JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

OPTIMIZATION OF CARBOXY METHYL CELLULASE PRODUCTION BY *CLADOSPORIUM* SP. WITH FOOD WASTE IN BAGASSE SOLID STATE FERMENTATION USING RESPONSE SURFACE METHODOLOGY

Rama Mohan Poludasu [1,2 *], *Obulam Vijaya Sarathi Reddy*[1], *Piyarat Boonsawang* [2], *Vani Mathakala* [3]

Address(es):
[1] SV University, Department of Biochemitry, Tirupati, India-517502.
[2] Prince of Songkla University, Faculty of Agro-Industry, Biotechnology Program, Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Hat Yai, Songkhla, Thailand, 90110.
[3] Sri Padmavati Mahila Visvavidyalayam (SPMVV), Department of Applied Microbiology, Tirupati, Andhra Pradesh 517502.

*Corresponding author: rammohanroyal@gmail.com

https://doi.org/10.55251/jmbfs.10132

ARTICLE INFO

Received 22. 5. 2023
Revised 12. 7. 2024
Accepted 16. 7. 2024
Published 1. 10. 2024

Regular article

OPEN ACCESS

ABSTRACT

Enzyme production costs should be reduced by using inexpensively available substrates. In this, we determine the effects of various medium components and food processing waste on *Cladosporium* sp. production of CMCase in SSF. Five factors were tested with a three-level Box-Wilson factorial, central composite design (CCD) to determine how they impacted *Cladosporium* sp. NCIM 901's ability to produce CMCase. For the model, the R^2 (coefficient of determination) value was 0.9349. Based on the process findings, the optimal medium for higher enzyme synthesis had wheat bran (4 g/L), $cacl_2$ (2 g/L), urea (7.5 g/L) and rice bran (8.76 g/L), with the medium's original pH at 5.5. The model's accuracy is confirmed by the experimental result of CMCase, which is 23.42 U/gds and equivalent to the expected yield of 22.31 U/gds. The accuracy of the current experimental design has been verified by experimental validation. The enzyme's properties indicated that its greatest activity occurred at pH 5.5 and that its ideal temperature was about 50 °C. The enzyme is more active toward CMC and less active toward filter paper. $CaCl_2$ and $MgCl_2$ increased the enzyme's activity. Enzymatic hydrolysis of waste-derived polysaccharides into simple sugars is necessary for the industrial manufacture of bioethanol. The efficacy of the produced *Cladosporium* sp. cellulase (CMCase) enzyme to saccharify pearl millet grass results shows that H_2SO_4 treated substrate gave more fermentable sugars compared to the untreated substrate. Results suggest that the indigenous produced-CMCase can be efficiently used for lignocellulosic biomass saccharification (55.40%) compared to the untreated sample (37.10%) in 48 h, which will eventually reduce the cost utilized for purchasing commercial enzymes.

Keywords: *Cladosporium* sp. NCIM 901, Sugarcane bagasse, Wheat and Rice bran, Solid State Fermentation, Central Composite Design, Carboxymethyl cellulase, Characterization, Pearl millet grass saccharification

INTRODUCTION

The abundant microorganisms in nature, especially fungi, play a vital role in agriculture by decomposing organic matter and managing waste. Researchers focus on newly discovered fungal species for their potential in producing essential cellulase enzymes crucial in industrial processes. Extensive research has focused on cellulases in biomass and waste conversion **(Naher *et al*., 2021)**.

The production of cellulases from less expensive substrates is an effective way to reduce costs. Cellulases, involved in the hydrolysis of cellulose, generally exist in three different kinds. Endoglucanases (EG) attack the carbohydrate chain inside and selectively hydrolyse substituted soluble celluloses like carboxymethyl cellulose (CMC), yet, they are little efficient towards strongly structured crystalline cellulose, for example, Avicel or cotton fibers. Cellobiohydrolases (CBH) can break down crystalline cellulose and produce cellobiose, nevertheless, they have no impact on substituted celluloses. Short cello-oligosaccharides and cellobioses are generated by the enzyme and are hydrolyzed by β-glucosidases to glucose **(Jayasekara and Ratnayake, 2019)**.

For the generation of enzymes, solid-state fermentation (SSF) is an alternative to the more traditional submerged culturing method **(Lopez-Gomez and Venus, 2021)**. Higher output per bioreactor volume, reduced budget and area requirements, simpler apparatus and easy access to downstream processing are some positive aspects of SSF **(Srivastava *et al*., 2020)**. The development of microbes is restricted by different factors. For microbial development and complex cellulolytic enzyme synthesis during SSF, working parameters including pH, temperature and moisture content are essential **(Farinas, 2018)**. The optimization of crucial factors that influence microbial development and product formation is also essential for an efficient scale-up plan. Optimization of several parameters is sometimes a difficult and time-consuming operation. Response surface methodology (RSM) can be used to assess the importance of a range of variables, mainly when there are complex relationships between the individual components **(Inger *et al*., 2019)**. RSM based on design of experiments is a set of statistical and mathematical tool for designing experiments and optimizing the effect process variables. The BBD offers optimal results with few experimental runs when compared with other response surface methodology techniques **(Maran *et al*., 2013)**. Many studies employed RSM design for the cellulase enzyme production **(Shajahan *et al*. 2017; Ahmad *et al*., 2020)**.

Agro-industrial byproducts can be used in a variety of ways, including wheat straw, cassava waste, sugar beet pulp, maize cobs, wheat bran and apple pomace by using SSF for the stimulation of both cellulolytic and hemicellulolytic enzymes **(Sadh *et al*., 2018)**. Wheat bran is a compositionally intricate material, comprising cellulose (40–50% of dry matter), hemicellulose (25–35% of dry matter), and lignin (15–20% of dry matter). It represents a valuable source for the production of platform molecules and bioethanol **(Celiktas *et al*., 2014)**. An important benefit of utilizing wheat bran is that it inherently contains sufficient nutrients, obviating the need for additional carbon and nitrogen sources **(Katileviciute *et al*., 2019)**. Rice bran constituents vary due to the various processing techniques and machinery utilized, but it typically makes up 5–5% (by weight) of regular rice. Oil normally makes up 14–24% of rice bran, whereas protein is 16–18%, carbohydrate is 33–35% (about 20% crude fiber), water is 7–14% and ash is 8–12%. Additionally, it is abundant in protein, vitamins B and E and other minerals **(Satter *et al*., 2014)**. Rice bran, wheat bran, and wheat straw were used for cellulase and xylanase production **(Kumar *et al*., 2016; Devi *et al*., 2022)**.

The key issue for commercial use is the endoglucanase used in different biotechnological applications, also the high price of manufacture and little titers of this enzyme. As a result, studies on how to enhance the enzyme productivity and the capability of microbial strains to utilise cheap substrate have been conducted. In the current study, an effort was made to improve the medium conditions to increase the enzyme production. The utilization of bagasse, rice bran and wheat bran as the fermentation substrate during SSF while using *Cladosporium* sp. NCIM 901 for CMCase has not received much investigation. Wheat and rice bran are byproducts from the processing of wheat and rice. They have the potential to be utilized as substrates for industrial fermentation since they are commonly obtainable.

MATERIALS AND METHODS

Fungal Strain

The National Collection of Industrial Microorganisms (NCIM) in Pune, India, provided the *Cladosporium* sp. NCIM 901 was employed in this investigation to produce an enzyme employing SSF. The fungus was developed, cultivated and then stored at 4 °C on potato dextrose agar (PDA, Himedia, India) slants.

Sugarcane bagasse preparation

Following **Mohan *et al*. (2012)** approach, the bagasse was prepared and pretreated. Both rice and wheat bran were continually dried at 65 °C for 6 to 8 h before being ground and utilized as a powdery form in the SSF.

Fungal inoculum preparation

After growing on PDA slants at room temperature for 4–5 days, the *Cladosporium* sp. culture was suspended in sterile distilled water consisting of Tween-80 (0.1%, w/v). The obtained spore solution was utilized as the inoculum (1×10^8 spore/mL) for SSF after being passed through a cotton wool filter to remove mycelia and counted directly under the microscope in a Neubauer counting chamber **(Zhang *et al*., 2020)**.

CMCase production via SSF

SSF experiment performed in 250 mL conical flasks consisting of prepared bagasse (5 g) to serve as a natural carbon source and Mary Mandel's mineral medium (MM) (15 mL) with the below constitution (g/L); peptone, 5; NaCl, 5; Urea, 2; NH_4NO_3, 5; $CaCl_2$, 1; $MgSO_4$, 15; KH_2PO_4, 5; $(NH_4)_2SO_4$, 4.5; Tween-80, 0.5 mL along with trace elements: $CoCl_2$, 0.0002; $ZnSO_4•7H_2O$, 0.001; $MnSO_4•7H_2O$, 0.001 and $FeSO_4•7H_2O$, 0.005). Medium pH was modified according to design model recommendations earlier to autoclave sterilization. After being sterilized at 121°C under 15 psi of pressure for at least 15 min, the flasks were permitted to cool to ambient temperature. After adding the inoculum at a level of 2 mL/g, each flask's moisture level was balanced and maintained at a maximum of 75% by incubating it for five days at 35-37 °C in a water-saturated environment. By combining 50 mL of 50 mM sodium-citrate buffer (pH 6.0) with the experimental flasks and leaving them at 20 ºC for an 1 h while gently shaking, the enzyme preparation was extracted from the flasks. A muslin cloth was used to extract the resulting liquid suspension, which was then centrifuged (10,000× g) for 10 to 12 min. The collected supernatant, of which 10 mL was utilized as a crude cellulase source, was collected. During the preparation of crude cellulase, sterile conditions were maintained.

Optimized process parameters

Different process variables, such as media consequents and environmental conditions had an impact on the SSF production of CMCase on pretreated bagasse. Among these, the medium parameters pH, $CaCl_2$, Urea, wheat bran and rice bran were shown to be important variables. The quantities of these elements were optimized in accordance with the design model for enhanced CMCase enzyme production. Utilizing 0.1 N HCl/0.1 N NaOH enables the initial medium pH to be kept constant.

Characterization of CMCase

Optimum temperature

The CMCase activity was assessed by conducting the test at different temperatures between 30 and 90 °C to determine its optimal temperature. The ideal temperature is determined to be that at which the maximum amount of activity is occurring.

Optimum pH

The pH ranges of the enzymes were estimated by allowing them to incubate at 50 °C for 10 min in the relevant 50mM buffers- sodium acetate (pH 3-4.5), trisodium citrate (pH 5-5.5) and sodium phosphate (pH 6-9) buffer. The ideal pH is referred to as the level at which maximal activity is achieved.

Heat stability

By heating CMCase for 15 minutes at various temperatures (40-90 °C) and assessing its activity, the enzyme thermostability was studied. The thermostability of the material was estimated by measuring the percentage of the initial activity that remained following a 15-min heat treatment at 90 °C.

Substrate specificity assay

Through conducting the test with phosphoric acid swollen cellulose (PASC), CMC, microcrystalline cellulose, Filter paper, cotton, chitin, starch and cellulose, the substrate specificity of the CMCase was assessed. Each substrate was utilized at 10 mg/mL concentration. For 30 min at 50 °C, 10 mM of para nitro phenyl -D-glucopyranoside (PNPG) and cellobiose were added to sodium citrate (pH 5.5) buffer. After the experiment, the enzyme activity was measured.
Filter paper hydrolyzing CMCase activity was carried out by mixing a little piece of grade No.1 Whatman cellulose filter paper (size 1 cm x 6 cm) with 0.5 mL of extracted enzyme solution in 0.5 mL of trisodium citrate buffer (10mM) with pH 5.5. The entire solution was then incubated for 60 min at 50 °C. The DNS method was utilized to estimate how much reducing sugar was produced during the reaction **(Miller, 1959)**. The enzyme considers the maximum concentration of sugar released from a given substrate to be its specific substrate.

Impact of different reagents on CMCase activity

In the presence of metal ions, the effects of different additives on enzyme activity were investigated. CMC substrate was used after the CMCase was incubated at ambient temperature for 30 min in a solution containing 5 mM (end concentration) of $MnCl_2$, $CaCl_2$, $MgCl_2$, EDTA, $HgCl_2$ and DTT. At a pH of 5.5, 10 mM of EDTA and DTT were used. The activity that was determined when no reagents or metal ions were present was reported as 100%. A reagent is considered successful if it increased the activity more than it would have without that.

CMCase (endoglucanase) assay

By incubating 1 mL of process mixture comprising 0.5 mL of the substrate (1% CMC in 0.1 M sodium-citrate buffer, pH 5.0) and 0.5 mL of well-diluted enzyme mixture for 1 h at 40ºC, the CMCase activity was estimated. Similar conditions were used to simultaneously incubate reagent and enzyme blanks. In the end, 3 mL of the 3, 5-Dinitrosalicylic acid reagent (DNS) was mixed into every tube. Using glucose as a standard, the liberated reducing sugars were quantified colorimetrically (540 nm) by the DNS technique **(Miller, 1959)**. Therefore, the amount of enzyme released from CMC at a rate of 1 mol of glucose per milliliter per minute is equal to one international unit (IU) of CMCase activity.

Response surface methodology (RSM)

According to the RSM study, design-expert software was applied for the statistical design and experimental analysis (version 8.0.6.1, Stat-Ease InC., USA). To identify the optimal variables in SSF for the CMCase (U/gds) production, a central composite design (CCD) with a quadratic model was utilized **(Box and Wilson, 1951)**. Each of the five variables is presented in a three-level structure with four concentric point combinations **(Table 1)**. A model composed of 25 CCD factorial points, 10 axial points and 8 central points was employed to inspect the total impact of 5 independent variables on enzyme production.
The following equation (Eq.1) may be utilized to calculate the general quadratic (second-order) polynomial regression connection between the dependent response (Y, CMCase U/gds) and the 5 process components.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 \quad (1)$$

For assessing the CMCase activity in SSF, the statistical experimental design for the process components, including pH (3–8), wheat bran (2–6 g/L), $CaCl_2$ (1-3 g/L), urea (5–10 g/L) and rice bran (2–6 g/L), was taken into consideration. In all, 50 trials **(Table 2)** were planned and performed by the model. For every variable in **Table 1**, the selection range of lowest and highest values, the present model was assessed. Using Design-Expert software, an F-test for analysis of variance (ANOVA) and 2D response surface contour plots were produced. The numerical optimization software of Design-Expert was used to identify the maximum activities indicated by the optimal levels of five independent variables.

Pretreatment of pearl millet grass

The samples of chopped, milled pearl millet grass were immersed in 2% H_2SO_4 solution at a 1:10 (solid: liquid) ratio for two hours at ambient temperature. Further samples were autoclaved for 60 minutes at 120°C. Following autoclaving, the samples were filtered and any solids that remained were pH-neutralized.

Studies on saccharification

Pearl millet grass is saccharified 25 mL of culture filtrate with a CMCase activity of 23.42 U/gds and 1% pretreated Pearl millet grass was placed in a 500 ml flask and stirred at a speed of 120 rpm for 8 h in a water bath shaker at 50 °C. After the enzymatic hydrolysis was finished, the material was centrifuged (10,000 rpm) for 10 min. For an examination of the sugar content, the supernatant was extracted.

The saccharification (%) rate was calculated by following the methodology of **Irfan *et al*. (2016)**.

RESULTS

To enhance the enzyme production in SSF of sugarcane bagasse, the best significant factors were chosen and their optimization was attempted. The prepared bagasse made greater cellulose surface area accessible for extracellular enzyme activation. **Table 1** displays the variables' actual and coded levels for CCD.

Table 1 Actual and coded levels of variables of central composite design (CCD)

Factor	Name	Units	Low level	Middle level	High level	Low coded	Middle code	High coded
A	pH		3	5.5	8	–1	0	1
B	Wheat bran	g/L	2	4	6	–1	0	1
C	$CaCl_2$	g/L	1	2	3	–1	0	1
D	Urea	g/L	5	7.5	10	–1	0	1
E	Rice bran	g/L	2	4	6	–1	0	1

Table 2 Experimental design with coded values of variables and experimental and predicted responses of the central composite design (CCD) matrix model

Std	A:pH	B.Wheat bran g/L	C:$CaCl_2$ g/L	D:Ureag/L	E.Rice bran g/L	CMCase (U/gds) Predicted	Actual
1	3	2	1	5	2	11.2	11.05
2	8	2	1	5	2	14.8	13.7
3	3	6	1	5	2	8.64	8.15
4	8	6	1	5	2	12.08	11.36
5	3	2	3	5	2	11.8	12.02
6	8	2	3	5	2	16.2	15.41
7	3	6	3	5	2	10.42	11.61
8	8	6	3	5	2	15.24	15.57
9	3	2	1	10	2	10.53	11.24
10	8	2	1	10	2	9.24	9.85
11	3	6	1	10	2	11.02	11.23
12	8	6	1	10	2	10.8	10.39
13	3	2	3	10	2	9.26	9.93
14	8	2	3	10	2	10.05	9.28
15	3	6	3	10	2	11.24	12.41
16	8	6	3	10	2	12.8	12.32
17	3	2	1	5	6	10.6	10.67
18	8	2	1	5	6	15.03	15.41
19	3	6	1	5	6	6.23	7.73
20	8	6	1	5	6	14.56	13.03
21	3	2	3	5	6	9.06	9.92
22	8	2	3	5	6	16.2	15.41
23	3	6	3	5	6	9.86	9.48
24	8	6	3	5	6	15.34	15.53
25	3	2	1	10	6	12.6	12.02
26	8	2	1	10	6	13.8	12.72
27	3	6	1	10	6	10.26	11.98
28	8	6	1	10	6	13.24	13.23
29	3	2	3	10	6	7.06	8.99
30	8	2	3	10	6	10.02	10.44
31	3	6	3	10	6	11.24	11.45
32	8	6	3	10	6	12.54	13.45
33	3	4	2	7.5	4	3.26	0.022
34	8	4	2	7.5	4	3.02	5.55
35	5.5	2	2	7.5	4	9.24	9.47
36	5.5	4	2	7.5	4	10.54	9.6
37	5.5	4	2	7.5	4	10.2	11.05
38	5.5	4	2	7.5	4	14.02	12.46
39	5.5	4	2	7.5	4	9.86	10.86
40	5.5	4	2	7.5	4	10.31	8.61
41	5.5	4	2	7.5	4	21.02	21.43
42	5.5	4	2	7.5	8.76	23.42	22.31
43[a]	5.5	4	2	7.5	4	16.89	16.69
44[a]	5.5	4	2	7.5	4	16.89	16.69
45[a]	5.5	4	2	7.5	4	16.46	16.69
46[a]	5.5	4	2	7.5	4	16.28	16.69
47[a]	5.5	4	2	7.5	4	16.89	16.69
48[a]	5.5	4	2	7.5	4	16.89	16.69
49[a]	5.5	4	2	7.5	4	16.78	16.69
50[a]	5.5	4	2	7.5	4	16.89	16.69

Std: standard run order
[a]: Central run values.

Impact of method variables on CMCase activity

RSM based on CCD was used to optimize the CMCase enzyme production and 50 experimental trials using various combinations of the five parameters were conducted. The proposed model's quadratic equation (Eq.1) and data analysis revealed the interactive effects of free variables including pH, concentration of wheat bran, $CaCl_2$, urea and rice bran on CMCase activity and also explains the ideal conditions for enzyme activity. **Table 2** provides the observed and also

predicted experimental response for each trial based on the multiple regression equation (Eq.1).

Regression analysis with several variables was utilized to analyze the experimental results and to calculate the regression coefficients. To represent CMCase enzyme production as an experimental activity of independent factors, a quadratic equation (Eq.1) was acquired.

$$Y = 16.69 + 1.16 X_1 + 0.02 X_2 + 0.3 X_3 - 0.47 X_4 + 0.19 X_5 - 2.46 X_1^2 - 1.26 X_2^2 - 0.87 X_3^2 - 1.23 X_4^2 + 0.92 X_5^2 + 0.14 X_1X_2 + 0.19 X_1X_3 - 1.01 X_1X_4 + 0.52 X_1X_5 + 0.63 X_2X_3 + 0.72 X_2X_4 - 8.13 X_2X_5 - 0.57 X_3X_4 - 0.43 X_3X_5 + 0.29 X_4X_5 \quad (2)$$

In the above equation, X1, X_2, X_3, X_4 and X_5 represent coded values of free variables like pH, wheat bran, $CaCl_2$, urea and rice bran, respectively and Y is the predicted response (CMCase U/gds).

The significant, beneficial impact of these factors on the CMCase activity is explained by the linear coefficient of pH and $CaCl_2$, which have elevated values of 1.16 and 0.3, respectively (Eq. 2). Because pH and $CaCl_2$ enhanced the total enzyme activity as compared to wheat bran content, this shows that less wheat bran is needed. The negative linear coefficient of other variables in the ranging assay demonstrates an improvement in enzyme activity despite early increases in these variables. After a certain point, the total variable had an inhibitory influence on enzyme activity, as represented by the negative quadratic coefficient for factors.

Table 3 Analysis of variance of quadratic model of CMCase

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	719.77	20	35.99	20.81	< 0.0001
A-pH	58.53	1	58.53	33.84	< 0.0001
B-Wheat bran (g/L)	0.031	1	0.031	0.018	0.895
C-$CaCl_2$ (g/L)	3.77	1	3.77	2.18	0.1504
D-Urea (g/L)	9.69	1	9.69	5.6	0.0248
E-Rice bran (g/L)	1.49	1	1.49	0.86	0.3613
AB	0.62	1	0.62	0.36	0.5535
AC	1.12	1	1.12	0.65	0.428
AD	32.72	1	32.72	18.92	0.0002
AE	8.74	1	8.74	5.05	0.0324
BC	12.5	1	12.5	7.23	0.0118
BD	16.68	1	16.68	9.64	0.0042
BE	2.11E-03	1	2.11E-03	1.22E-03	0.9724
CD	10.42	1	10.42	6.02	0.0203
CE	5.87	1	5.87	3.39	0.0758
DE	2.71	1	2.71	1.57	0.2203
A^2	335.61	1	335.61	194.04	< 0.0001
B^2	88.82	1	88.82	51.36	< 0.0001
C^2	42.24	1	42.24	24.42	< 0.0001
D^2	84.04	1	84.04	48.59	< 0.0001
E^2	46.56	1	46.56	26.92	< 0.0001
Residual	50.16	29	1.73		
Lack of Fit	49.75	22	2.26	39.21	< 0.0001
Pure Error	0.4	7	0.058		
Cor Total	769.93	49			

[CMCase]: R-squared: 0.9349; Adj R-squared: 0.8899; Pred R-squared: 0.7219; C.V. = 10.57%; Mean: 12.44

Fig. 1 displays the residuals' normal plot. The software program statistically examined the significance of this model using the F-test of an ANOVA and **Table 3** shows the results. The F statistic (f value) often indicates a high significance level for the polynomial regression model **(Rene *et al.*, 2007)** with a smaller computed probability (P-value). The current polynomial design is considerable with a calculated F-value of 20.81, suggesting that the present model is considerable (P<F is below 0.05) and there exists a 0.01% probability that the "Model F-Value" might have been caused by noise **(Design-Expert, 2018)**. The present model could clarify 93.49% of the variability, depending on the R^2 value of 0.9349, but it could only account for 6.51% of the overall difference. The R^2-value closer to 1 specifies a higher degree of correlation between actual and predicted values. The plotted dots arranged themselves across the diagonal line, demonstrating the strong fit of the current model, as seen by the relationship between the observed and predicted values **(Fig. 2)**. The low coefficient of variation (CV) value of about 10.57% demonstrates the observed values' high level of accuracy and reliability. **Table 3** provides the P-values for each independent variable, as well as its squared and interaction terms. The P-values of individual variables are typically used to determine the importance of those variables, with smaller P-values indicating more significant terms. The Prob>F value must be lower than 0.05 to demonstrate that the present model terms are important. The model variables A, D, AD, AE, BC, BD, CD, A^2, B^2, C^2, D^2 and E^2 are important in this situation. However, the design terms were not considered significant if the values are higher than 0.10 **(Design-Expert, 2018)**.

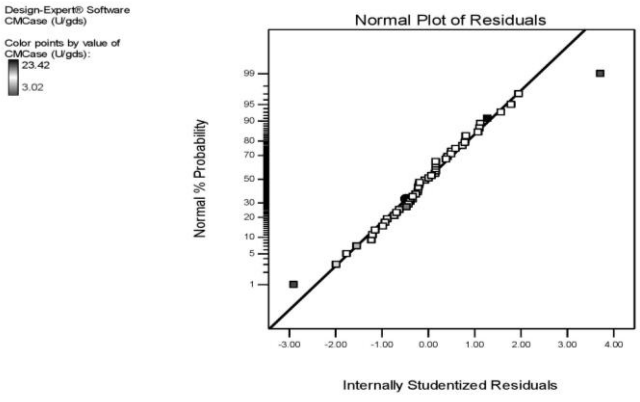

Figure 1 Normal plot of residuals.

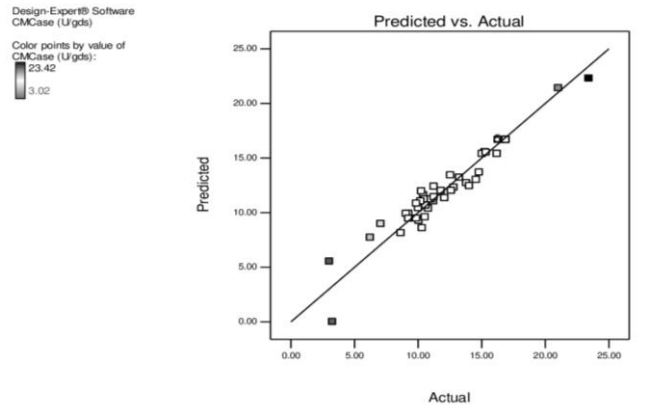

Figure 2 Predicted vs. actual observation run values.

The response was plotted and the best values for every variable for maximum CMCase activity were determined using contour plots. The association among two independent variables and the availability of additional factors functioning at a static level had an impact on the selection of the response surface designs. The two-dimensional (2D) contour plot outline indicates the nature and strength of interactions **(Fig. 3)**.

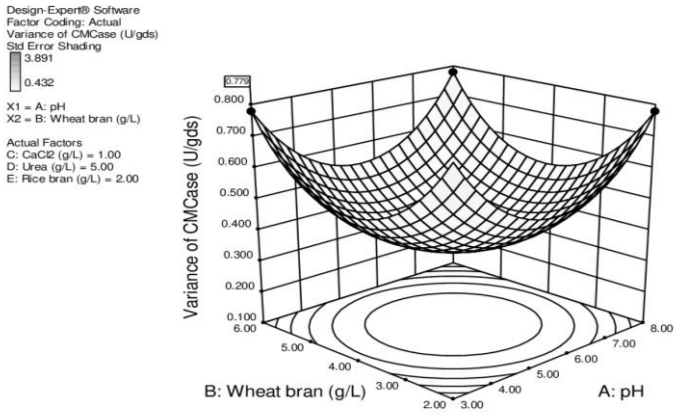

Figure 3 Contour plot of interactive effect of variables on CMCase of *Cladosporium* sp.

Impact of pH and urea on CMCase activity

The CMCase production was strongly impacted by pH ($P<0.005$) and also the correlation between pH and urea ($P<0.005$). Concerning squared terms, the pH and urea were both significantly different ($P<0.005$). The negative interaction coefficient (Eq. 2) indicates that while keeping the medium pH at 5.5, reducing the urea content enhances the enzyme activity. The response contour plot **(Fig. 3a)** demonstrated that urea concentrations between 7.0 and 8.0 g/L, with the optimum at 7.5 and the values of pH between 5.5 and 7.0, at the ideal pH of 5.5, could be utilized to provide the best CMCase activity (23.42 U/gds). Production of enzymes is encouraged when all other factors are working at their optimal level. Because of the enzyme's instability in various pH environments, enhancing or reducing the pH level leads to a decline in enzyme activity. However, widely separated contours along the pH axis imply that pH variations are less important than changes in urea concentration.

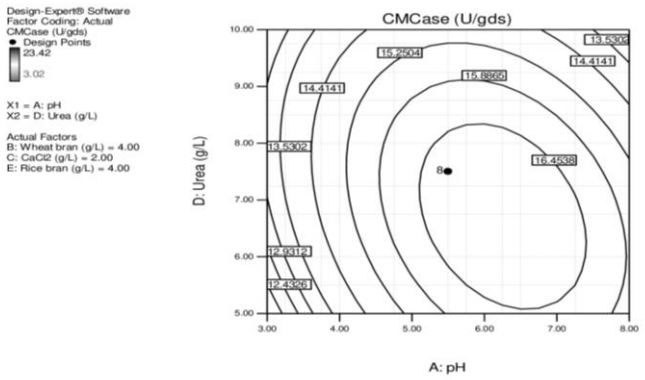

Figure 3a Contour plot of the interactive effect of pH and urea on the CMCase activity.

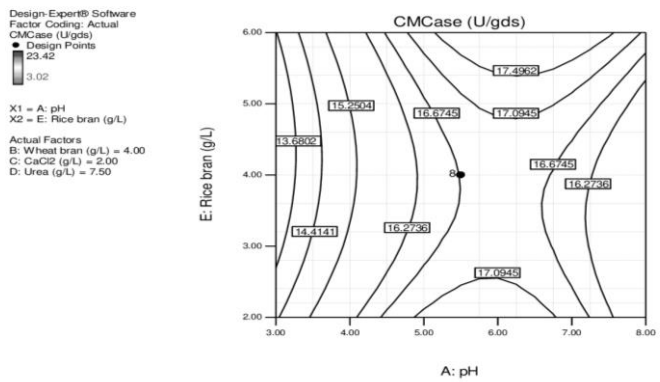

Figure 3b Contour plot of the interactive effect of pH and rice bran on the CMCase activity.

Impact of pH and rice bran on CMCase activity

There is a significant relationship between these two parameters for maximal enzyme activity ($P<0.05$). The isoprene contour plot **(Fig. 3b)** shows that the maximum CMCase activity can be reached at pH 5.5 and rice bran concentration of 8.0. By holding other variables at a constant level, these factors' positive interaction coefficient (Eq. 2) on the CMCase activity shows that their action is detected, because both of their higher levels signify inadequate responses. 22.31 U/gds was the highest predicted enzyme activity that could be measured. The remaining variables were evaluated at their optimum midpoint. According to 2D-contour plots, the activity peaked between pH ranges of 5 and 6, after which it decreased but did so gradually as the rice bran concentration increased. Regardless of pH, as rice bran concentration rises, the enzyme activity modifies.

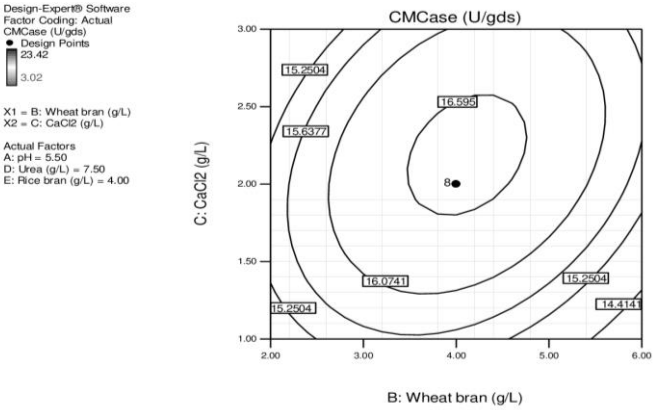

Figure 3c Contour plot of the interactive effect of wheat bran and $CaCl_2$ on CMCase activity.

Impact of wheat bran and $CaCl_2$ on CMCase activity

Although the individual P values of wheat bran and $CaCl_2$ are not significant, they both had a linear effect on enzyme production ($P>0.05$). However, the formation of CMCase should be affected by both wheat bran and $CaCl_2$ ($P<0.005$) having

substantial squared terms and a significant interaction between them. By keeping other variables constant at their fixed values, these two variables had a significant impact on the enzyme activity, as revealed by the positive interaction coefficient (Eq. 2) of these two variables. Through the contour plot in **Fig. 3c**, the ideal values of $CaCl_2$ and wheat bran were identified to be between 1.8 and 2.2 g/L and 2.0 g/L, respectively to produce the highest levels of CMCase activity. The highest yield of enzyme activity that could be predicted was 22.31 U/gds. The values of the other variables during testing were optimal. The $CaCl_2$ concentration is very important than the irregularly spaced contour along the X-axis showing the changes in wheat bran content.

Impact of wheat bran and urea on CMCase activity

In Figure 3d, the association between wheat bran and urea on the formation of CMCase is contour plotted. Wheat bran considerably affected the synthesis of enzymes in squared terms ($P<0.005$) and there was a significant interaction between urea and wheat bran ($P<0.005$). The principal effect of wheat bran (Eq. 2) has a positive value, in contrast to the urea concentration, which has a negative value. This indicates that the variable affects the synthesis of enzymes and that an increased concentration is needed for enzyme activity. Increases in CMCase activity are shown to be correlated with increases in both independent variables on a 2D contour plot of the two variables.

It is shown that CMCase activity is constant up to a certain wheat bran level to urea ratio; however, above that level, an increase in urea level suppresses enzyme activity, revealing the inhibitory effect of urea levels in the medium. When a variable reduces enzyme activity over the optimal level, its value is negative. The significant value of 0.004 for the interactive coefficient **(Table 3)** indicates that there is an interaction between wheat bran and urea. The activity of the enzyme may change in response to changes in wheat bran concentration. According to the contour plot **(Fig. 3d)**, the urea concentration should be between 7.0 and 8.0 g/L, the starting medium should have a pH of 5.5, $CaCl_2$ at 2.0 g/L and rice bran at 4.0 g/L to produce the highest CMCase activity (23.42 U/gds). The widely separated contour plots on the X-axis of the contour plot demonstrate how variations in the quantity of wheat bran in the fermentation medium have an impact on enzyme activity.

Impact of $CaCl_2$ and urea on CMCase activity

The synthesis of CMCase was considerably affected by $CaCl_2$ and urea, respectively ($P<0.0005$ in squared terms and $P<0.05$ in linear terms) and also by their interaction ($P<0.05$) **(Table 3)**. The CMCase activity was impacted by these factors up to a point above this the enzyme activity was adversely affected by them, as indicated by the negative interaction coefficient (Eq. 2) of these process variables. The positive linear coefficient of $CaCl_2$ level was suggested to be more strongly correlated with the CMCase activity by the negative interaction coefficient impact of both variables (Eq. 2). The 2D contour plot **(Fig. 3e)** shows that changing the concentrations of urea and $CaCl_2$ in the fermentation medium changed the enzyme activity. The contours show that when urea levels were raised, enzyme activity declined and that $CaCl_2$ levels had a significant impact. The enzyme activity is adversely affected by urea's non-significant value in terms of the linear and squared components (Eq. 2). CMCase activity maximum at 23.42 U/gds when $CaCl_2$ (2 g/L) and urea (7.5 g/L) were present, with all other factors functioning at their optimum values.

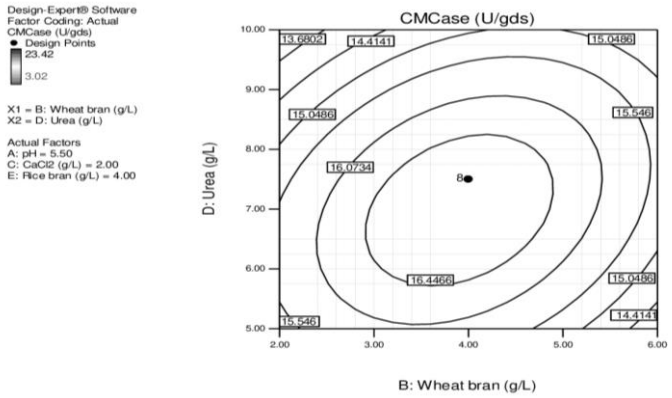

Figure 3d Contour plot of the interactive effect of wheat bran and urea on CMCase activity.

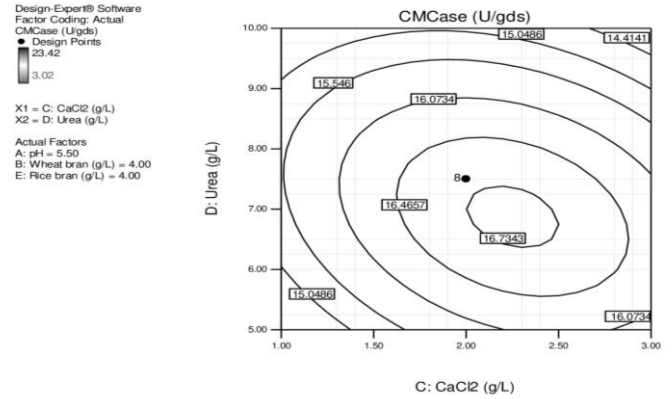

Figure 3e Contour plot of the interactive effect of $CaCl_2$ and urea.

Experimental validation

Three verification trials in total were carried out within the test range shown in **Table 4** to evaluate the model's suitability. In order to determine the correlation between the actual and predicted values, the results from the validation runs were statistically examined as well. It was found that the R^2 between the experimental and expected results was 0.9401, indicating that a particular way to assess the model's accuracy is how well the experimental and predicted values fit together.

Table 4 Model validation experiments

Number	pH	Wheat bran (g/L)	$CaCl_2$ (g/L)	Urea (g/L)	Rice bran (g/L)	Experimental values (U/gds)	Predicted values (U/gds)
1	3	2	1	5	2	10.6	11.4
2	3	6	3	5	2	10.4	11.6
3	8	6	3	10	6	11.8	12.4
4	3	6	3	10	2	10.2	11.8
5	5.5	4	2	7.5	4	16.2	16.9

Characterization studies

To identify the conditions in which it exhibited its highest level of activity, the produced crude CMCase enzyme was characterized.

Optimum pH of the enzyme

Fig. 4 shows how the enzyme activity is impacted by different pH values. The maximum level of CMCase activity was observed at pH 5.5. The enzyme activity was significantly reduced once the medium's pH was raised. Even so, as the pH increased further to 7.5, the activity gradually decreased. Similar to this, a declining pattern was observed when the CMCase activity was determined at pH levels less than 4.5.

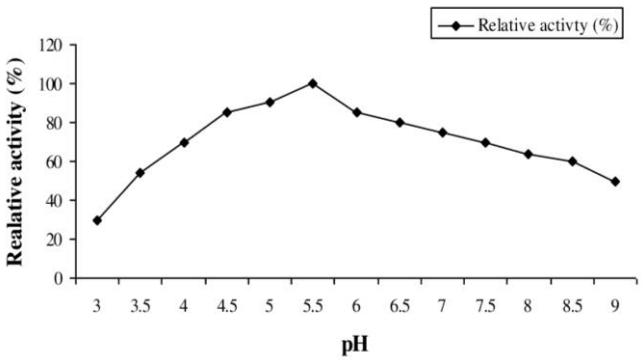

Figure 4 Effect of pH on the CMCase activity.

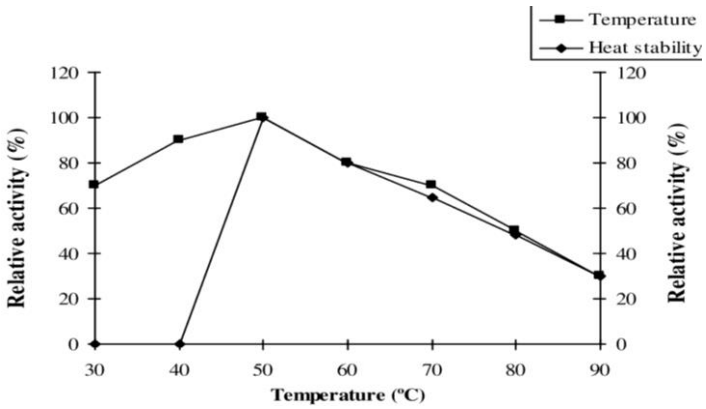

Figure 5 Effect of different temperature and Heat stability of crude CMCase.

Optimum Temperature of the enzyme

Fig. 5 displays the relationship between CMCase activity and temperature. The enzyme's activity was found to increase at 50 ºC. However, the enzyme's activity was adversely affected and gradually decreased as the temperature is raised above 50 ºC. When the enzyme activity was measured below 50 ºC, the CMCase activity gradually decreased. Since enzymatic proteins undergo denaturation at high temperature, at a specific degree of temperature increase (beyond 50 ºC), the enzyme activity is significantly reduced.

Heat stability of the crude CMCase enzyme

The CMCase activity **(Fig. 5)** shows that the association between enzyme stability and temperature increase is not linear. In this observation, enzyme stability was highest at 50 °C temperature. The enzyme stability was considerably decreased by temperature increases above this point; at 90 ºC, it showed only 30% stability. The denaturation of enzymes at high temperatures can result in decreased enzyme activity.

Substrate specificity of CMCase

Table 5 presents the hydrolyzing activity on several cellulosic substrates used to ascertain the crude CMCase substrate specificity. Compared to other substrates, the enzyme activity is more on the CMC substrate with β-1, 4-linkage. Filter paper and phosphoric acid swollen cellulose (PASC) exhibited less enzyme activity. Because the enzyme has a poor affinity for crystalline cellulose, it cannot hydrolyse crystalline cellulosic material like cellulose powder. There was no amylolytic or β-glucosidase activity observed for the enzyme. Additionally, the PNPG substrate was not degraded by the enzyme. These results imply that an endotype of cellulase would be a more suitable classification for this enzyme.

Table 5 Substrate specificity of the crude CMCase of *Cladosporium* sp. NCIM 901

Substrate	Linkage	Enzyme activity (IU/mg protein $^{-1}$)
CMC	β-1,4	14.6
Filter paper	β-1,4	8.2
Microcrystalline cellulose	β-1,4	4.3
Phosphoric acid swollen cellulose	β-1,4	9.2
Cotton	β-1,4	0.8
Chitin	β-1,4	0.0
Starch	α-1,4	0.0
Cellobiose	β-1,4	0.0
Cellulose	β-1,4	0.0
PNPG	β-1,4	0.0

*The data presented are averages and standard errors of two independent experiments.

Impact of different reagents on CMCase activity

The impact of different additives on the CMCase activity was examined when each addition was present at a 5mM concentration. **Table 6** shows how most of the other additives, except $CoCl_2$ and $HgCl_2$, increased the CMCase activity. Hg^{+2} significantly reduced the crude enzyme's CMCase activity. Hg^{2+} ions attach to and interact with the thiol and carboxyl groups or the tryptophan residue of amino acids in the enzyme, which is where the enzyme is blocked **(Lamed *et al.*, 1994)**. However, CMCase activity increased by $CaCl_2$ and $MgCl_2$. Dithiothreitol (DTT) and ethylene diamine tetraacetic acid (EDTA) measured at pH 5.5 did not affect it.

Table 6 Effect of different reagents on the activity of CMCase

Effector	Concentration (mM)	Relative enzyme activity (%)
None	-	100 ±0
$CaCl_2$	5	110 ± 8
$MgCl_2$	5	120 ± 3
$MnCl_2$	5	82 ± 12
$CoCl_2$	5	64 ± 4
$HgCl_2$	5	62 ± 3
EDTA	10	102 ± 4
DTT	10	102 ± 12

*The data presented are averages and standard errors of two independent experiments.

Pearl millet grass saccharification

The efficacy of the produced *Cladosporium* sp. cellulase (CMCase) enzyme to saccharify pearl millet grass and produce fermentable sugars was investigated. An indigenously produced cellulase (CMCase) enzyme was used to saccharify untreated and H_2SO_4-treated pearl millet grass. According to the findings (**Fig. 6**), pearl millet grass treated with H_2SO_4 had the highest saccharification rate (55.40%) compared to the untreated sample (37.10%). With longer incubation times, the level of total sugars produced during the saccharification process increased. With an indigenous enzyme and grass samples that had been treated with 2% H_2SO_4, 48 h of incubation time released the highest amount of total sugars.

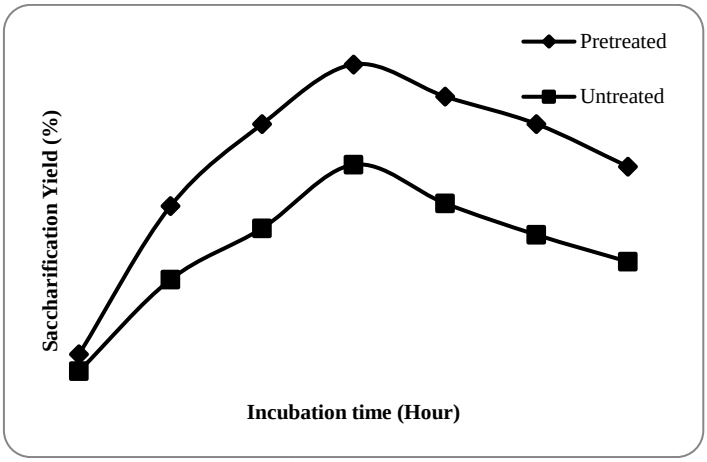

Figure 6 Saccharification Yield (%) in Pearl millet grass.

DISCUSSION

To prepare the SSF medium for the CMCase production, RSM was performed. Pretreatment of lignocellulosic materials is crucial because it makes it simpler for microbes or enzymes to reach the cellulose substrate. Peracetic acid (PAA) pretreatment of the bagasse was used in the current investigation to increase the substrate's accessibility to enzyme activity.

The basis of the production process for an enzyme like cellulase may be considered as the pH level of the substrate. pH changes have a considerable influence on cellulase synthesis. The exoglucanase showed its highest activity at a pH of 4.0, whereas the optimum pH for endoglucanase and β-glucosidase ranged between 4.0 and 5.0 **(Sulyman *et al.*, 2020)**. In the current work, 5.5 is the optimal initial pH of the medium. **Navaneethapandian *et al.* (2021)** utilized *Aspergillus flavus* SB04 strain in SSF employing rice bran served as the solid substrate and optimal conditions of moisture level 75%, temperature 33 °C and pH 6. They achieved similar findings. When the microbial culture medium's initial moisture content was 70.5% the highest enzyme activity was attained. The generation of cellulolytic enzymes when wheat bran (WB) was used as the carbon supplement has been said to benefit from similar moisture levels **(Kumar *et al.*, 2016)**. The denaturation of the enzyme and pH changes during fermentation may be the causes of the activity reduction after five days. The highest enzyme activity of *Penicillium* sp. AKB-24 was observed after a 7-day incubation period at 30°C and pH 7, with a moisture content of 77.5%, supplemented with 1.2% yeast extract and 0.1% sodium dodecyl sulphate. The peak activities recorded were as follows: endoglucanase at 134 IU/gds, FPase at 3 FPU/gds, β-glucosidase at 6 IU/gds, and xylanase at 3592 IU/gds (**Kumar *et al.*, 2016).**

In the current investigation, the greatest level of rice bran (8 g/L) and bagasse were used to increase the maximum enzyme activity. The ideal concentration of wheat bran was 4 g/L. Because of its protein content, wheat bran is an excellent source of nitrogen and when it is added to soybean hulls; it increases the C/N ratio, which promotes the growth of fungi and the production of cellulase. Moreover, it is a good source of hemicellulose rich in arabinans in addition to xylose and arabinose, a sugar that resembles glucose **(Sanchez-Bastardo *et al.*, 2017)** that provides an

excellent source of soluble sugar. Hemicellulose itself is a powerful inducer for the cellulolytic enzyme complex system **(Jia *et al*., 2021)**. It is widely known that adding WB to a growth medium causes the induction of cellulolytic and hemicellulolytic activities. Previous studies show that, the highest xylanase production (141.28 U/mL) by *Bacillus safensis* XPS7 occurred at 45°C, pH 9, with a 24-hour incubation period. This was achieved using a 2% (w/v) blend of wheat straw and wheat bran as the carbon source, supplemented with 1.5% (w/v) ammonium nitrate as the nitrogen source in a modified Riviere's medium. These results emphasize the cost-effectiveness and abundant potential of combining wheat straw and wheat bran as a valuable carbon source, positioning it as a promising choice for scaling up xylanase production within the industrial sector **(Devi *et al*., 2022)**. Under submerged and in solid-state conditions of WB and straw mixtures, the fungi have been found to synthesize greater amounts of lignocellulose degrading enzymes **(Devi *et al*., 2022; Kumar *et al*., 2016)**. In earlier research, it was noted that the highest levels of xylanase (49.3 IU g^{-1}), CMCase (14.7 IU g^{-1}), and endoglucanase (0.68 IU g^{-1}) activities were significantly elevated when utilizing a mixed culture of rice straw and wheat bran in a 1:1 ratio. Conversely, greater amounts of β-glucosidase (8.5 IU g^{-1}) and exoglucanase (0.93 IU g^{-1}) were observed in a rice straw and wheat bran ratio of 7 RS : 3 WB **(Sherief *et al*., 2010).** Beyond the optimum concentration, the substrate concentration lowered the enzyme activity. Additionally, compared to bagasse, rice bran (RB) has more enhanced nutrients **(Naik *et al*., 2023)**. During their study, **Navaneethapandian *et al*., (2021)** detected the highest cellulase production (45.2 mg/ml) in submerged fermentation of rice bran by using glucose as the carbon source and yeast extract as the nitrogen source. The culture conditions were optimized to achieve a higher yield of the cellulase enzyme, and the maximum enzyme activity was observed after a 14-day incubation period.

Nitrogen acts as a catalyst for promoting fungal cell growth, enhancing both biomass formation and the production of cellulase enzymes. The production of cellulase enzymes involved the utilization of diverse nitrogen sources such as sodium nitrate, yeast, potassium nitrate, aqueous ammonia, ammonium sulfate and urea **(Elisashvili *et al*., 2008)**. The maximum predicted enzyme activity in the current study, when urea was utilized as the nitrogen source for optimization, was estimated to be around 22.31 U/gds and an actual enzyme yield of 23.42 U/gds was observed in response, demonstrating a fine association among the actual and predicted values with a considerable impact on enzyme production. It was noted that the activities of CMCase and FPase were higher, at 68.8 and 38.8 U/mL, respectively, in the culture filtrate of full-grown *A. niger* on urea **(Akula and Golla, 2018)**. In their study, **Pramanik *et al*. (2020)** determined that yeast extract was the superior nitrogen source for cellulase production by *Bacillus pseudomycoides* utilizing sugarcane bagasse substrate. **Abdullah *et al*. (2017)** found the peak cellulase activity at 4.5% urea (w/w), 1 mM MgCl2, and pH 3.5, achieving a maximum enzyme activity of 0.630 U/g.

CMCase was characterized to determine the ideal conditions for enzyme activity. The findings were contrasted with the results of **Olukunle *et al*. (2021)**, who claimed that CMCase works best at a pH 6.0. The optimal temperature range for cellulases from filamentous fungi typically falls between 30 and 55°C **(Xu *et al*., 2010)**. The temperature optimum for CMCase in the current investigation was 50 °C and this aligns with the findings for fungi, such as *P. funiculosum*, which display activity at temperatures exceeding 50°C. Specifically, *P. funiculosum* endoglucanase exhibited an optimum temperature of 58°C **(De Castro *et al*., 2010)**. In general, the stability of the enzyme was considerably influenced by temperature. An earlier study by **(Potprommanee *et al*., 2017)** also found a considerable decline in CMCase activity to 80 from 50 ºC, which is comparable to this study. The results also support the conclusions of **Thongekkaew *et al*. (2008)** described that heating to 90 °C only retained 50% of the maximal CMCase activity. Studies on substrate specificity revealed that CMC and PASC both increased the total activity of crude CMCase. The endocellulase has the same ability to break down soluble polysaccharides more quickly than crystalline celluloses as other thermophilic fungus isolates **(Sohail *et al*., 2022)**.

The results showed that treated substrates outperformed the control (untreated Pearl millet grass) in terms of sugar production and saccharification yield, proving that pretreatment was successful in reducing the lignin component and effectively exposed the most cellulose to enzymatic attack. This conclusion is also supported by an earlier study **(Zhang *et al*. 2019)**, which shows that pretreated samples degrade more quickly than untreated substrates. **Irfan *et al*. (2011)** claim that commercial enzymes and indigenous enzyme from *Trichoderma reesie* cultivated in SSF is used for the pretreated bagasse enzymatic hydrolysis. Results revealed that when H_2O_2 concentration increased, the saccharification process increased. For sugarcane bagasse, the greatest saccharification rate (41.9%) was recorded at 5% (v/v) H_2O_2. By comparing the above stated result in the existing study the saccharification rate was greater and showed better enzyme activity.

CONCLUSION

Environmental problems may eventually be reduced by using readily available, inexpensive lignocellulosic substrates for enzymatic fermentation for example, bagasse, wheat bran and rice bran. The price of microbial growth medium would be lesser if byproducts from the food processing sector were utilized in the fermentation method. The production of enzymes was significantly impacted economically by the optimization of fermentation conditions. The optimal experimental actual and predicted response (CMCase) activity of 23.42 U/gds and 22.31 U/gds, respectively are in good agreement. This demonstrated the applicability of the design used and the positive result of RSM in achieving the best possible conditions for the synthesis of enzymes from pretreated bagasse. CMCase was characterized and found to be thermally and pH stable at 50 ºC and 5.5, respectively. Saccharification studies using pearl millet grass showed that the produced CMCase enzyme results in a higher saccharification rate in H_2SO_4 treated biomass and indicates that the enzyme will be applied for lignocellulosic biomass substrates effectively.

Acknowledgment: The support from UGC is highly acknowledged.

Conflict of interest: Authors declare no conflict of interest.

REFERENCES

Abdullah, A., Hamid, H., Christwardana, M., & Hadiyanto, H. (2018). Optimization of cellulase production by *Aspergillus niger* ITBCC L74 with bagasse as substrate using response surface methodology. *HAYATI J Biosci*, 25(3): 115. https://doi.org/10.4308/hjb.25.3.115

Akula, S., & Golla, N. (2018). Optimization of cellulase production by *Aspergillus niger* isolated from forest soil. *The Open Biotechnol Let*, 12: 256-269. https://doi: 10.2174/1874070701812010256

Ahmad, T., Sharma, A., Gupta, G., Mansoor, S., Jan, S., Kaur, B., Paray, B.H., & Ahmad, A. (2020). Response surface optimization of cellulase production from *Aneurinibacillus aneurinilyticus* BKT-9: An isolate of urban himalayan freshwater. *Saudi J Biol Sci*, 27(9): 2333-2343. https://doi:10.1016/j.sjbs.2020.04.036

Box, G.E.P., & Wilson, K.B. (1951). On the experimental attainment of optimum conditions. *J R Sta Soc*, 13: 1-45. https://www.jstor.org/stable/2983966

Celiktas, M. S., Kirsch, C., & Smirnova, I. (2014). Cascade processing of wheat bran through a biorefinery approach. *Energy Convers, Manage*. 84, 633-639. https://doi: 10.1016/j.enconman.2014.04.039.

De Castro AM, de Albuquerque de Carvalho ML, Leite SG, Pereira N Jr. (2010). Cellulases from *Penicillium funiculosum*: production, properties and application to cellulose hydrolysis. *J Ind Microbiol Biotechnol*, 37(2):151-158. https://doi: 10.1007/s10295-009-0656-2

Design-Expert® Software. (2018). Version 8 user's guide.

Devi, S., Dwivedi, D., & Bhatt, A.K. (2022). Utilization of agroresidues for the production of xylanase by *Bacillus safensis* XPS7 and optimization of production parameters. *Fermentation*,8: 221. https://doi.org/10.3390/fermentation8050221

Elisashvili, V., Kachlishvili, E., & Penninckx, M. (2008). Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes. *Ind Microbiol Biotechnol*, 35:1531–1538. https://doi: 10.1007/s10295-008-0454-2

Farinas, C.S. (2018). Chapter 9 - Solid-state fermentation for the on-site production of cellulolytic enzymes and their use in the saccharification of lignocellulosic biomass. *Current Developments in Biotechnology and Bioengineering*, 169-183. http://dx.doi.org/10.1016/B978-0-444-63990-5.00009-8

Inger, M., Dobrzynska-Inger, A., Rajewski, J., & Wilk, M. (2019). The use of response surface methodology in ammonia oxidation reaction study. *J Chem*, 2641315. https://doi: 10.1155/2019/2641315

Irfan, M., Gulsher, M., Abbas, S., Syed, Q., Nadeem, M., & Baig, S. (2011). Effect of various pretreatment conditions on enzymatic saccharification. *S J Sci Technol*, 33 (4): 397-404.

Jayasekara, S., & Ratnayake, R. (2019). Microbial cellulases: an overview and applications. Cellulose. *Intech Open*, 1-130. http://dx.doi.org/10.5772/intechopen.84531

Jia, W., Ge, M., Zhang, Z., Zhang, D., & Gao., L.(2021). Mechanism study of novel inducer combinations containing laver powder for *Penicillium piceum* lignocellulolytic enzyme production. *GCB Bioenergy*, 13(4): 656-664. https://doi.org/10.1111/gcbb.12811

Katileviciute, A., Plakys, G., Budreviciute, A., Onder, K., Damiati, S., & Kodzius, R. (2019). A sight to wheat bran: high value-added products. *Biomolecules*. 17, 9(12):887. https://doi: 10.3390/biom9120887.

Lamed, R., Tormo, J., Chirino, A.J., Morag, E., & Bayer, E.A. (1994). Crystallization and preliminary X-ray analysis of the major cellulose-binding domain of the cellulase from *Clostridium thermocellum*. *J Mol Biol*, 244: 236-7. https://doi.org/10.1006/jmbi.1994.1721

Lopez-Gomez, J.P., & Venus, J. (2021). Potential role of sequential solid-state and submerged-liquid fermentations in a circular bio-economy. *Fermentation*, 7(2): 76. https://doi.org/10.3390/fermentation7020076

Maran, J.P., Manikandan, S., & Mekala, V. (2013). Modeling and optimization of betalain extraction from *Opuntia ficusindica* using Box–Behnken design with desirability function. *Ind Crops Prod*, 49:304-311. https://doi.org/10.1016/j.indcrop.2013.05.012

Miller, G.L. (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*, 31: 426–428. https://doi.org/10.1021/ac60147a030

Mohan, P.R., Ramesh, B., & Reddy, O.V.S. (2012). Production and optimization of ethanol production from pretreated sugarcane bagasse using *Saccharomyces bayanus* in simultaneous saccharification and fermentation. *Microbiol J*, 2: 52-63. https://dx.doi.org/10.3923/mj.2012.52.63

Naher, L., Fatin, S.N., Sheikh, M.A.H., Azeez, L.A., Siddiquee, S., Zain, N.M., & Karim, S.M.R. (2021). Cellulase enzyme production from filamentous fungi *Trichoderma reesei* and *Aspergillus awamori* in submerged fermentation with rice straw. *J Fungi (Basel)*, 7(10):868. https://doi: 10.3390/jof7100868

Naik, B., Kumar, V., Rizwanuddin, S., Chauhan, M., Gupta, A.K., Rustagi, S., Kumar, V., & Gupta, S. (2023). Agro-industrial waste: a cost-effective and eco-friendly substrate to produce amylase. *Food Prod Process and Nutr*, 5: 30. https://doi.org/10.1186/s43014-023-00143-2

Navaneethapandian, U., Kumar, G., Liduja, K., Jayachithra, R., & Gopakumaran, N. (2020). Biocatalyst: cellulase production in solid state fermentation (SSF) using rice bran as substrate. *Biointerface Res. Appl. Chem*, 11(1): 7689-7699. https://doi.org/10.33263/BRIAC111.76897699

Olukunle, O. F., Ayodeji, A. O., & Akinloye, P. O. (2021). Carboxymethyl Cellulase (CMCase) from UV-irradiation mutated *Bacillus cereus* FOA-2 cultivated on Plantain (*Musa parasidiaca*) stalk-based medium: production, purification and characterization. *Scientific African*, 11: e00691. https://doi:10.1016/j.sciaf.2020.e00691

Potprommanee, L., Wang, X.Q., Han, Y.J., Nyobe, D., Peng, Y.P., Huang, Q., Liu, J.Y., Liao, Y.L., & Chang, K.L. (2017). Characterization of a thermophilic cellulase from *Geobacillus* sp. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass. *PLoS One*,12(4):e0175004. https://doi: 10.1371/journal.pone

Pramanik, S.K., Mahmud, S., Paul, G.K., Jabin, T., Naher, K., Uddin, M.S., Zaman, S., & Saleh, M.A. (2020). Fermentation optimization of cellulase production from sugarcane bagasse by *Bacillus pseudomycoides* and molecular modeling study of cellulase. *Curr Res Microb Sci*, 27(2):100013. https://doi: 10.1016/j.crmicr.2020.100013

Sadh, P.K., Duhan, S., & Duhan, J.S. (2018). Agro-industrial wastes and their utilization using solid state fermentation: a review. *Bioresour Bioprocess*, 5: 1. https://doi.org/10.1186/s40643-017-0187-z

Sanchez-Bastardo, N., Romero, A., & Alonso, E. (2017). Extraction of arabinoxylans from wheat bran using hydrothermal processes assisted by heterogeneous catalysts. *Carbohydr Polym*, 160:143-152. https://doi:10.1016/j.carbpol.2016.12.035

Satter, M.A., Ara, H., Jabin, S.A., Abedin, N., Azad, A.K., Hossain, A., & Ara, U. (2014). Nutritional composition and stabilization of local variety rice bran BRRI-28. *I J Sci Technol*, 3(5): 306-313.

Shajahan, S., Moorthy, I. G., Sivakumar, N., & Selvakumar, G. (2017). Statistical modeling and optimization of cellulase production by *Bacillus licheniformis* NCIM 5556 isolated from the hot spring, Maharashtra, India. *J King Saud Uni Sci*, 29(3), 302-310. https://doi.org/10.1016/j.jksus.2016.08.001

Sherief, A.A., El-Tanash, A.B., & Atia, N. (2010). Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. *Res J Microbiol*, 5: 199-211. https://scialert.net/abstract/?doi=jm.2010.199.211

Sohail, M., Barzkar, N., Michaud, P., Jahromi, T.S., Babich, O., Sukhikh, S., Das, R., & Nahavandi, R. (2022). Cellulolytic and xylanolytic enzymes from yeasts: properties and industrial applications. *Molecules*, 27(12): 3783. https://doi: 10.3390/molecules27123783

Srivastava, N., Mishra, P.K., & Upadhyay, S.N. (2020). Microbial cellulase production. In: Industrial enzymes for biofuels production. *Elsevier*, 19-35. http://dx.doi.org/10.1016/B978-0-12-821010-9.00002-4

Sulyman, A.O., Igunnu, A., & Malomo, S.O. (2020) Isolation, purification and characterization of cellulase produced by *Aspergillus niger* cultured on *Arachis hypogaea* shells. *Heliyon*, 76(12): 05668. https://doi: 10.1016/j.heliyon.2020.e05668

Thongekkaew, J., Ikeda, H., Masaki, K., & Iefuji, H. (2008). An acidic and thermostable carboxymethyl cellulase from the yeast *Cryptococcus* sp. S-2: Purification, characterization and improvement of its recombinant enzyme production by high cell-density fermentation of *Pichia pastoris*. *Protein Expres Puri*, 60: 140-146. https://doi.org/10.1016/j.pep.2008.03.021

Xu, Q., Adney, W.S., Ding, SY., Michael, H.E. (2007). Cellulases for biomass conversion. In: Polaina, J., MacCabe, A.P. (eds) Industrial Enzymes. Springer, Dordrecht. https://doi.org/10.1007/1-4020-5377-0_3

Zhang, H., Huang, S., Wei, W., Zhang, J., & Xie, J. (2019). Investigation of alkaline hydrogen peroxide pretreatment and Tween 80 to enhance enzymatic hydrolysis of sugarcane bagasse. *Biotechnol Biofuel*, 3(12): 107. https://doi: 10.1186/s13068-019-1454-3

Zhang, M., Gu, L., Zheng, P., Chen, Z., Dou, X., Qin, Q., & Cai, X. (2020). Improvement of cell counting method for Neubauer counting chamber. *J Clin Lab Anal*, 34(1): e23024. https://doi: 10.1002/jcla.23024