POTENTIAL OF MELON PEELS FOR THE PRODUCTION OF CELLULOSE DEGRADING ENZYMES FROM BACILLUS SUBTILIS STRAIN 2I IN SUBMERGED FERMENTATION

AQSA TAHIR,[*] ATIA AFZAL,[*] IRFAN AHMAD,[**] DALIA SUKMAWATI,[***] MARCELO FRANCO,[****] TRISANTI ANINDYAWATI[*****] and MUHAMMAD IRFAN[*]

[*]*Department of Biotechnology, University of Sargodha, Sargodha Pakistan*
[**]*Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia*
[***]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jalan Rawamangun Muka Jakarta Timur, Indonesia 13220*
[****]*Department of Exact Science, State University of Santa Cruz, Ilheus, Bahia Brazil*
[*****]*Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), KST Soekarno, Jl. Raya Bogor Km. 46 Cibinong 16911, Indonesia*
✉ *Corresponding author: M. Irfan, irfan.ashraf@uos.edu.pk*

Received October 8, 2024

Cellulase is known to be an important enzyme in the field of industrial biotechnology. In the present study, cellulase production by *Bacillus subtilis* strain 2I was statistically optimized using melon peels in submerged fermentation. During initial optimization by OFAT, the highest CMCase (40.892 IU/mL/min) and FPase (98.398 IU/mL/min) activity were obtained at 24 h incubation time, 3% substrate concentration and 2% inoculum size. Six nutritional variables (X_1-X_6) in the cellulase production medium were screened through the Plackett–Burman design (PBD), out of which two variables were identified as significant for each CMCase and FPase. Further optimization by response surface methodology (RSM) through CCD indicated that K_2HPO_4 (0.25%) and KH_2PO_4 (0.5%) were significant for CMCase, while yeast extract (0.1%) and ammonium sulphate (0.275 %) were significant for higher FPase production, respectively. Characterization revealed cellulases displayed maximum activity with 1% substrate at 50 ℃ and pH 7. Furthermore, higher activities were observed in the presence of Ca^{+2} and Fe^{+2}. Among solvents, n-hexane, ethyl-alcohol and butanol enhanced cellulase activity, while SDS showed inhibitory effects. Cellulases showed activation energy (Ea) of -11.013 and -10.53 kJ/mol, enthalpy change (ΔH) of 8.32 and 7.84 kJ/mol, and entropy (ΔS) of -16.50 and -15.54 kJ/mol for CMCase and FPase, respectively. Additionally, cellulases produced in the current investigation could be utilized for cheap and effective enzymatic saccharification of lignocellulosic biomass.

Keywords: cellulase, CMCase, FPase, *Bacillus subtilis*, melon peels, optimization, response surface methodology, central composite design

INTRODUCTION

Fruits and vegetables are essential for human nutrition and well-being. Also, for such commodities, a significant demand increase has been observed due to increasing population growth and changing dietary habits. The overall food wastage along the food supply chain, such as during production, post-harvesting, processing, distribution and consumption by the final consumer, is referred to as "food waste". Annually, 1/3rd (app. 1.3 BMT) of the global food produced is lost or squandered.[1] However, this wastage, which is literally referred to as food waste, cannot be regarded as waste because of its unique biochemical nature. For example, fruit peels are rich in nutrients, such as vitamins, minerals, fats, proteins, anti-oxidants, growth-promoting factors and an abundance of both simple and complex sugars, *e.g.* cellulose. So, the eco-friendly utilization of these nutritionally rich peels is their bioconversion into various industrially valuable enzymes, especially cellulase, using potential microbes under specified fermentation conditions.[2]

Cellulase is a consortium of hydrolytic enzymes consisting of endo-1,4-β-D-glucanase (EC 3.2.1.4), β-glucosidase (EC 3.2.1.21) and exo-1,4-β-D-glucanase (EC 3.2.1.74) that act synergistically on cellulose to convert it into simpler reducing sugars. Cellulase is widely used in various industrial applications as most developing countries are transitioning toward urbanization, leading to the establishment of industries that mostly use cheap and abundantly available lignocellulosic raw material to lower the cost of final products.[3]

In recent years, melon peels have attracted the attention of researchers around the globe due to their rich nutritional content, including higher levels of carbohydrates, proteins, minerals, antioxidants and phenolic compounds. Melon peels have been regarded as the gold mine of valuable bioactive compounds.[4] Considering their biochemical profile, melon peels have been recently used in biofuel production,[5] enzyme synthesis,[6] and cancer treatment.[7] They have also been used to prepare dietary fiber,[8] fortified foods[9] and value-added drinks.[10] In Pakistan, the massive production and wastage of melon necessitates its appropriate utilization. So, in the present study, the potential of melon peels as a substrate for cellulase production was investigated using *Bacillus subtilis* strain 2I in submerged fermentation. Optimization of culture conditions and medium composition through OFAT and response surface methodology was performed. The cellulase produced was characterized and applied for saccharification of lignocellulosic biomass.

EXPERIMENTAL

Selection of microbe

The pre-isolated and pre-characterized strain 2I of *Bacillus subtilis* was obtained from the Microbiology Laboratory of the Biotechnology Department, University of Sargodha, Pakistan. *B. subtilis* was revived on nutrient agar slants. These slants were maintained and stored at 4 ℃ for use throughout the process.[2]

Substrate collection

Melon peels were collected from household waste, local fruit shops, and fruit processing industries in the district of Sargodha, Pakistan. The collected peels were brought to the laboratory in polythene bags. These peels were then sterilized by repeated washing with distilled water, followed by oven drying (at 60 ℃), grinding, sieving, and preservation in sterile airtight jars.[11]

Inoculum preparation

The culture of *B. subtilis* was taken on a sterile inoculum loop from the prepared nutrient agar slants. It was then incubated in sterile nutrient broth vessels at 37 ℃ for 24 h. The turbid appearance of broth demonstrated the successful preparation of inoculum that can be used as stock during study.[2]

Fermentation technique

General fermentation media containing 1% substrate was taken in a 250 mL Erlenmeyer flask and sterilized in an autoclave at 121 ℃ and 15 Psi. After sterilization, flasks were allowed to cool at room temperature and then inoculated with 1% fresh vegetative culture of *B. subtilis* under a sterilized environment. These flasks were placed in a shaking incubator at 35-37 ℃ and 120 rpm for 24 h. After incubation, the enzyme was harvested by centrifugation of fermentation media at 10,000 rpm for 20 minutes. The supernatant obtained was used for further analysis.[12]

Cellulase assay

CMCase (carboxymethyl cellulase) and FPase (filter paper activity) assays were performed using the DNS method.[2] CMCase activity was estimated by incubating 0.5 mL of 1% CMC with 0.5 mL of crude enzyme for 30 minutes in a water bath heated at 50 ℃. Meanwhile, FPase activity was accessed by incubating 500 µL of the crude enzyme with 1 x 6 cm strips of filter paper (Whatman No. 1) dipped in 0.5 mL of sodium citrate buffer (0.05 M and pH 5) in a water bath maintained at 50 ℃ temperature for 30 minutes. After incubation, the hydrolytic reaction was stopped by adding 1.5 mL of DNS and incubating the mixture for 10 minutes in boiling water. At last, spectrophotometric analysis was performed at 540 nm. Samples were studied in duplicates, and 1% glucose was used as standard.

Optimization of process parameters

The process parameters are classified into culture and nutritional parameters in enzyme production. OFAT optimized culture parameters, while nutritional parameters were first screened by PBD and then optimized using the CCD of response surface methodology. The statistical optimization of these parameters was performed to maximize the titers of cellulase produced with minimum resources being used and lesser costs of the final product. The process optimization was done in the steps described below.

Optimization by OFAT

The one factor at a time approach involves the classical monothetic analysis of certain process parameters. This method is mostly used by researchers to optimize enzyme production.[13] OFAT was used in this study for initial optimization of enzyme production. In the present study, some cultural parameters were investigated, *i.e*., incubation time of fermentation media

(24, 48, 72 and 96 h), substrate concentration (0.5-4%), and inoculum size (0.5-4%).

Screening media by PBD

Placket-Burman Design (PBD) is an efficient tool for identifying and screening the key influencing factors among many different process variables.[14] In this study, PBD was used to screen the significant factors that were promoting increased cellulase production. A total of six variables were studied, which include some nutritional parameters of media like X_1 = yeast extract, X_2 = NaCl, X_3 = $MgSO_4$, X_4 = $(NH_4)_2SO_4$, X_5 = K_2HPO_4 and X_6 = KH_2PO_4, as shown in Table 1. This PBD study was conducted in 12 runs with the range of every variable in Table 3.

Optimization by RSM

Response surface methodology (RSM) is a powerful approach to the statistical optimization of enzyme production. It is a combination of statistical and mathematical techniques that allow the interactive cumulative analysis of process variables involved in enzyme production.[15] After identifying significant variables by PBD, the Central Composite Design (CCD) with 3-levels was employed for the evaluation of optimal concentrations of each variable. Based on PBD, only two variables were identified as significant. For further optimization studies, the values assigned to each variable are given in Table 2.

Table 1
Nutritional variables screened by Placket-Burman design

Variables	Symbols	Coded values	
		+1	-1
Yeast extract (%)	X1	0.5	0.1
NaCl (%)	X2	0.09	0.03
$MgSO_4$ (%)	X3	0.3	0.1
$(NH_4)_2SO_4$ (%)	X4	0.5	0.05
K_2HPO_4 (%)	X5	0.75	0.25
KH_2PO_4 (%)	X6	0.75	0.25

Table 2
Values of significant variables assigned by CCD

CMCase				
Variables (%)	Symbols	Coded values		
		+1	0	-1
K_2HPO_4	X_5	0.75	0.5	0.25
KH_2PO_4	X_6	0.75	0.5	0.25
FPase				
Variables (%)	Symbols	Coded values		
		+1	0	-1
Yeast extract	X_1	0.5	0.3	0.1
$(NH_4)_2SO_4$	X_4	0.5	0.275	0.05

Characterization of crude cellulase

Temperature: The activity and stability of crude cellulase at different temperatures was assessed by incubating the enzyme at various temperatures under study. Cellulase activity was checked by incubating crude cellulase + substrate (CMC or filter paper) separately at 20, 30, 40, 50, 60 and 70 °C for 30 minutes in water bath. Whereas enzyme stability was checked by the pre-incubating the enzyme separately at the above temperature range for 2 hours. This pre-incubated enzyme was then analyzed by the cellulase assay described above. However, in case of stability, enzyme activity is expressed in terms of relative or residual enzyme activity.

pH: Cellulase activity and stability was checked by incubating the enzyme in buffers of different pH, including 4, 5, 6.2, 7, 10 and 11, during enzyme assay. While performing cellulase assay, buffers of the above-mentioned pH were used to prepare substrates (CMC solution for CMCase and filter paper in buffer for FPase). After the addition of crude cellulase separately in these different substrates, it was incubated and then analyzed for its activity according to rest of the steps of cellulase assay. In contrast, the stability of cellulase was checked by pre-incubating the enzyme separately with the substrates (prepared in buffers of different pH as explained earlier) for 2 hours. After the pre-incubation, this enzyme was then analyzed for its activity by performing the remaining steps of cellulase assay. Again, in stability studies, residual cellulase activity (contrast of activity of pre-incubated enzyme at different pH with that of the activity of non-pre incubated enzyme) was evaluated.

Substrate concentration: The activity and stability of the enzyme were simultaneously checked by incubating it with a range of different substrate concentrations (0.2-1%) of CMC solution for CMCase, while different dimensions of filter paper were used for FPase during the assay.

Metal ions: Cellulase activity and stability were checked by pre-incubation of enzyme with metal solutions ($CaCl_2$, $MgSO_4$, NH_4Cl_2, $FeSO_4$, NaCl, $CuSO_4$, $MnSO_4$, $ZnSO_4$, KCl and EDTA) of varied concentrations, *i.e.* 1 mM, 3 mM, and 5 mM. Relative enzyme activity was then checked using this pre-incubated enzyme in the cellulase assay as per the standard procedure described above.

Solvents: Simultaneous evaluation of cellulase activity and stability was done by pre-incubating crude cellulase with solvents like SDS, Tween-80, butanol, n-hexane, ethyl alcohol, isopropanol, and methanol, separately. Different concentrations, *i.e.* 10%, 20%, and 30% of each solvent were used for better characterization. This pre-incubated enzyme was then analyzed by cellulase assay for evaluation of relative cellulase activity.

Measurement of kinetic and thermodynamic parameters

Kinetic parameters

The kinetic parameters of the cellulolytic reaction, such as V_{max} (maximal velocity) and K_m (Michaelis–Menton constant), were calculated by a Lineweaver-Burk plot (LB) from the optimal assay conditions by conducting a series of tests with varying initial substrate concentrations (0.2-1%).[16]

The Lineweaver-Burk plot is the graphical representation of the Michaelis-Menton equation that explains enzyme-substrate relationship. The reciprocal equation of the LB plot modified from the Michaelis-Menton equation is as follows:

$$1/V_0 = K_m/V_{max} * 1/S + 1/V_{max} \quad (1)$$

In addition, the linear slope equation is:

$$Y = m\,x + b \quad (2)$$

As the Line-weaver-Burk plot shows, the linear correlation of enzyme activity with its substrate concentration, the values of kinetic parameters V_{max} and K_m were derived by comparing Equations 1 and 2 in the following manner: $y=1/V_0$, $m=K_m/V_{max}$, $b=1/V_{max}$. These kinetic parameters can be easily evaluated by the slope equation of the LB plot in Excel. However, results with greater statistical accuracy can be obtained using the Graph Pad Prism that was used in this kinetic analysis.

Thermodynamic parameters

Thermodynamic parameters like entropy and enthalpy were evaluated on the basis of cellulase activity at different temperatures (20-70 °C). The activation energies (Ea) for both CMCase and FPase were first estimated using the Arrhenius method, which was later used to calculate thermodynamic parameters like entropy (ΔS) and enthalpy change (ΔH):[16]

$$Ea = -slope * R \quad (3)$$

$$\Delta H = Ea - RT \quad (4)$$

The following Equation (6) was used to calculate ΔS entropy change:

$$Ln\ Vmax/T = Ln\ (KB/h) + \Delta S/R - \Delta H/R.\ 1/T \quad (5)$$

$$\Delta S = R\ (Ln(Vmax/T) - Ln\ (KB/h) + \Delta H/R\ .\ 1/T \quad (6)$$

where the Boltzmann constant $R = 8.314\ J/K^{-1}.mol^{-1}$, the gas constant KB= 1.38*10–23 $J.K^{-1}$, Planck's constant h = 6.63*10-34 J.s, and T is the absolute temperature.

Application of crude cellulase in biomass saccharification

Crude indigenous cellulase with CMCase and FPase activity of 39.773 and 98.191 IU/mL/min, respectively, was used for lignocellulosic biomass hydrolysis. The mixture containing a hundred millilitres of crude cellulase and 5 g alkaline pretreated Bermuda grass (*Cynodon dactylon*) in 100 mL of citrate buffer was incubated at 50 °C for various time periods. Samples were withdrawn every 2 hours, and sugar analysis was performed to determine saccharification percentage.

Statistical analysis

All the experiments were performed in triplicates, and their mean values were employed for further analysis. All experimental data were graphically represented by Excel 2016 and statistically analyzed using ANOVA and multiple regression. The comparison of correlation coefficients was done to determine the significance of the model.

RESULTS AND DISCUSSION

Melon peels were surprisingly found as a gold mine of various biochemical components, comprising 84.81% carbohydrates, 34.90% protein content, 11.5% ash content, 29.59% crude fiber, 61% iron, 84% copper, 27.68% cellulose, 8.2% hemicelluloses, 26.46% lignin, phenolic compounds (0.69 to 2.96 mg of gallic acid equivalent/g extract), flavonoids 262 µg catechin as equivalent (CA)/100 g and antioxidants 0.13 to 0.26 (in mg ascorbic acid equivalents/mL extract).[7,8,17,18,19] This enriched biochemical profile of melon peels intrigued researchers for their utilization as a perfect carbon and energy source in fermentation processes for production of various bio-products and valuable secondary metabolites, *e.g.* enzyme production.[6] The present study involves optimizing several independent culture and nutritional parameters with a special focus on maximizing cellulase production. *Bacillus subtilis* was used as a producer microbe, and melon peels were used as a carbon and energy source. Recent

studies showed *Bacillus subtilis* as an excellent cellulase producer.[11,12,20-23]

Optimization by OFAT

Microbial cellulase production is greatly affected by the culture parameters of the medium. OFAT optimized three parameters, primarily by amending each variable under study, while keeping all other factors constant.

Incubation time

Fermentation media were incubated for 24, 48, 72, and 96 h, and cellulase activity was checked periodically. At 24 h of incubation time, a maximum CMCase activity of 17.592 IU/mL/min and a maximum FPase activity of 35.082 IU/mL/min were observed. A gradual decrease in cellulase activity was observed from 24 to 96 h, as illustrated in Figure 1. Most researchers reported 24 and 48 h of fermentation time for *Bacillus subtilis* in submerged fermentation.[12,24,25]

Substrate concentration

The culture medium was further optimized by adding 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4% substrate in fermentation media in separate flasks. After incubation, the activity of the cellulase enzyme was checked. Cellulase activity gradually increased by increasing substrate concentration up to a certain point (0.5-3%), after which only negligible change was observed. CMCase and FPase showed maximum activity of 40.086 IU/mL/min and 97.046 IU/mL, respectively, at 3% substrate concentration, as demonstrated in Figure 2. Different microbes utilize varied substrate concentrations for optimal cellulase production. Afzal *et al.*[26] also reported a 3% substrate concentration for cellulase production. In another study, a 1% substrate concentration was reported for the maximum activity of cellulase by *Bacillus cereus.*[27]

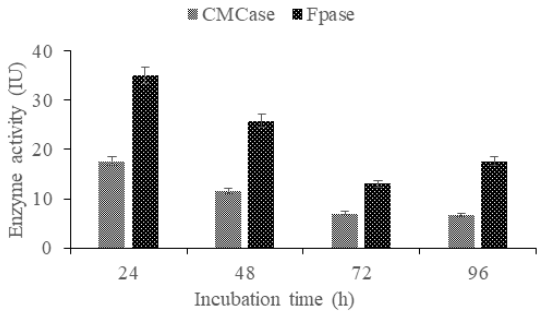


Figure 1: Optimization of incubation time by OFAT

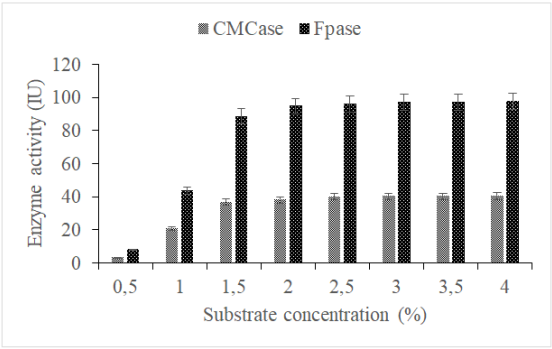


Figure 2: Substrate concentration optimized by OFAT

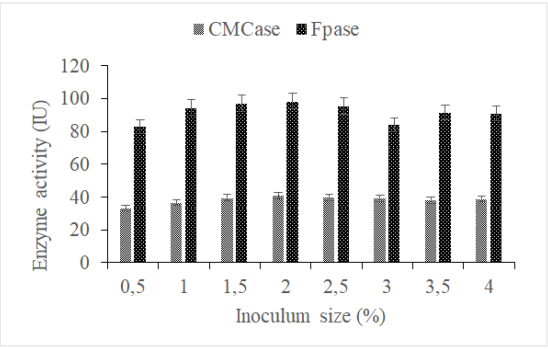


Figure 3: Optimizing inoculum size using OFAT approach

Inoculum size

Inoculum size was optimized by adding 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4% inoculum of *Bacillus subtilis* in sterile fermentation media in separate flasks. After 24 h of submerged fermentation, cellulase assay showed maximum CMCase activity (40.892 IU/mL/min) and maximum FPase activity (98.398 IU/mL/min) at 2% (v/v) inoculum, as shown in Figure 3. Comparable findings were reported by some researchers where 2% v/v

inoculum size resulted in maximum cellulase production by various *Bacillus* sp.[28,29] Some reports showed maximum cellulase production at 3% inoculum size,[30] while Yang *et al*.[25] and Afzal *et al*.[26] reported that 4% inoculum size was best for cellulase production by *Bacillus*.

Screening media by PBD

Some nutritional parameters of fermentation media were screened by PBD. A 12-run experiment was conducted to screen 6 nutritional variables (X1-X6). The results obtained from this experiment are shown in Table 3. These were analyzed by multiple regression and its response showed that 2 variables, *i.e.* K_2HPO_4 (X_5) and KH_2PO_4 (X_6) for CMCase, and yeast extract (X_1) and ammonium sulphate (X_4) for FPase, were significantly affecting cellulase production, as demonstrated in Figure 4.

Optimization by RSM

For optimizing the concentrations of significant parameters, *i.e*., X_5 and X_6 (for CMCase) and X_1 and X_4 (for FPase), the CCD design of RSM was used with three levels of each variable. This optimization experiment was conducted in 9 runs; the results are listed in Table 4. The resulting response was further calculated by the 2nd-order polynomial regression. The results demonstrated that maximum CMCase (135.104 IU/mL/min) activity was recorded at 0.25% K_2HPO_4 and 0.5% KH_2PO_4, while the highest FPase (138.84 IU/mL/min) activity was observed at optimum concentrations of 0.1% yeast extract and 0.275% ammonium sulphate.

Table 3
Placket-Burman design applied to different variables to screen significant nutritional parameters for the production of cellulase enzyme using *Bacillus subtilis* 2I in submerged fermentation

Run no.	Variables						Responses (IU/mL/min)	
	X_1	X_2	X_3	X_4	X_5	X_6	CMCase	FPase
1	+1	+1	+1	+1	+1	+1	120.3617	125.836
2	-1	+1	-1	+1	+1	-1	118.1706	123.2474
3	-1	-1	+1	+1	+1	+1	119.7728	125.3467
4	+1	-1	-1	-1	+1	+1	119.4704	124.574
5	-1	+1	-1	+1	-1	+1	118.664	122.4875
6	-1	-1	+1	-1	+1	-1	117.9743	122.861
7	-1	-1	-1	-1	-1	+1	116.3084	120.7617
8	+1	-1	-1	+1	-1	-1	117.5499	124.3421
9	+1	+1	-1	-1	+1	-1	118.2926	123.1315
10	+1	+1	+1	-1	-1	+1	118.2184	123.1315
11	-1	+1	+1	-1	-1	-1	117.3218	123.1186
12	+1	-1	+1	+1	-1	-1	115.6877	124.4838

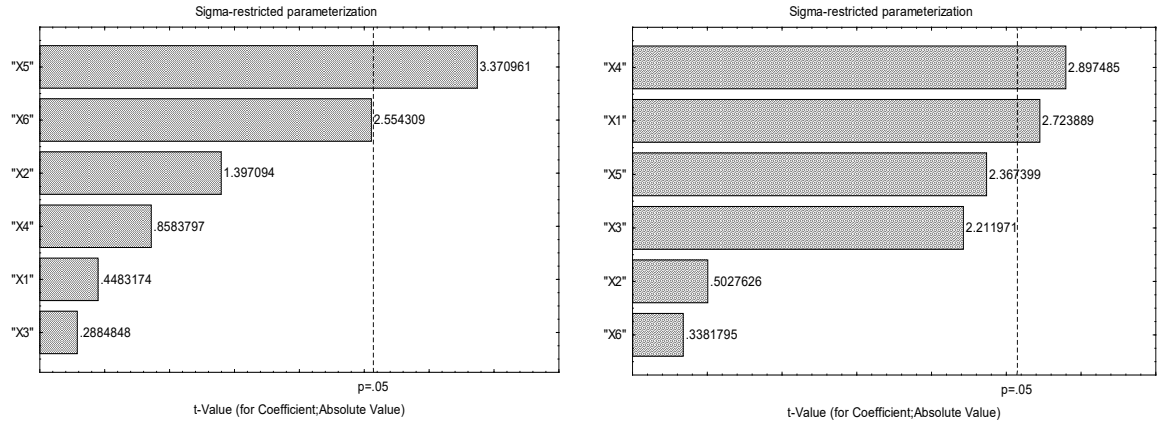


Figure 4: Pareto chart demonstrating significant variables for CMCase (left) and FPase (right)

The significant parameters screened through CCD were statistically analyzed by ANOVA (Table 5). The F and P values indicated by the model demonstrated the significance of the

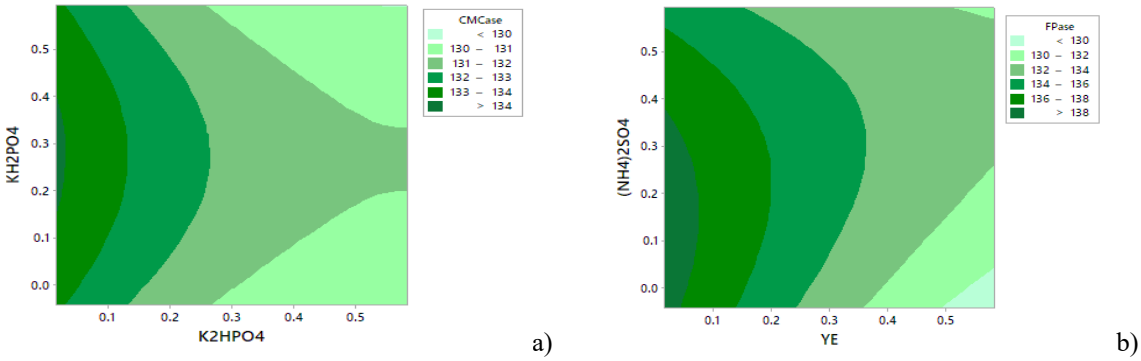
proposed model. In addition, the model showed multiple regression R^2 values of 53.48% and 79.60%, which indicate variability in the production of CMCase and FPase, respectively. The accuracy of results was demonstrated by the proximity between the values of predicted multiple R^2 (CMCase: 53.48%, FPase: 79.60%) and adjusted R^2 (CMCase: 20.26%, FPase: 65.03%).[31] If the R^2 score is close to 100%, it indicates a higher correlation between predicted and experimental values. In this study, its value was 79% in the case of FPase, which demonstrated the suitability of the model for FPase.

Table 4
CCD design for optimizing cellulase production

Runs	CMCase					FPase				
	Variables		Enzyme activity (IU/mL/min)			Variables		Enzyme activity (IU/mL/min)		
	X_5	X_6	Observed	Predicted	Residual	X_1	X_4	Observed	Predicted	Residual
1	-1	0	135.10400	134.2332	0.870758	-1	0	138.84000	138.6660	0.173991
2	-1	+1	132.34000	132.5887	-0.248659	-1	+1	135.29900	135.7825	-0.483453
3	+1	0	132.80400	133.1453	-0.341310	+1	0	134.78900	134.5036	0.285353
4	0	-1	131.74000	131.4582	0.281758	0	-1	134.19600	134.2277	-0.031675
5	-1	-1	132.16700	132.7891	-0.622099	-1	-1	138.05100	137.7415	0.309461
6	0	+1	131.51200	131.2643	0.247690	0	+1	132.78000	132.2890	0.491020
7	0	0	132.37300	132.9024	-0.529448	0	0	134.69300	135.1523	-0.459345
8	+1	-1	132.03500	131.6947	0.340341	+1	-1	133.28100	133.5588	-0.277786
9	+1	+1	132.15900	132.1580	0.000969	+1	+1	133.67200	133.6796	-0.007567

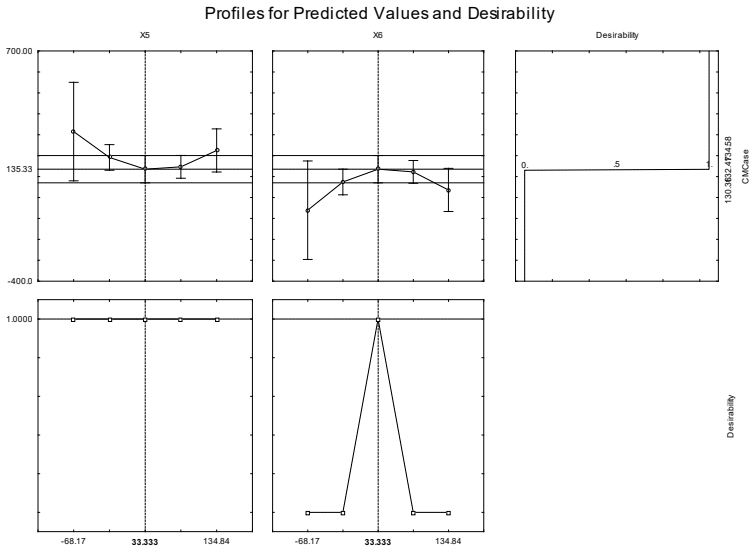
Table 5
Analysis of variance (ANOVA) of significant parameters for CMCase and FPase production

CMCase					
Source	df	SS	MS	F	P
Model	5	12.9992	2.59984	1.61	0.273
Linear	2	9.6329	4.81647	2.98	0.116
K_2HPO_4	1	9.6295	9.62946	5.96	0.045
KH_2PO_4	1	0.0035	0.00347	0.00	0.964
Square	2	3.3654	1.68268	1.04	0.402
K_2HPO_4* K_2HPO_4	1	1.1305	1.13050	0.70	0.430
KH_2PO_4* KH_2PO_4	1	1.8050	1.80496	1.12	0.326
2-Way Interaction	1	0.0009	0.00090	0.00	0.982
K_2HPO_4* KH_2PO_4	1	0.0009	0.00090	0.00	0.982
Error	7	11.3061	1.61516		
Lack-of-fit	3	10.2413	3.41378	12.82	0.016
Pure error	4	1.0648	0.26620		
Total	12	24.3053			
FPase					
Effect	df	SS	MS	F	P
Model	5	53.2404	10.6481	5.46	0.023
Linear	2	43.8192	21.9096	11.24	0.007
YE	1	43.8139	43.8139	22.48	0.002
(NH4)2SO4	1	0.0053	0.0053	0.00	0.960
Square	2	6.9405	3.4703	1.78	0.237
YE*YE	1	0.8583	0.8583	0.44	0.528
(NH4)2SO4*(NH4)2SO4	1	5.4024	5.4024	2.77	0.140
2-way interaction	1	2.4806	2.4806	1.27	0.296
YE*(NH4)2SO4	1	2.4806	2.4806	1.27	0.296
Error	7	13.6439	1.9491		
Lack-of-fit	3	13.6439	4.5480	*	*
Pure error	4	0.0000	0.0000		
Total	12	66.8843			

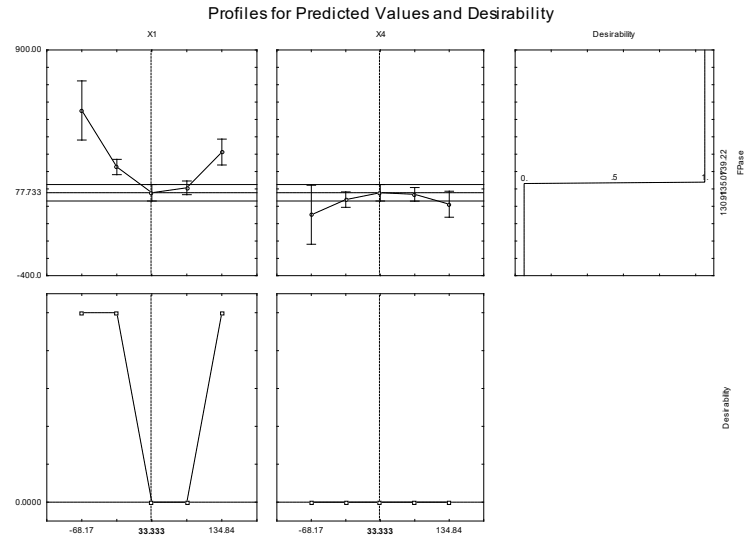


a) b)

Figure 5: Contour charts of significant factors for CMCase (a) and FPase (b) production



a)



b)

Figure 6: Desirability profile for CMCase (a) and FPase (b) production

The predicted and observed values of FPase showed a greater correlation between them. Different coefficient factors were responsible for dissimilarities.[11]

Figure 5 displays the interaction effect between yeast extract (X1) and ammonium sulphate (X4) on cellulase production. These surface plots demonstrate the significance of each parameter on cellulase production in submerged fermentation. The desirability charts for CMCase and FPase production are displayed in Figure 6. These charts described the validation of the predicted model through repeated experimentation. The model revealed that the obtained results were consistent with the predicted values. The regression equations for CMCase and FPase are as follows:

Y (CMCase activity, IU) = 133.58 - 11.44 K_2HPO_4 + 5.54 KH_2PO_4+ 10.1 K_2HPO_4*K_2HPO_4-10.06 KH_2PO_4 *KH_2PO_4 - 0.3 K_2HPO_4*KH_2PO_4 (7)

Y (Fpase activity, IU) =139.15 - 21.78 YE + 4.21 $(NH_4)2SO_4$+ 8.8 YE*YE - 17.4 $(NH_4)_2SO_4$*$(NH_4)_2SO_4$+ 17.5 YE*$(NH_4)_2SO_4$ (8)

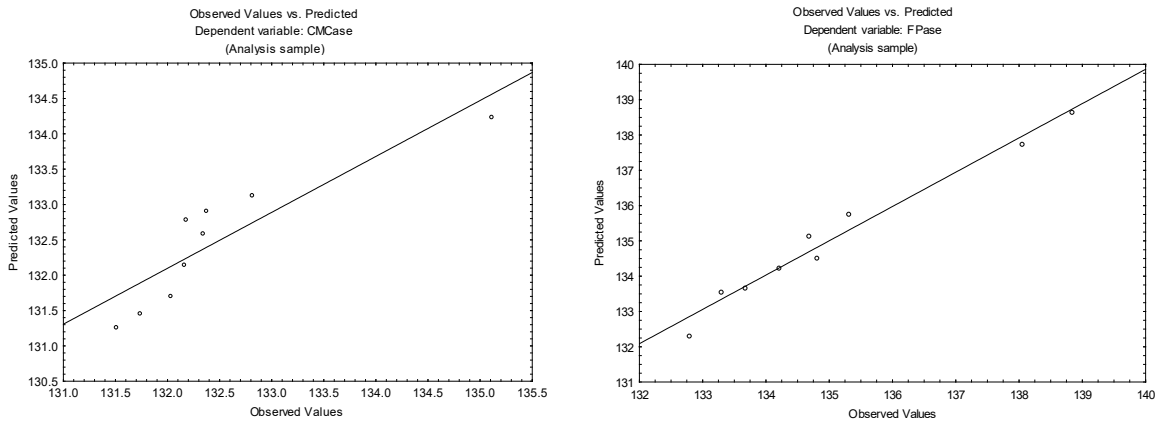


Figure 7: Graphs of observed *vs*. predicted values for CMCase and FPase

Figure 7 illustrates the graphs of observed *vs*. predicted values proposed by the model for CMCase and FPase.

These graphs showed the close proximity between observed and predicted values in the case of FPase, while a lesser correlation between these values was observed in the case of CMCase.

Characterization of crude cellulase

Effect of temperature

Along the temperature range under study (20-70 °C), both CMCase and FPase displayed maximum activities at 50 °C (Fig. 8), after which it declined slowly with increasing temperature. Listyaningrum *et al*.[21] reported similar results with the *Bacillus* strain. In addition, both FPase and CMCase were found stable at 40 °C, as indicated in Figure 9. Islam and Roy[32] also reported the maximum activity of cellulase produced by cellulase-producing bacteria in molasses at 40 °C. Deka *et al*.[33] also stated the maximum stability of cellulase within the temperature of 20-45 °C.

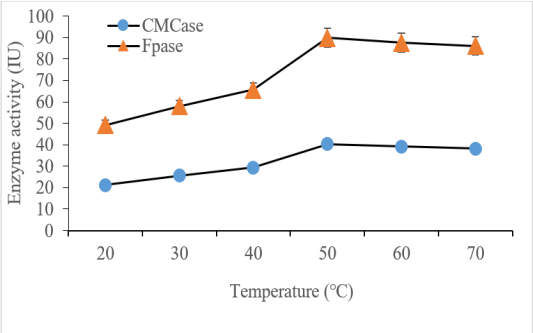


Figure 8: Effect of different temperatures on cellulase activity

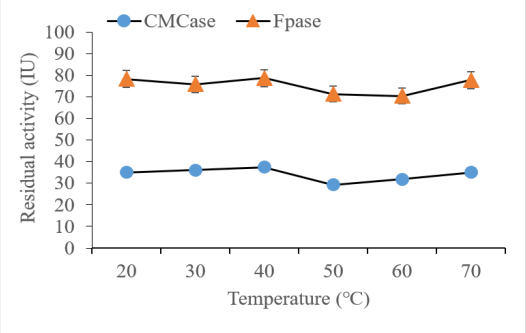


Figure 9: Cellulase stability at various temperatures

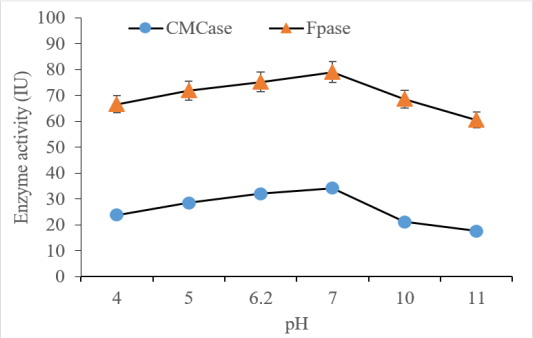


Figure 10: Effect of various pH on cellulase activity

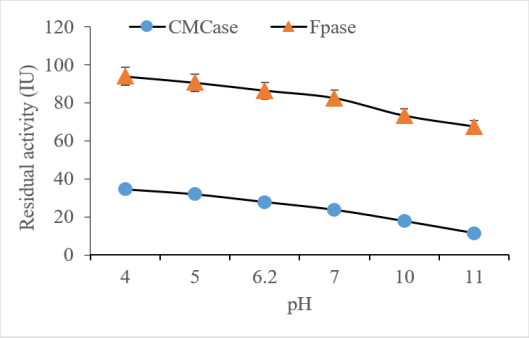


Figure 11: Stability of cellulase at different pH

Effect of pH

In the range of pH tested, both CMCase and FPase displayed maximum activity at pH 7 (Fig. 10), while maximum stability was recorded at pH 4 (Fig. 11). In literature, cellulase is most active and stable within a pH range of 4-7. Various scientists reported greater cellulase activities at pH 7.[32,34] At the same time, some others reported maximum activity and stability at pH 5.[21,23] Cellulase has also shown maximum activity at pH 5.5 and stability at pH 4-6.[16]

Effect of substrate

Among the tested range of substrate concentration (0.2-1%), both CMCase and FPase displayed exponential increases in their activities and were found most active at 1% substrate (Fig. 12). Islam and Roy[32] claimed corresponding results for CMCase, which showed maximum activity at 1% CMC. Nema *et al*.[27] and Sharif *et al*.[34] also indicated similar outcomes with cellulase using *Bacillus* species in their studies.

Effect of metal ions

Among various metals under study, $CaCl_2$ supplemented medium showed maximum CMCase activity at 3 mM, while maximum FPase activity at 5 mM. Followed by this, NH_4Cl_2 and $FeSO_4$ showed greater CMCase activity at 1 mM concentration. In contrast, $MnSO_4$ and $ZnSO_4$ showed minimum CMCase activity. On the other hand, after $CaCl_2$, greater FPase activity was shown by $FeSO_4$ (1 mM), $ZnSO_4$ (3 mM) and EDTA (5 mM), while $CuSO_4$ and $MnSO_4$ showed lower FPase activity (Table 6). Other researchers also reported maximum cellulase activity with $CaCl_2$.[16,29]

Table 6
Effect of different metals on cellulase activity and stability

Metals/ Chemical	CMCase relative activity (%)			FPase relative activity (%)		
	1 mM	3 mM	5 mM	1 mM	3 mM	5 mM
Control	100	100	100	100	100	100
$CaCl_2$	122.486	125.812	122.446	112.6	101.992	117.389
KCl	106.076	108.155	110.969	104.607	109.362	111.089
NH_4Cl_2	123.494	116.336	115.299	111.928	109.362	105.747
$MnSO_4$	101.542	101.487	101.159	101.771	102.887	102.066
$MgSO_4$	114.63	112.668	109.353	108.813	108.981	104.919
$CuSO_4$	107.628	111.629	105.718	104.336	103.113	106.106
$FeSO_4$	121.653	107.167	119.598	114.43	107.565	113.097
$ZnSO_4$	104.773	105.243	104.715	110.435	112.78	110.957
EDTA	106.437	108.688	115.833	103.189	106.026	112.151

Table 7
Effect of varied concentrations of different solvents on cellulase activity and stability

Solvents/ Chemical	CMCase relative activity (%)			FPase relative activity (%)		
	1 mM	3 mM	5 mM	1 mM	3 mM	5 mM
Control	100	100	100	100	100	100
SDS	100.576	108.453	106.882	100	100	100.194
Tween-80	122.438	116.524	122.243	105.4	102.629	103.954
Butanol	123.781	121.009	132.321	107.963	103.745	101.979
n-Hexane	123.225	129.901	114.575	107.602	113.48	112.048
Ethyl Alcohol	116.524	119.725	120.78	119.425	116.719	121.796
Iso-Propanol	114.405	116.524	113.76	102.303	104.587	103.62
Methanol	111.285	114.234	111.967	102.303	102.957	102.629

Effect of solvents

Among the tested solvents, butanol and n-hexane showed maximum CMCase activity at 30% and 20%, respectively, as depicted in Table 7. At the same time, ethyl-alcohol (at 30%) and n-hexane (at 20%) showed maximum FPase activity. In contrast, SDS showed minimum activity for both CMCase and FPase. In addition, Tween-80 showed greater enzyme production for CMCase, which resulted in lower titers of enzyme in the case of FPase. Various researchers demonstrated minimum cellulase activity with SDS and sometimes with Tween-80.[16,29]

Kinetic parameters

Figure 12 displayed the response of cellulase activity according to different substrate concentrations (0.2-1% of CMC and filter paper). The Y-value intercepts (~1/Vmax) for CMCase and FPase were 0.017482 and 0.0077942, respectively. The K_m values of 5.061 and 4.755 mg/mL and V_{max} of 57.20 and 128.3 IU/mL/min for CMCase and FPase, respectively.

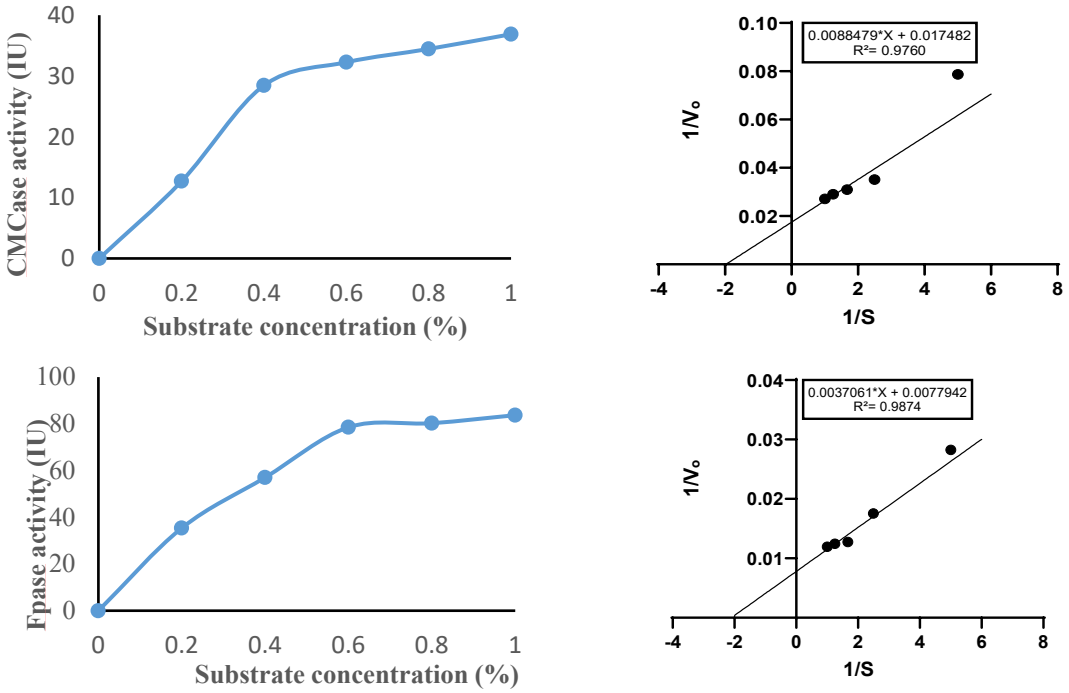


Figure 12: Lineweaver-Burk plot for CMCase (upper) and FPase (lower)

Different cellulases exhibit diverse kinetics depending on several factors, such as biochemical composition, 3D structures, interaction with substrate, temperature *etc*. Nisar *et al*.[16] similarly calculated these kinetic parameters and reported K_m as 0.63, and 28.56 mg/mL, while V_{max} as 82 and 80 U/mL/min for endoglucanases and betaglucosidases, respectively. In another study, a novel cellulase CelC307 was isolated from a thermophilic bacterium, *Cohnella* sp. A01

demonstrated K_m of 0.46 mM, while kcat 104.30×10^{-3} (S^{-1}) and kcat/Km as 226.73 (M^{-1} S^{-1}) using 1% CMC as substrate.[35] A comprehensive analysis of kinetic and thermodynamic stability under diverse conditions is crucial for the evaluation of its potential in industrial applications. The investigation of enzyme kinetics elaborates on the reaction rate, extent of reaction and energy requirements of the reaction.[36]

In this investigation, both CMCase and FPase demonstrated lower K_m values, expressing the higher affinity of indigenous cellulase for its potential substrates and greater reaction rates with minor substrate concentrations.

Thermodynamic parameters

The Arrhenius plot was used to evaluate the values of Ea, from which ΔH and ΔS were estimated using some mathematical expressions described above. The activation energy (Ea) of -11.013 and -10.53 kJ/mol, and enthalpy (ΔH) of reaction of 8.32 and 7.84 kJ/mol were evaluated for CMCase (Fig. 13 (A)) and FPase (Fig. 14 (A)) respectively. In addition, the entropy (ΔS) was estimated by the plot of $\ln(V_{max}/T)$ against 1/T. Hence, ΔS values of -16.50 kJ/mol for CMCase and -15.54 kJ/mol for FPase were calculated (Fig. 13 (B) and Fig. 14 (B)). Nisar *et al*.[16] elaborated this method for estimating thermodynamic parameters for endoglucanases and betaglucosidases. The activation energy (Ea), enthalpy (ΔH) and entropy (ΔS) of these enzymes were estimated as 44.55, 50.02 kJ/mol; 42.20, 47.70 kJ/mol, and 5.1, 5.7 kJ/mol, respectively. Another study reported the thermodynamic analysis of cellulase CelC307, which demonstrated 25.36 kJ/mole of Ea, 22.75 kJ $mole^{-1}$ of ΔH and 114.51 J $mol\ k^{-1}$ of ΔS.[35]

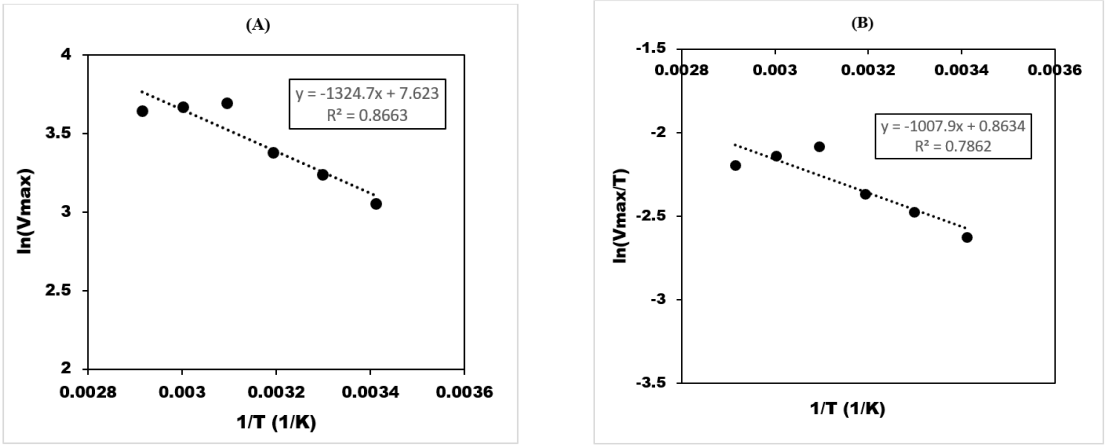


Figure 13: Arrhenius plot (A) and estimation of entropy change ΔS (B) for CMCase

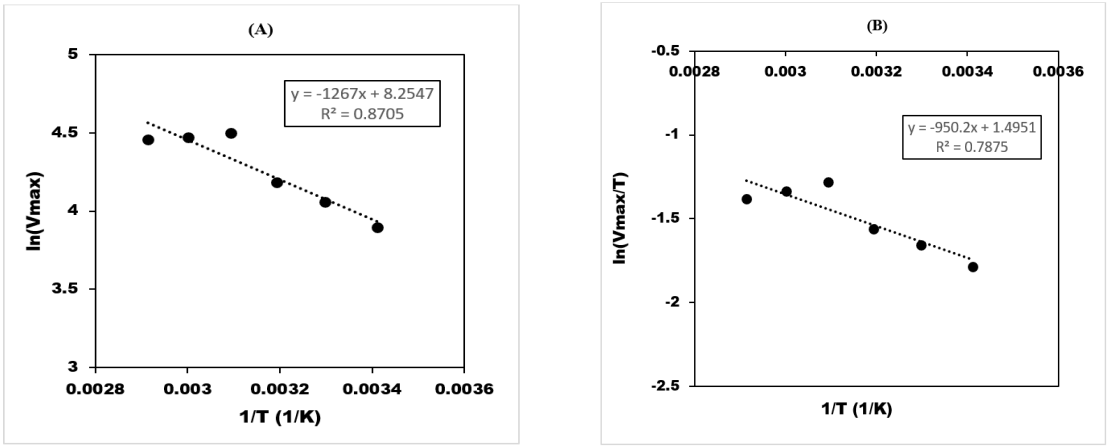
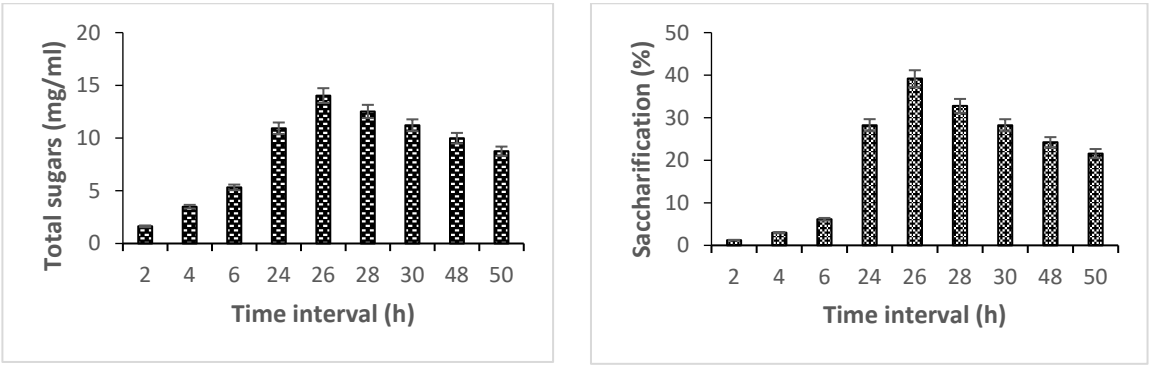


Figure 14: Arrhenius plot (A) and evaluation of entropy change ΔS (B) for FPase



A) B)

Figure 15: Estimation of total sugars (A) and percent saccharification (B) during hydrolysis of lignocellulosic biomass

The activation energy Ea refers to the energy requirements of a reaction to proceed smoothly by making a strong enzyme substrate complex (ESC) that ultimately leads to product formation. The current study showed smaller Ea values for CMCase and FPase indicated stronger ES complex, hence minimum energy requirements. Enthalpy (ΔH) generally refers to the difference in the total heat content of the system. In this case, positive values of ΔH demonstrated the endothermic behavior of the cellulolytic reaction. Lastly, entropy change (ΔS) determining the dysfunction of the enzymatic system had negative values, indicating a decrease in entropy or randomness of the system. The entropy of the system may be explained on the basis of thermal denaturation of the enzyme, which caused non-covalent linkages in the enzymatic structure to disrupt, hence increased the enthalpy of reaction. This disruption in the enzymatic structure allowed the interaction of the substrate with the active sites of the enzyme. Hence, the entropy and the enthalpy of a reaction are interrelated concepts.

Application of cellulase in saccharification of lignocellulosic biomass

Our indigenous cellulase demonstrated significant enzymatic hydrolysis of pretreated lignocellulosic biomass, yielding maximum total sugars of 14.02896 mg/mL (Fig. 15 (A)) and reducing sugars of 9.79968 mg/mL at 26 hours incubation time, resulting in a 39.19% percent saccharification rate as indicated in Figure 15 (B). Tabssum *et al*.[28] used the indigenous cellulase produced from *Bacillus cereus* for saccharification of raw and pre-treated poplar biomass and reported 11.50% saccharification for raw/untreated poplar biomass. Ghazanfar *et al*.[37] utilized the cellulase produced by *Bacillus aerius* in their research for the hydrolysis of raw seed pods of *Bombax ceiba*. The saccharification of 38% of KOH-steam pretreated *B. ceiba* confirmed its industrial exploitation in biofuel production.

CONCLUSION

The current investigation involved cellulase production from already isolated *Bacillus subtilis* 2I using melon peels as a carbohydrate-rich substrate in submerged fermentation. The optimum physiological parameters for cellulase production were determined. The present study also highlighted the statistical efficiency of CCD for process optimization. The saccharification outcomes depicted that cellulase produced in the current study could be exploited as a convenient candidate for efficient and cost-effective lignocellulosic biomass hydrolysis in bioethanol production processes.

ACKNOWLEDGEMENT: The authors are thankful to the Deanship of Research and Graduate Studies, King Khalid University, Abha, Saudi Arabia, for financially supporting this work through the Large Research Group Project under Grant no. R.G.P.2/517/45.

REFERENCES

[1] M. I. Hussain, I. Ijaz, M. A. Mustafa, N. Sabar, S.M.U. Kazmi *et al*., *Pak. J. Med. Health Sci*., **17**, 43 (2023), https//doi.org/10.53350/PJMHS202317143

[2] M. Irfan, J. Bakhtawar, S. Sadia, H. A. Shakir, M. Khan *et al*., *Int. J. Biol. Chem*., **13** (2020), https//doi.org/10.26577/ijbch.2020.v13.i2.11

[3] P. Maravi and A. Kumar, *J. Appl. Biol. Biotechnol*., **9**, 142 (2021), https//doi.org/10.7324/JABB.2021.9213

[4] S. Namet, M. R. Khan, M. A. Shabbir, A. Din, Z. F. Bhat *et al*., *Biomass Convers. Biorefin*., (2023), https//doi.org/10.1007/s13399-023-05147-z

[5] A. Chaudhary, I. Hussain, Q.-A. Ahmad, Z. Hussain, A. M. Akram *et al*., *Biomass Convers. Biorefin.*, **14**, 3463 (2024), https//doi.org/10.1007/s13399-022-02687-8
[6] M. O. Baltaci, M. A. Omeroglu, S. Albayrak, G. Adiguzel and A. Adiguzel, *Ann. Acad. Bras. Cienc.*, **94**, (2022), https//doi.org/10.1590/0001-3765202220220151
[7] P. M. Rolim, G. P. Fidelis, C. E. A. Padilha, E. S. Santos, H. A. O. Rocha *et al*., *Braz. J. Med. Biol. Res.*, **51**, (2018), https//doi.org/10.1590/1414-431X20176069
[8] H. M. A. Al-Sayed and A. R. Ahmed, *Ann. Agric. Sci.*, **58**, 83 (2013), https//doi.org/10.1016/J.AOAS.2013.01.012
[9] F. M. Vella, R. Calandrelli, D. Cautela and B. Laratta, *Foods*, **12** (2023), https//doi.org/10.3390/FOODS12132523
[10] S. Namet, M. R. Khan, R. M. Aadil and M. A. Zia, *J. Food Process. Preserv.*, **2023**, 1 (2023), https//doi.org/10.1155/2023/6631784
[11] N. Iram, H. A. Shakir, M. Irfan, M. Khan, S. Ali *et al*., *Acta Sci. Tech.*, **43**, e50538 (2021), https//doi.org/10.4025/actascitechnol.v43i1.50538
[12] M. Irfan, Q. Mushtaq, F. Tabssum, H. A. Shakir and J. I. Qazi, *AMB Exp.*, **7** (2017), https//doi.org/10.1186/S13568-017-0331-3
[13] Z. H. Kheiralla, N. S. El-Gendy, H. A. Ahmed, T. H. Shaltout and M. M. D. Hussein, *Energ. Sources A: Rec. Util. Environ. Eff.*, **40**, 1877 (2018), https//doi.org/10.1080/15567036.2018.1487485
[14] S. Shajahan, I. G. Moorthy, N. Sivakumar and G. Selvakumar, *J. King Saud Univ. Sci.*, **29**, 302 (2017), https//doi.org/10.1016/j.jksus.2016.08.001
[15] T. Ahmad, A. Sharma, G. Gupta, S. Mansoor, S. Jan *et al*., *Saudi J. Biol. Sci.*, **27**, 2333 (2020), https//doi.org/10.1016/j.sjbs.2020.04.036
[16] K. Nisar, R. Abdullah, A. Kaleem, M. Iqtedar, M. Aftab *et al*., *Saudi J. Biol. Sci.*, **29**, 103483 (2022), https//doi.org/10.1016/j.sjbs.2022.103483
[17] R. Gómez-García, D. A. Campos, A. Oliveira, C. N. Aguilar, A. R. Madureira *et al*., *Food Chem.*, **335**, 127579 (2021), https//doi.org/10.1016/j.foodchem.2020.127579
[18] S. M. Ganji, H. Singh and M. Friedman, *J. Food Sci.*, **84**, 1943 (2019), https//doi.org/10.1111/1750-3841.14666
[19] R. Gómez-García, M. Sánchez-Gutiérrez, C. Freitas-Costa, A. A. Vilas-Boas, D. A. Campos *et al*., *Food Res. Int.*, **154** (2022), https//doi.org/10.1016/J.FOODRES.2022.111045
[20] A. Kazemi, S. Rasoul-Amini, M. Shahbazi, A. Safari and Y. Ghasemi, *Prep. Biochem. Biotechnol.*, **44**, 107 (2014), https//doi.org/10.1080/10826068.2013.792276
[21] N. P. Listyaningrum, A. Sutrisno and A. K. Wardani, *IOP Conf. Ser. Earth Environ. Sci.*, **131**, (2018), https//doi.org/10.1088/1755-1315/131/1/012043
[22] I. Q. M. Padilha, L. C. T. Carvalho, P. V. S. Dias, T. C. S. L. Grisi, F. L. Honorato da Silva *et al*., *Braz. J. Chem. Eng.*, **32**, 35 (2015), https//doi.org/10.1590/0104-6632.20150321S00003298
[23] C. P. Sreena and D. Sebastian, *J. Gen. Eng. Biotech.*, **16**, 9 (2018), https//doi.org/10.1016/j.jgeb.2017.12.005
[24] A. S. Kumar, A. Kumar, V. Kumar and B. Singh, *Prep. Biochem. Biotechnol.*, **51**, 697 (2021), https//doi.org/10.1080/10826068.2020.1852419
[25] W. Yang, F. Meng, J. Peng, P. Han, F. Fang *et al*., *Elect. J. Biotech.*, **17**, 262 (2014), https//doi.org/10.1016/J.EJBT.2014.08.002
[26] I. Afzal, A. A. Shah, Z. Makhdum, A. Hameed and F. Hasan, *Minerva Biotec.*, **24**, 101 (2012)
[27] N. Nema, L. Alamir and M. Mohammad, *Int. Food Res. J.*, **22**, 1831 (2015)
[28] F. Tabssum, M. Irfan, H. A. Shakir and J. I. Qazi, *J. Biol. Eng.*, **12**, (2018), https//doi.org/10.1186/S13036-018-0097-4
[29] T. Shankar, *Middle East J. Sci. Res.*, **8**, 40 (2011)
[30] A. K. Ray, *Acta Ichthyol. Piscat.*, **37**, 47 (2007), https//doi.org/10.3750/AIP2007.37.1.07
[31] K. A. Hassan, *Int. J. Sci. Environ. Technol.*, **4**, 1 (2015)
[32] F. Islam and N. Roy, *BMC Res. Notes*, **11** (2018), https//doi.org/10.1186/s13104-018-3558-4
[33] D. Deka, M. Jawed and A. Goyal, *Prep. Biochem. Biotechnol.*, **43**, 256 (2013), https//doi.org/10.1080/10826068.2012.719849
[34] S. Sharif, A. Jammu, K. Asad, H. Shah, K. A. Fariq *et al*., *ResearchSquare Preprints* (2023), https//doi.org/10.21203/rs.3.rs-2885168/v1
[35] S. Mohammadi, H. Tarrahimofrad, S. Arjmand, J. Zamani, K. Haghbeen *et al*., *Sci. Rep.*, **12**, 10301 (2022), https//doi.org/10.1038/s41598-022-14651-7
[36] M. Salehi, *Int. J. Biol. Macromol.*, **254**, 127782 (2024), https//doi.org/10.1016/j.ijbiomac.2023.127782
[37] M. Ghazanfar, M. Irfan, H. A. Shakir, M. Khan, M. Nadeem *et al*., *Cellulose Chem. Technol.*, **56**, 321 (2022), https//doi.org/10.35812/CelluloseChemTechnol.2022.56.28