0.7376		
A^2	341.89	273.06	1	1	341.89	273.06	12.04	11.01	0.006	0.0078		
B^2	209.2	148.74	1	1	209.2	148.74	7.37	6	0.0218	0.0343		
C^2	124.14	75.22	1	1	124.14	75.22	4.37	3.03	0.063	0.1123		
AB	4.5	0.28	1	1	4.5	0.28	0.16	0.011	0.6989	0.9173		
AC	15.4	1.13	1	1	15.4	1.13	0.54	0.045	0.4783	0.8357		
BC	23.81	2.21	1	1	23.81	2.21	0.84	0.089	0.3814	0.7717		
Residual	283.93	248.1	10	10	28.39	24.81						
Lack of fit	234.58	222.65	5	5	46.92	44.53	4.75	8.75	0.0561	0.0163	not significant	significant
Pure Error	49.35	25.45	5	5	9.87	5.09						
Cor Total	973.99	743.37	19	19								

*U: Untreated: P: Pre-treated

Table 6. ANOVA analysis for xylanase enzyme from pine needles biomass using response surface for central composite design

Source	Sum of Square		df		Mean Squares		F value		p-value prob>F			
	U	P	U	P	U	P	U	P	U	P	U	P
Model	94787.6	96410.69	9	9	10531.96	10712.3	2.73	4.06	0.0666	0.0198	not significant	Significant
A	12292.61	12617.11	1	1	12292.61	12617.11	3.19	4.78	0.1044	0.0537		
B	2069.55	1368.44	1	1	2069.55	1368.44	0.54	0.52	0.4805	0.488		
C	4430.75	11364.25	1	1	4430.75	11364.25	1.15	4.31	0.3088	0.0647		
A^2	15027.8	15225.92	1	1	15027.8	15225.92	3.9	5.77	0.0765	0.0372		
B^2	34112.86	30883.79	1	1	34112.86	30883.79	8.85	11.7	0.0139	0.0065		
C^2	1574.51	71.82	1	1	1574.51	71.82	0.41	0.027	0.537	0.8723		
AB	15255.68	16065.28	1	1	15255.68	16065.28	3.96	6.09	0.0746	0.0333		
AC	10771.45	11408.05	1	1	10771.45	11408.05	2.8	4.32	0.1255	0.0643		
BC	248.09	85.15	1	1	248.09	85.15	0.064	0.032	0.8048	0.861		
Residual	38532.57	26394.01	10	10	3853.26	2639.4						
Lack of Fit	30039.55	19284.44	5	5	6007.91	3856.89	3.54	2.71	0.0959	0.1487	not significant	not significant
Pure Error	8493.02	7109.57	5	5	1698.6	1421.91						
Cor Total	1.33E+05	1.23E+05	19	19								

*U: Untreated: P: Pretreated

temperature and incubation time, interaction between temperature and pH and interaction between incubation time and pH, respectively. Fig. 3a & 3b represent normal plots of *Trichoderma* sp. for internally studentized residual response production in CCD using untreated and pretreated pine needles biomass for laccase production.

The statistical significance of Eqns. 3 and 4 was evaluated for cellulase production by *F*-test and the analysis of variance (ANOVA) of the response surface quadratic model for *Trichoderma* sp. for cellulase is presented in Table 5 as well as ANOVA for xylanase production is represented in Table 6.

Enzyme Activity 3 (Cellulase Untreated) = 32.55 - 2.10 × A + 1.05 × B - 0.022 × C - 4.87 × A2 - 3.81 × B2 - 2.94 × C2 + 0.75 × A × B - 1.39 × A × C - 1.73 × B × C **(Eqn. 3)**

Enzyme Activity 4 (Cellulase Pre - treated) = 29.04 - 1.66 × A + 1.42 × B - 0.46 × C - 4.35 × A2 - 3.21 × B2 - 2.28 × C2 + 0.19 × A × B - 0.37 × A × C - 0.53 × B × C **(Eqn. 4)**

3D surface presentation under CCD for cellulase production by *Trichoderma* sp. using untreated and pretreated pine needles biomass with interactions between different variables are represented in Fig. 4a(i-iii) & 4b(i-iii), where interaction between incubation time and pH, interaction between temperature and pH and interaction between incubation time and temperature respectively. The Fig. 5a & 5b represents normal plot of *Trichoderma* sp. for internaly studentized residulas response production in CCD by using untreated (Fig. 5a) and pretreated (Fig. 5b) pine needles biomass for cellulase production.

The statistical significance of equations 5 and 6 were also analyzed for reducing sugar production by *F*-test and the analysis of variance (ANOVA) of the response surface quadratic model for *Trichoderma* sp. for xylanase is presented in Table 7.

Enzyme Activity 5 (Xylanase Untreated) = 286.18 - 30.00 × A + 12.31 × B - 18.01 × C - 32.29 × A2 - 48.65 × B2 + 10.45 × C2 + 43.67× A × B + 36.69 × A × C + 5.57 × B × C **(Eqn. 5)**

Enzyme Activity 6 (Xylanase Pretreated) = 276.13 - 30.40 × A - 10.01 × B - 28.85 × C - 32.50 × A2 - 46.29 × B2 + 2.23 × C2 - 44.81 × A × B + 37.76 × A × C + 3.26 × B × C (**Eqn. 6)**

3D surface presentation under CCD for xylanase production by *Trichoderma* sp. using untreated and pretreated pine needles biomass, with interactions between different variables are represented in Fig. 6a (i-iii) & 6b (i-iii), where interaction between incubation time and temperature, interaction between incubation time and pH and interaction between pH and temperature, respectively. The Fig. 7a & 7b represents a normal plot of *Trichoderma* sp. for internally studentized residuals response production in CCD by using untreated and pretreated pine needles biomass for xylanase production.

Table 7. ANOVA for reducing sugars from pine needles biomass using response surface for central composite design

Source	Sum Squares		df		Mean Squares		F value		p-value prob>F			
	U	P	U	P	U	P	U	P	U	P	U	P
Model	821.36	820.36	9	9	91.26	91.24	3.75	3.74	0.0257	0.0257	Significant	significant
A	321.89	318.89	1	1	321.89	318.89	13.22	13.2	0.0046	0.0044		
B	31.63	30.63	1	1	31.63	30.63	1.3	1.2	0.281	0.281		
C	1.08	1.06	1	1	1.08	1.06	0.044	0.044	0.8372	0.8372		
A^2	391.2	390.2	1	1	391.2	390.2	16.06	16.04	0.0025	0.0025		
B^2	25.89	24.89	1	1	25.89	24.89	1.06	1.04	0.3268	0.3264		
C^2	23.89	22.89	1	1	23.89	22.89	0.98	0.97	0.3454	0.3452		
AB	28.39	27.39	1	1	28.39	27.39	1.17	1.16	0.3056	0.3054		
AC	29.53	28.53	1	1	29.53	28.53	1.21	1.2	0.2966	0.2964		
BC	0.68	0.65	1	1	0.68	0.65	0.028	0.028	0.8708	0.8706		
Residual	243.53	240.52	10	10	24.35	24.35						
Lack of Fit	196.13	194.12	5	5	39.23	39.23	4.14	4.12	0.0726	0.0724	not significant	not significant
Pure Error	47.4	46.4	5	5	9.48	9.48						
Cor Total	1064.89	1052.79	19	19								

*U: Untreated: P: Pretreated

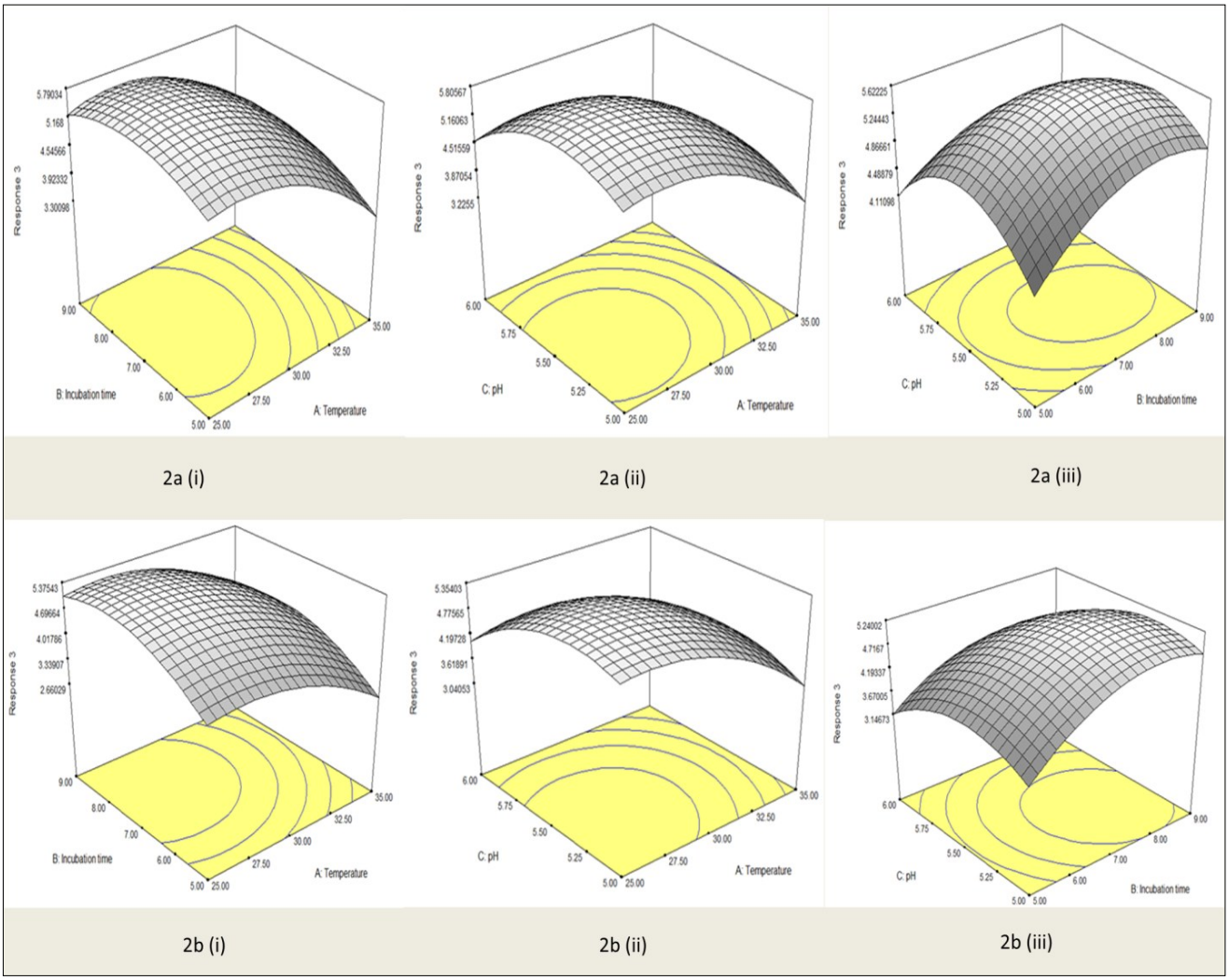

Fig. 2. 3D surface graphs using Central Composite Design for laccase production by *Trichoderma* sp. using untreated and pretreated pine needles biomass: **2ai.** Interaction between temperature and incubation time; **2aii.** Interaction between temperature and pH; **2aiii.** Interaction between incubation time and pH; **2bi.** Interaction between temperature and incubation time; **2bii.** Interaction between temperature and pH; **2biii.** Interaction between incubation time and pH.

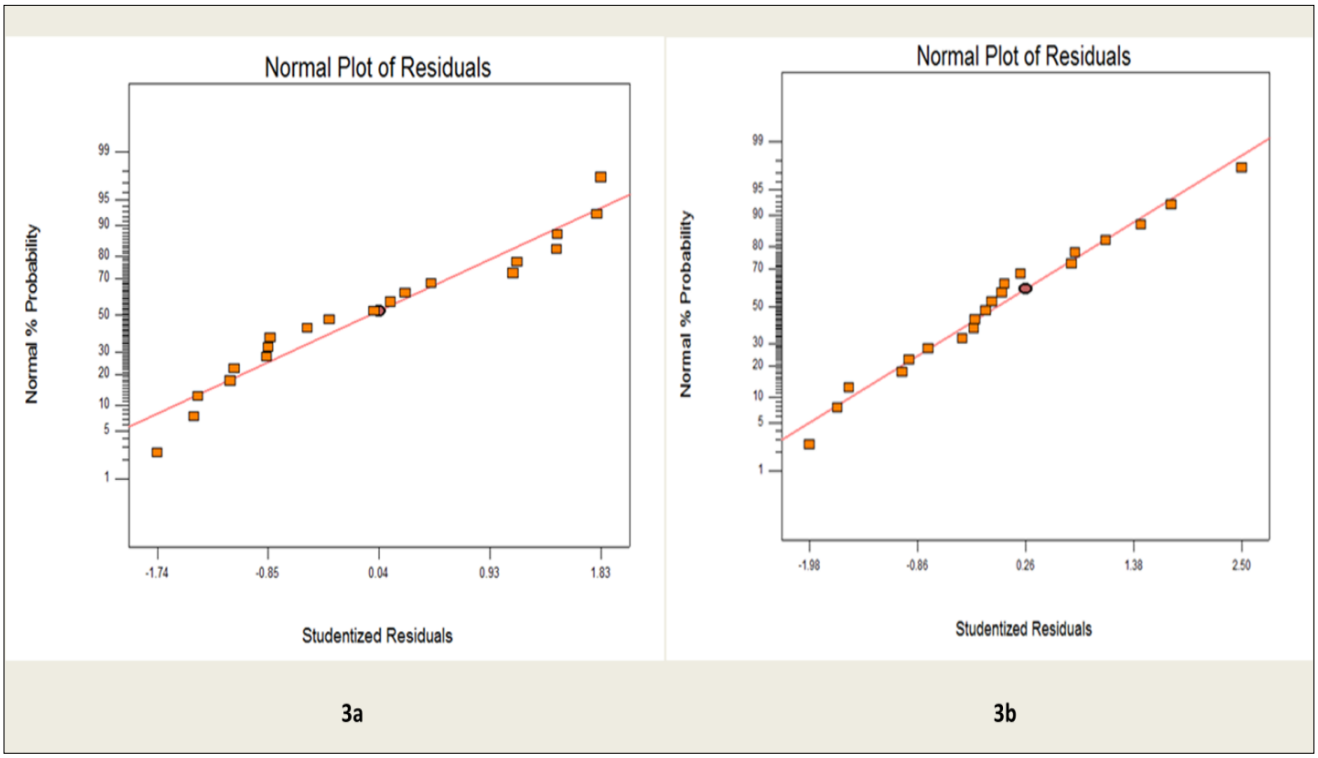

Fig. 3. Normal plot of *Trichoderma* sp.for internally studentized residuals response production in CCD by using: **3a.** Untreated & **3b.** Pretreated pine needles biomass for laccase production.

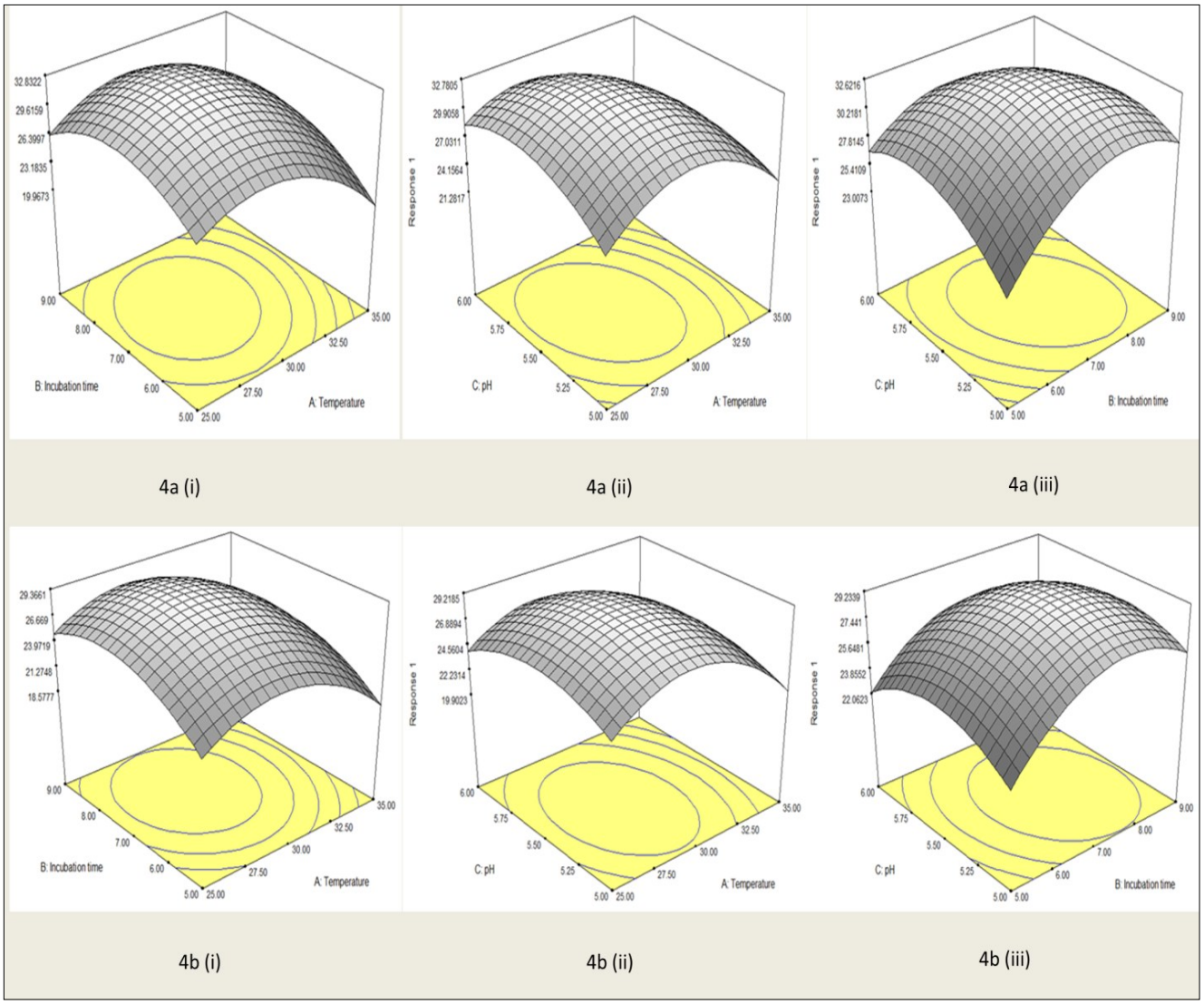

Fig. 4. 3D surface graphs using Central Composite Design for cellulase production by *Trichoderma* sp. using untreated and pretreated pine needles biomass: **4ai.** Interaction between temperature and incubation time; **4aii.** Temperature and pH; **4aiii.** Incubation time and pH; **4bi.** Temperature and incubation time; **4bii.** Temperature and pH; **4biii.** Incubation time and pH.

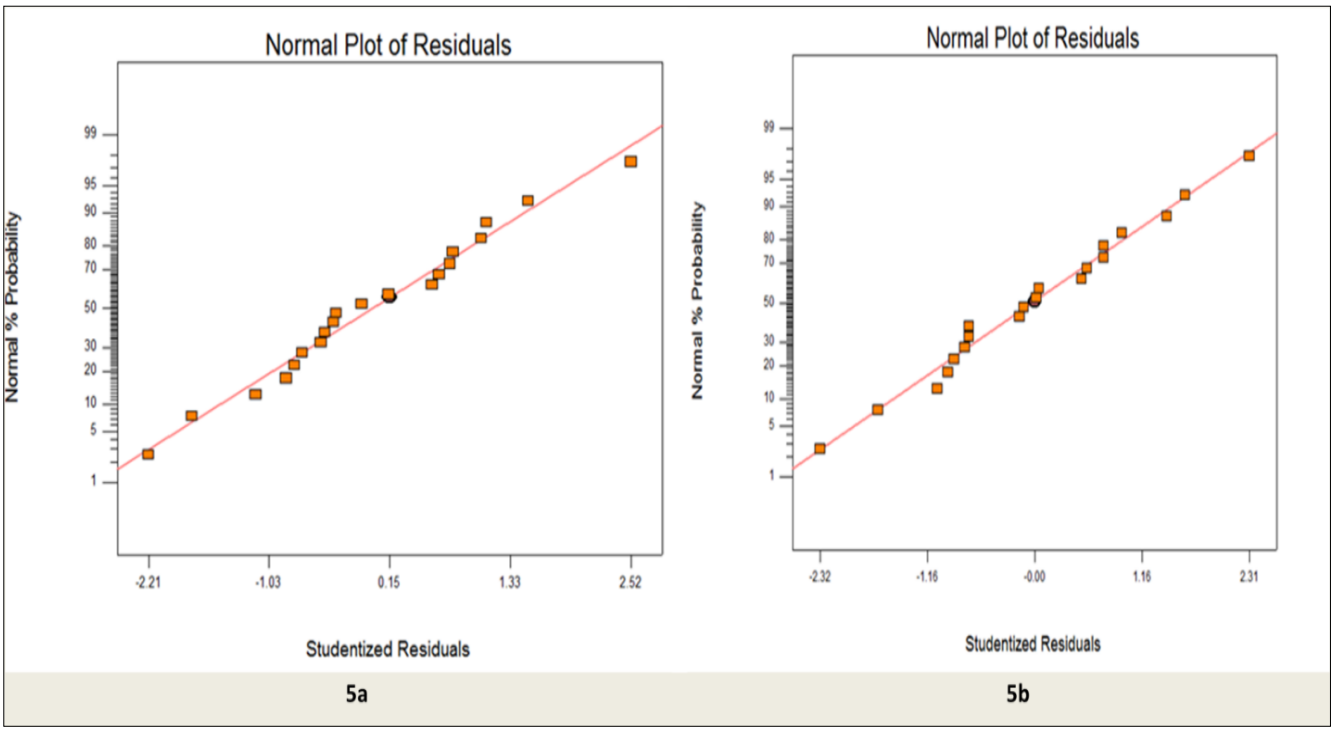

Fig. 5. Normal plot for cellulase production from *Trichoderma* sp. for internally studentized residuals response production in CCD by using **5a.** Untreated & **5b.** Microwave pretreated pine needles biomass.

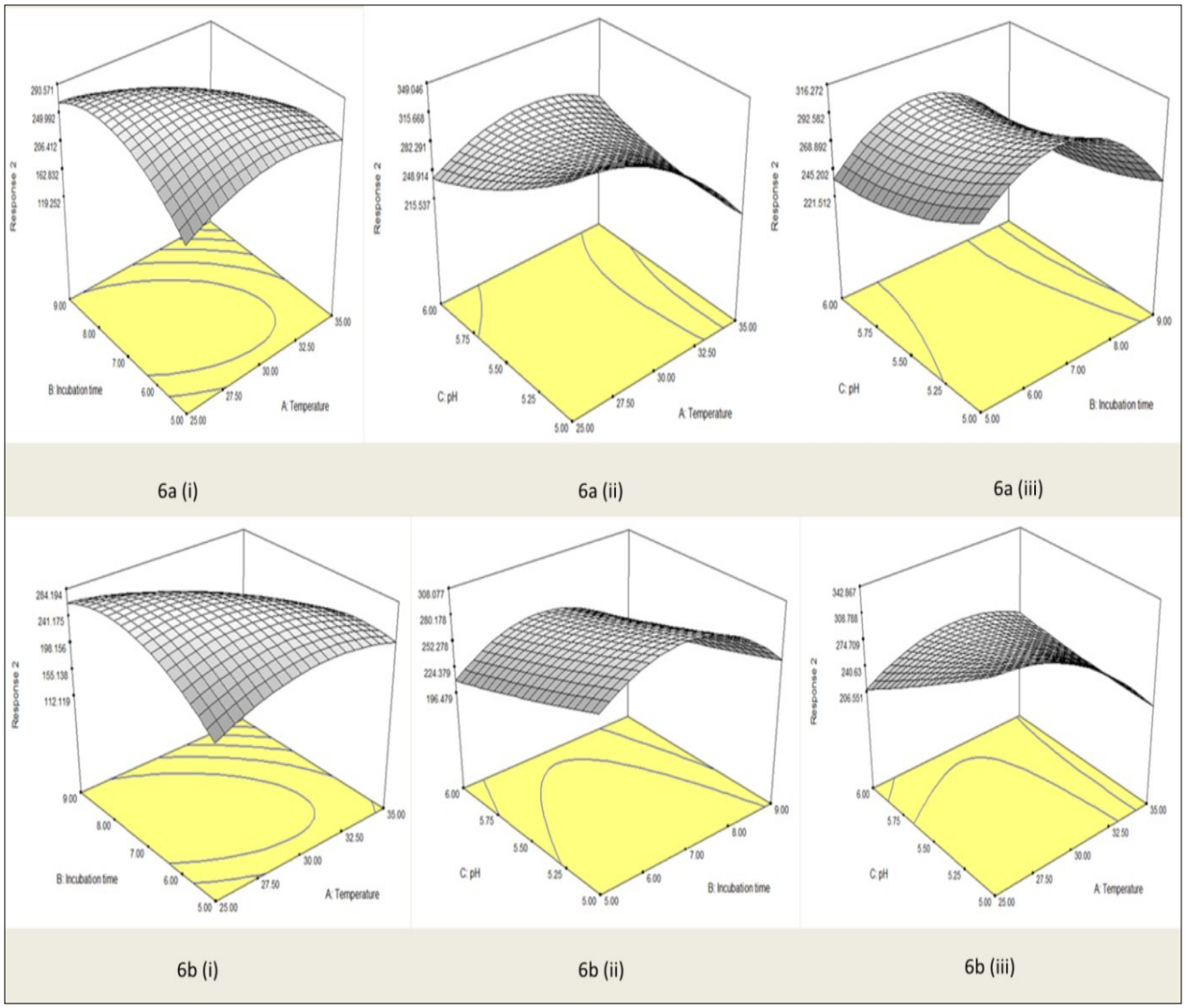

Fig. 6. 3D surface graphs using Central Composite Design for xylanase production by *Trichoderma* sp. using untreated and pretreated pine needles biomass: **6ai.** Interaction between temperature and incubation time; **6aii.** Temperature and pH; **6aiii.** Incubation time and pH; **6bi.** Temperature and incubation time; **6bii.** Temperature and pH; **6biii.** Incubation time and pH.

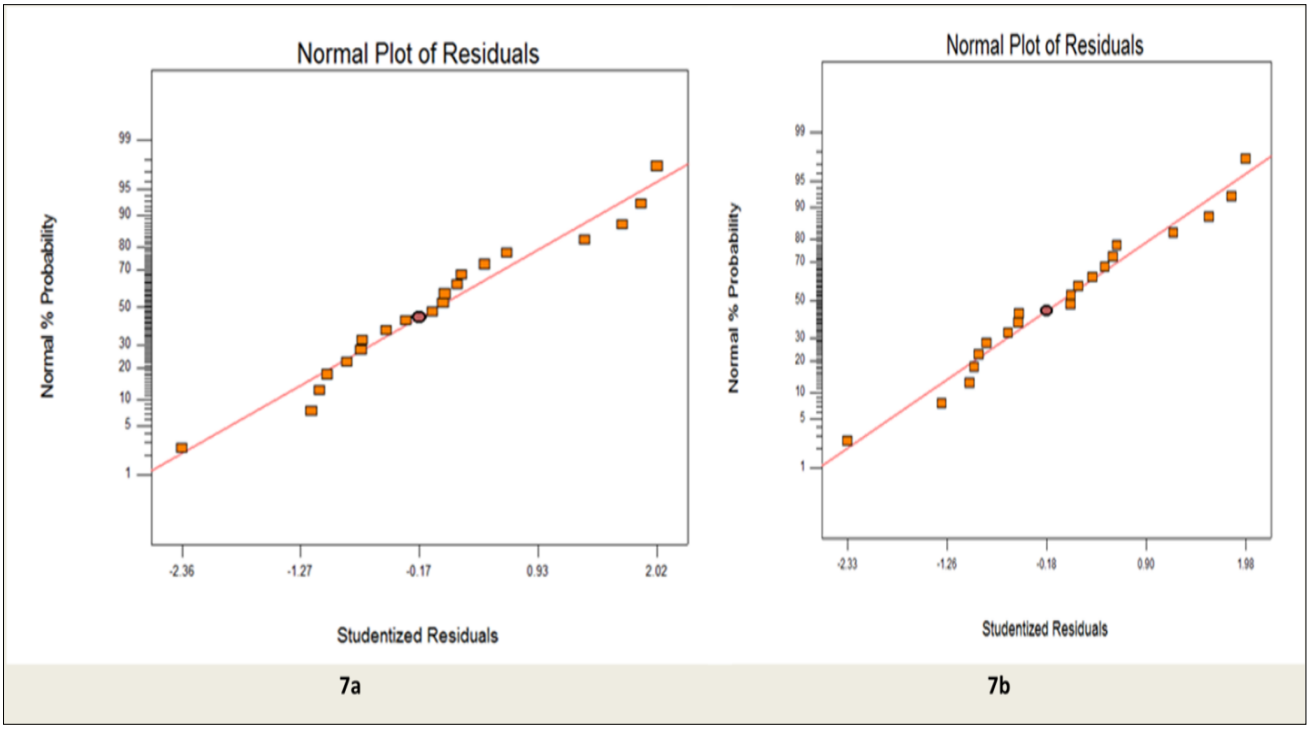

Fig. 7. Normal plot for xylanase production from *Trichoderma* sp. for internally studentized residuals response production in CCD by using: **7a.** Untreated & **7b.** Pretreated pine needles biomass.

The statistical significance of Eqns. 7 & 8 were also analyzed for reducing sugar production by *F*-test and the analysis of variance (ANOVA) of the response surface quadratic model for *Trichoderma* sp. for reducing sugars is presented in Table 7.

Reducing sugar (Untreated) = 31.85 - 4.85 × A-1.52 × B-0.28 × C -5.21 × A2 - 1.34 × B2+ 1.29 × C2 - 1.88 × A × B + 1.92 × A × C + 0.29 × B × C (**Eqn. 7)**

Reducing sugar (Pretreated) = 28.85 - 3.85 × A - 1.42 × B - 0.22 × C - 5.11 × A2 - 1.24 × B2 + 1.25 × C2 - 1.78 × A × B + 1.84 × A × C+ 0.20 × B × C (**Eqn. 8)**

3D surface presentation under CCD for reducing sugars production by *Trichoderma* sp. using untreated and pretreated pine needles biomass with interactions between different variables are represented in Fig. 8a (i-iii) & 8b (i-iii), where interaction between incubation time and temperature, interaction between temperature and pH and interaction between incubation time and pH, respectively. The Fig. 9a & 9b represents a normal plot of *Trichoderma* sp. for internally standardized residual response production in CCD by using untreated (Fig. 9a) pine needles and pretreated (Fig. 9b) pine needles biomass for reducing sugars production.

The closer R^2 (correlation coefficient) measures the linear relationship between two variables is to 1.0. The valueof R= 0.9903 (Eq. 1), 0.6190 (Eq. 2), 0.9280 (for Eq. 3), 1.3255 (Eq. 4), 0.8375 (for Eq. 5), 0.3123 (for Eq. 6), 0.4908 (for Eq. 7) and 0.4890 (for Eq. 8) for untreated and pretreated biomass indicated a close compliance between the experimental values and the predicted values of the model equations. An adequate precision value for laccase, cellulase, xylanase and reducing sugar production by untreated pine needles biomass for *Trichoderma* sp. was 4.583, 4.595, 5.481 and 7.399, respectively, whereas adequate precision in pretreated pine needles biomass, i.e. 4.817, 4.290, 6.298 and 7.232, respectively, was measured. The coefficient of variation (CV) designates the degree of precision between the treatments that were compared. A higher coefficient of variation (CV) was observed for untreated pine needle biomass, with values of 23.62 (laccase), 21.64 (cellulase), 26.08 (xylanase) and 18.62 (reducing sugar). In contrast, the pretreated biomass exhibited CV values of 23.72, 22.32, 22.95 and 17.56, respectively, indicating improved precision and reliability of the experiment. The P-values were analyzed to determine the significance of the coefficients, which help in understanding the mutual interactions between independent and dependent variables. A lower P-value indicates a higher significance of the corresponding coefficient. For laccase, cellulase, xylanase and reducing sugar production by *Trichoderma* sp., the parameter estimates and P-values suggested that the independent variables A (temperature), B (incubation time) and C (pH)- significantly influenced enzyme production. Additionally, the quadratic terms of all variables were found to be statistically significant. In terms of interactions: For untreated pine needle biomass, significant model terms included: Laccase: A, A^2, C^2, Cellulase: A^2, B^2, Xylanase: B^2, Reducing sugar: A, A^2, For pretreated pine needle biomass, significant interactions were: Laccase: A, A^2, B^2, C^2, Cellulase: A^2, B^2, Xylanase: A^2, B^2, AB, Reducing sugar: A, A^2, B^2. These results indicate that temperature, incubation time and pH play crucial roles in enzyme production, with specific quadratic and interaction terms contributing significantly to the response in both untreated and pretreated biomass.

Optimum conditions localization

Three-dimensional (3D) surface plots and corresponding contour plots generated through the regression model were utilized to assess the effects of temperature, incubation time and pH on the production of hydrolytic enzymes and reducing sugars by *Trichoderma* sp. These plots help visualize the interactions between pairs of independent variables, with the third variable held constant at its central value. The contour plot configuration provides insights into the significance of mutual interactions among the independent variables.. In the contour plots, elliptical configurations indicated statistically significant interactions between variables, while circular patterns suggested weaker or negligible interactions. This graphical representation confirmed that the mutual influence of temperature and pH, as well as incubation time and pH, had a considerable effect on enzyme synthesis and sugar release. Fig. 2-9 depicts the predicted values for laccase, cellulase, xylanase and reducing sugar production by *Trichoderma* sp., showing the correlation among temperature, incubation time and pH along the X-axis. The 3D plots indicate a moderate interaction among these variables, contributing to the enhanced production of laccase, cellulase, xylanase and reducing sugars. These findings highlight the importance of optimizing process parameters to maximize enzyme activity and yield. This supports the utility of RSM in identifying critical zones of operation for maximizing enzyme yields in solid-state fermentation systems.

Differentiation between observed and predicted values of laccase, cellulase, xylanase enzyme activities and reducing sugar

A regression-based model derived from RSM was used to predict enzyme activity (laccase, cellulase, xylanase) and reducing sugar yield under various conditions using *Trichoderma* sp. The accuracy of this model was evaluated by comparing observed experimental values to predicted values, as visualized in Fig. 2-9.

The predicted R^2 values for untreated pine needle biomass were 0.9903 (laccase), 0.9280 (cellulase), 0.8375 (xylanase) and 0.4908 (reducing sugar), which showed reasonable agreement with the adjusted R^2 values of 0.4609, 0.4461, 0.4509 and 0.5655, respectively. Similarly, for pretreated biomass, the predicted R^2 values were 0.6190, 1.3255, 3.123 and 0.4890, aligning with the adjusted R^2 values of 0.5432, 0.3659, 0.5916 and 0.5545 for laccase, cellulase, xylanase and reducing sugar, respectively. These results demonstrate a strong correlation between experimental and predicted values, validating the model's effectiveness in estimating enzyme production.

The adjusted R^2 corrects the standard R^2 value by considering sample size and the number of model terms. When the number of terms is high and the sample size is low, the adjusted R^2 tends to be significantly lower than the predicted R^2. The model F-values for untreated biomass were 2.81 (laccase), 2.70 (cellulase), 2.73 (xylanase) and 3.75 (reducing sugar), whereas for pretreated biomass, the F-values were 3.51, 2.22, 4.06 and 3.65, respectively. The p-values (Prob > F < 0.05) confirmed that the model terms were statistically significant. These figures further validate the accuracy of the predicted data for *Trichoderma* sp. as derived from Eqn. 1-8.

Several studies have reported that the production of hydrolytic enzymes such as cellulases, xylanases and laccases from microorganisms utilizing lignocellulosic waste requires

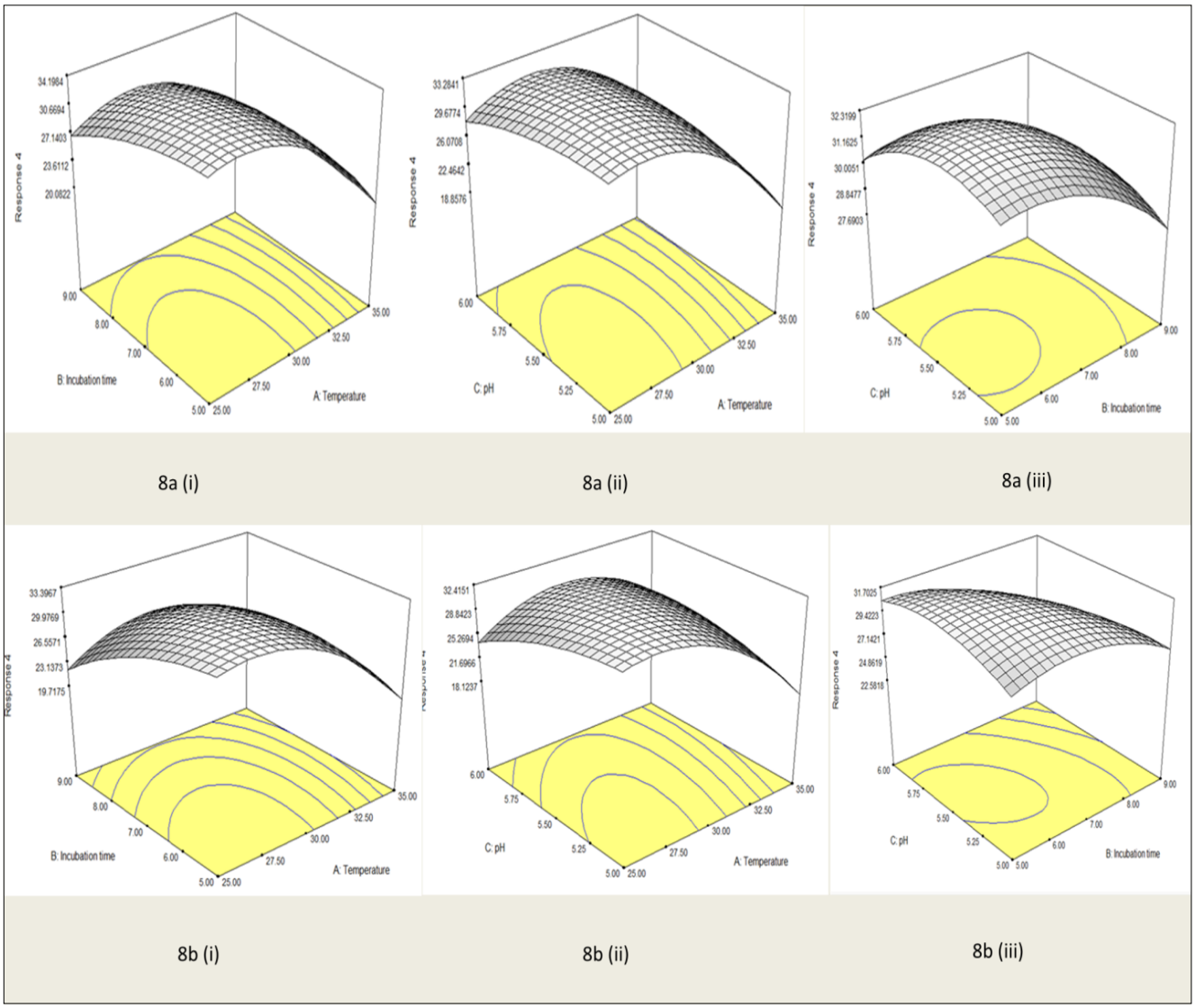

Fig. 8. 3D surface graphs using Central Composite Design for reducing sugar production by *Trichoderma* sp. using untreated and pretreated pine needles biomass: **8ai.** Interaction between temperature and incubation time; **8aii.** Interaction between temperature and pH; **8aiii.** Interaction between incubation time and pH; **8bi.** Interaction between temperature and incubation time; **8bii.** Interaction between temperature and pH; **8biii.** Interaction between incubation time and pH.

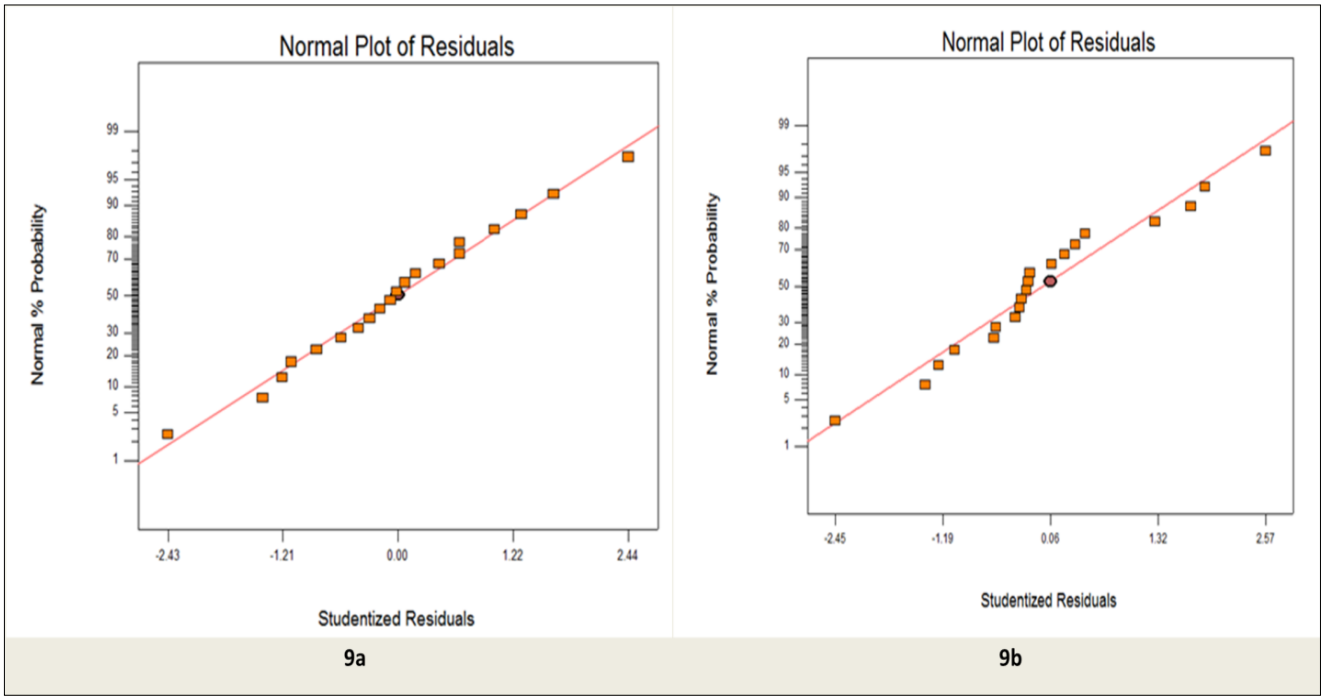

Fig. 9. Normal plot for reducing sugar production from *Trichoderma* sp. for internally studentized residuals response from: **9a.** Untreated and **9b.** Microwave pretreated pine needle biomass.

prolonged incubation periods (27). However, the presence of negative predicted R^2 values (e.g., -0.82) suggests that the model may require further refinement. Incorporating higher-order models could potentially yield more accurate results (28).

Checking model adequacy

To ensure the fitted regression models accurately represent the underlying biological system, a thorough model adequacy check was performed. This involved examining the residuals from the least squares regression, which serve as indicators of how well the model predicts actual outcomes. One of the key aspects of model adequacy assessment is analyzing the residuals from the least squares fit. These residuals play a crucial role in determining the model's validity. To check the normality assumption, a normal probability plot of residuals was constructed (Fig. 3a, 5a, 7a & 9a) for untreated pine needle biomass and and pretreated biomass (Fig. 3b, 5b, 7b & 9b). The results confirmed that the normality assumption was satisfied, as the residuals approximated a straight-line pattern, indicating a significant and valid model. Furthermore, the random scattering of residuals across the display suggests that the variance of the original observations remains constant for all values of the predicted response in both untreated and pretreated biomass. This consistency validates the empirical model, confirming its adequacy in representing laccase, cellulase and xylanase and reducing sugar activity within the response surface methodology (RSM) framework.

Model validation

The statistically optimal variable values were determined by analyzing both the major and minor axes of the contour, where the response at the center point yielded the maximum production of laccase, cellulase, xylanase and reducing sugars. These findings were further validated through canonical analysis of the response surface, which identified a minimum region for the model. The stationary point, representing the highest enzyme activity for *Trichoderma* sp., was observed at critical values of 6.90, 37.86, 398 and 47.98 U/g for untreated pine needle biomass, whereas for pretreated biomass, the critical values were 6.70, 36.80, 392.10 and 45.65 U/g. These optimal values were achieved at a temperature of 30 ℃, pH 5.5 and an incubation period of 7 days. To ensure maximum enzyme and reducing sugar production, statistical optimization was performed to identify the best combination of factors. The most suitable targets for each variable and response were selected and customized weightings were applied to refine the desirability function, ensuring an optimal balance of conditions for enhanced enzyme production.

Discussion

Pine needles are the potential source of carbohydrate polymers composed of cellulose, hemicellulose and lignin for fermentation into simple sugars. Pretreatments, i.e. physical, chemical or biological, are necessary for better degradation of pine needles (13). In the present study, physical pretreatment, i.e., microwave irradiation at 400 W for 2 min, was given to pine needles, which is one of the latest and feasible methods supposed to simplify three-dimensional structural polymers of lignocellulosic biomass. Degraded woody biomass is the best source of lignocellulolytic hydrolytic enzymes producers (bacteria, fungi). Thus, isolation of hyperligninolytic, hypercellulolytic and hyper xylanolytic fungi had been done from the rotten wood believed it a valuable habitat for fungi for biodegradation.

The maximum production of hydrolytic enzymes and reducing sugar was observed at 30 ℃. Temperatures more and less than this optimum level had been unfavourable for enzyme production due to minimal growth of isolated mesophilic fungal isolates, thus consistently dipping their enzyme activity. On the other hand, optimum temperature is highly suitable for the secretion of extracellular hydrolytic enzymes in culture supernatant, probably due to their rapid growth and vigorous metabolic activities. Research indicates that the maximum laccase activity by *Trichoderma versicolor* was observed at 28 ℃ using pretreated *Partenium* biomass, i.e. 277.16 U/g (29). Previous studies noticed maximum cellulase and xylanase production in *Aspergillus terreus* using pretreated and untreated pine needle biomass, i.e. 2.26, 118.54 U/g activities were attained at 30 ℃ (30). The maximum cellulase and xylanase production was found at 25 ℃ in *Trichoderma* sp. using pretreated pods of *P. juliflor,* whereas in untreated biomass, the maximum activity was observed at 30 ℃ (31).

Moisture content is a requisite factor for the growth of microbes. The water activity (a_w) requirement of all the strains is varied and requires different substrate: moisture ratios. Higher or lower than the optimum level of this ratio indicates inadequate water activity, thus decreasing the yield of the enzyme and reducing sugar production from the substrate.

Incubation time is a vital factor among different physical variables to affect enzyme activity as well as released reducing sugars. Overall, the incubation period of 7 to 10 days in the present study is the best for enzyme and reducing sugar production. Incubation time more than the optimum days had resulted in decreased yield of enzyme and reducing sugar, probably due to the exhausted nutrient resources, creating a stressed environment for microbial growth. Additionally, product inhibition can also be a possible factor in to release of sugar. Most of the fungal strains grow and produce enzymes at a lower pH. Here, the optimum pH for enhanced enzyme and sugar production was found to be 5.5, which confirmed its acidophilic nature. Higher pH more than 5.5 might have resulted in an adverse environment for the growth of these isolates, thus in turn inhibiting the production of enzymes as well as sugars.

To integrate and optimize the various factors influencing enzyme production, Response Surface Methodology (RSM) was employed. This statistical technique enabled the construction of a predictive model and identification of optimal conditions for maximum production of laccase, cellulase and xylanase (32). The use of laccase from microbial origin for the complete saccharification/hydrolysis of biomass is considered an eco-friendly and sustainable process. The maximum cellulase production achieved through RSM was observed at 37 ℃ and pH 7.0 for 72 hr with enzyme activites of CMCase (0.589 µmol/min), FPase (1.22 µmol/min) and amylase (0.92 µmol/min). By using the SDS-PAGE technique molecular weight of the enzyme was found to be 65 kDa, capable of producing cellulase enzymes, which was reported as thermostable by using zymogram analysis (33). The combination of one-factor-at-a-time (OFAT) and Response Surface Methodology (RSM) has proven effective in maximizing enzyme production by systematically optimizing process parameters. OFAT allows

initial identification of key variables, while RSM statistically models their interactions for optimal yield. Recent studies on microbial transglutaminase (33) and pectinase (34) have demonstrated significant yield improvements using this sequential approach. RSM designs like Central Composite Design ensure robust statistical validation, with high R^2 values and minimal prediction error. This hybrid method offers a reliable, reproducible framework for enzyme optimization in industrial and research settings (34, 35). Previous research optimized designs for pectinases and MTG enzymes, validated this combined approach, leading to reliable, predictable yield gains (35). The co-production of multiple enzymes by *Trichoderma* sp., particularly under optimized SSF conditions, demonstrates the organism's potential for cost-effective and eco-friendly lignocellulosic biomass degradation. The results underline the promise of microbial enzymatic systems in bioethanol production, biopulping and waste management industries, contributing significantly to the development of sustainable biotechnological processes.

Conclusion

This study successfully demonstrated the potential of *Trichoderma* sp. for enhanced production of hydrolytic enzymes under solid-state fermentation (SSF), optimized using Response Surface Methodology (RSM). The systematic optimization of key parameters-temperature, pH and incubation time-led to significant improvements in the yields of laccase, cellulase and xylanase, which are pivotal for the efficient depolymerization of lignocellulosic biomass. The optimized conditions derived from the RSM provided a robust and efficient framework for enzyme production, showcasing significant improvements over traditional, non-optimized methods. This optimization not only resulted in higher enzyme yields but also contributed to better enzyme activity for industrial applications. The success of this approach highlights the potential of *Trichoderma* sp. as a prolific producer of hydrolytic enzymes, which are essential in converting lignocellulosic biomass into an appreciable amount of fermentable sugars. This conversion is a key step in the development of sustainable biofuels and other bioproducts, offering a greener alternative to fossil fuels and aiding in the mitigation of environmental concerns such as climate change and resource depletion. Overall, this work lays the foundation for scaling up microbial enzyme production systems for biotechnological and industrial applications, particularly in the bioenergy, pulp and paper and waste management sectors.

Authors' contributions

NS conducted the research work, co-conceptualised the study, contributed to original draft writing and participated in manuscript review and editing. CT was involved in conceptualising the study and drafting the original manuscript. YKA co-conceptualised the study, contributed to original draft preparation and assisted in manuscript review and editing. HM carried out corrections, performed software analysis and prepared figures. VC contributed to corrections, software analysis and figure preparation. SS participated in corrections, software analysis and figure preparation. KB reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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