Pak. J. Bot., 56(4): 1585-1591, 2024. DOI: http://dx.doi.org/10.30848/PJB2024-4(21)

RESPONSE SURFACE METHODOLOGY FOR ENHANCED CMCase AND BIOMASS PRODUCTION BY *BACILLUS LICHENIFORMIS* TLW-3

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Abstract

Bacillus licheniformis recognized as safe and has unique genetic characteristics that make it important for industrial enzyme production. cellulase enzyme demand has increased globally due to its various applications in different industrial setups. The current era of cellulase application is the bioconversion of agro-industrial wastes into valuable products, which are then fermented in to ethanol. Previously, the OVAT (one variable at a time) approach was used, but this method is unable to provide information regarding interaction effects from different factors. Response surface method (RSM) is provides a solution to study the interaction of multiple parameters using a small number of experiments. During projected research, the central composite design (CCD) was designed with Minitab software (version17) using six factors (concentration of carboxymethyl cellulose (CMC), peptone, and yeast extract, incubation time, pH, and temperature) that were determined by OVAT. A total of 53 experiments were designed and conducted. Results analysis was done in the same software. The obtained model was found to be good fit with non significant error, which indicates the model's fitness. According to the response and contour plots, good interactions were found among the studied factors. Finally, biomass production was found to be 110g/ml and enzyme yield was increased up to 640.86IU/ml/min using the RSM.

Key words: Statistical optimization, OVAT, Minitab, Contour plot, Experiment design.

Introduction

There has been an ever-increasing demand for bioenergy (globally) because of the due concern regarding fossil fuel scarcity, the necessity of economic sustainability, and a pollution-free environment. Biomass of cellulosic components is one of the most plentiful and inexpensive renewable biological resources around the globe (Afzal *et al*., 2019; Ghazanfar *et al.*, 2022). The process involving bioethanol and biochemical end products carries financial as well as technical issues; however, heat-stable CMCase may exhibit high capability to overcome these drawbacks (Miller and Blum, 2010; Bhalla *et al*., 2013). Thermostable cellulase hydrolyzing enzymes serve as perfect catalysts for such processes for the reason that enzymatic degradation usually works more efficiently at high temperatures (Paes *et al*., 2006). Endoglucanases constitute the enzymes of the cellulase complex that randomly cleave the internal bond (β-1, 4-glucosidic bond) in the cellulose complex (Singhal *et al*., 2022).

There are various factors that affect enzymatic degradation of cellulosic biomass, including enzyme and substrate activity and conditions of the reaction environment (incubation temperature, medium composition, pH, incubation time, etc.). These production conditions significantly influence the enzyme production cost, which is the main hurdle in hydrolytic processes (Kumar *et al*., 2022). For chemical and physical parameter screening, OVAT is generally a method of choice for enzymes production optimization in order to get their maximum yield. Yet, it is time-consuming because of excessive experimental trials and has data reproducibility issues due to the lack of interactive effects studies on enzyme production (Shajahan *et al*., 2017). Such and similar problems can be overcome by applying statistics-based analysis methods that were planned and employed for reliable and fast outputs. RSM is the statistical method applied to study the interactions of the independent variables (Nayak *et al*., 2023). *Bacillus licheniformis* is considered an industrially important microbial flora for enzyme production because it is a safe and genetically diverse strain. This strain is widely applied in the fermentation, food, and waste treatment industries. This study was aimed to improve the previously optimized (by OVAT approach) production conditions to get the highest CMCase yield by the *B. licheniformis* TLW-3 strain (Kiran *et al*., 2018).

Material and Methods

Strain and culture conditions: *B. licheniformis* TLW-3 strain (KY440432 accession number) was previously isolated by Kiran *et al*., 2015. Culture was maintained on slants (LB agar) as working stock, and 80% glycerol stock of culture was prepared for long term preservation.

B. licheniformis TLW-3 Growth and CMCase Production Optimization using RSM

Designing of the experiments: For the experimental design of growth and CMCase production optimization, six factors (incubation time, pH, temperature, concentration of CMC, yeast extract, and peptone) were selected through OVAT optimization (Kiran *et al*., 2018). These factors were chosen in the range of 5-9 pH, 30-70ºC temperature, 24-120hrs incubation period and 5-25g/l CMC, peptone, and yeast extract concentrations. These investigations were done at a distance of $\alpha = 2$ from the design center consisting of 12 replicates from the axial points and 9 from the central points. These levels were coded as: low level = -1 and -2, middle level = 0 and high level +1 and +2 (Table 1).

Table 1. Factors and their levels used in designing of experiments.

Factor	Name	Levels				
		-2	-1	0	+1	+2
X1	pH	5	6	7	8	9
X2	Temperature (°C)	30	40	50	60	70
X3	Incubation time (hours)	24	48	72	96	120
X4	Peptone (g/l)	5	10	15	20	25
X5	Yeast Extract (g/l)	5	10	15	20	25
X6	Substrate concentration (g/l)	5	10	15	20	25

The number of experiments were calculated using the following equation:

$$N= 2^k + 2k + Xo$$

where N= number of runs, k= total variables and Xo= central points (for this design k= 6 and Xo= 9). Thus, the 53 runs were suggested by software as represented in (Table 2).

Experiments: Experiments were conducted in 25ml media (composed as direct by design), and after inoculation, incubation was done under the conditions as per design (Table 2). After fermentation, centrifugation (for 20 min at 4°C and 11448xg) was done for the extraction of crude CMCase enzyme. Afterwards, enzyme assay was conducted and enzyme units were calculated (Kiran *et al*., 2018). After centrifugation, the biomass was determined in mg/ml in a pre-weighted microfuge tube (Asad *et al*., 2021).

Enzyme assay: an enzyme assay was conducted for enzyme activity quantification. For this purpose, 100µl of 1% w/v CMC citrate buffer (50mM) was added to the 100µl extract of CMCase. The mixture was kept at 50°C for the period of 30 min. After incubation, dinirosalicylic acid (300µl) was added. Then, boiling was carried out for 15 min. After cooling to room temperature, absorbance was measured using a spectrophotometer (at 540nm). One enzyme unit is defined as the amount of CMCase required to produce 1.0mg of glucose by CMC utilization at 50°C in 30min.

Analysis of the results

The results analysis was done using the Response Surface Regression method in order to fit the below mentioned full polynomial second-order equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_6 x_6 + \beta_{11} x_1 x_1 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{16} x_1 x_6 + \beta_{22} x_2 x_2 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{26} x_2 x_6 + \beta_{33} x_3 x_3 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{36} x_3 x_6 + \beta_{44} x_4 x_4 + \beta_{45} x_4 x_5 + \beta_{46} x_4 x_6 + \beta_{55} x_5 x_5 + \beta_{56} x_5 x_6 + \beta_{66} x_6 x_6$$

where Y represents response, β_0 shows intercept, linear coefficients are β_1, β_2, and squared coefficients are shown by β_3, β_{11}, β_{22}, and β_{33} while interaction coefficients are presented as β_{12}, β_{13}, and β_{23}. The validity of the model was confirmed by ANOVA (analysis of variance). The Fisher test (F-test) was also applied for the confirmation of model significance. The quality and adequacy of the quadratic equation were further verified statistically by the R2 (coefficient of determination) and the adjusted R2. The 3D contour plots and their relevant surface curves were plotted to study the relationship between the experimental levels (of each independent variable) and its response, i.e., enzyme and biomass production (Mousa *et al*., 2022).

Experiment validation: The enzyme unit and biomass production were validated by keeping the biomass and CMCase production to the conditions provided by the software.

Results and Discussions

The large-scale commercial production cost of the cellulase enzyme is very high. Hence, there is a need to search for new isolates that have the potential to produce a high quantity of cellulase enzyme cost-effectively (Dina *et al*., 2023). *Bacillus licheniformis* TLW-3 strain used in this study has a promising ability to produced the highest CMCase yield. Although, according to the reported results *B. licheniformis* has secure and safe utilization, the statistical growth optimization information is lacking. RSM is a successful exploratory method for investigating the combined effect of multiple parameters on enzyme and biomass production. The preliminary optimization (for highest CMCase and biomass production) of the strain *B. licheniformis* was performed through the OVAT approach (Kiran *et al*., 2018). Accordingly, six factors (mentioned above) were selected. Software Minitab 17 was used to prepare the CCD design to get the interactive response of selected factors on growth (biomass) and enzyme production. The run order was followed, and experiments were performed. Finally, the analysis of results was done using the above mention software.

The adequacy and fitness of the model were determined through ANOVA and Fisher's *F*-test. Results for CMCase and biomass production are presented in (Table 3a and 3b). Accordingly, the model was found to be significant with a 0.000 and 0.001 p value ($p<0.05$ at 95% confidence level) for CMCase and biomass production, respectively which shows the significance of the model. Previously, p= 0.127 and F= 2.99 lack of fit were reported by Singh *et al*., (2014) for the production of CMCase.

The value for the coefficient of determination, i.e., R^2 was 84.69% with 68.15% of R^2-adj hence showing that the 85% variability can be explained by this experimental design. Different values of R^2 and R^2-adj were reported (Shankar and Isaiarasu, 2012; Utharalakshmi *et al*., 2015).

The random line in the residual vs. predicted response and the straight line in the normal probability plot also confirmed that both models are significant (Fig. 1a and 1b).

Table 2. Randomized design table.

Run	Blk	X_1	X_2	X_3	X_4	X_5	X_6
1	1	0	0	0	0	-2	0
2	1	-1	1	1	-1	1	1
3	1	1	-1	1	-1	-1	-1
4	1	0	0	0	0	0	0
5	1	-1	-1	1	1	1	1
6	1	1	-1	-1	1	-1	-1
7	1	1	1	1	-1	1	-1
8	1	0	0	0	0	0	0
9	1	-1	1	1	1	-1	1
10	1	1	1	-1	1	-1	1
11	1	0	0	-2	0	0	0
12	1	-2	0	0	0	0	0
13	1	1	1	-1	-1	-1	-1
14	1	1	-1	-1	1	1	1
15	1	1	1	1	1	-1	-1
16	1	1	-1	-1	-1	-1	1
17	1	0	0	0	0	0	0
18	1	-1	1	-1	1	1	1
19	1	2	0	0	0	0	0
20	1	0	0	0	2	0	0
21	1	-1	-1	1	-1	-1	1
22	1	1	-1	1	1	1	-1
23	1	0	0	2	0	0	0
24	1	-1	1	-1	-1	1	-1
25	1	-1	-1	1	1	-1	-1
26	1	-1	-1	-1	-1	1	1
27	1	-1	1	1	1	1	-1
28	1	-1	1	-1	1	-1	-1
29	1	-1	1	1	-1	-1	-1
30	1	0	0	0	0	0	0
31	1	-1	-1	-1	-1	-1	-1
32	1	1	1	-1	-1	1	1
33	1	-1	-1	1	-1	1	-1
34	1	1	1	1	-1	-1	1
35	1	0	0	0	0	0	0
36	1	-1	1	-1	-1	-1	1
37	1	0	-2	0	0	0	0
38	1	0	0	0	0	2	0
39	1	1	1	-1	1	1	-1
40	1	1	-1	1	1	-1	1
41	1	0	2	0	0	0	0
42	1	-1	-1	-1	1	-1	1
43	1	0	0	0	0	0	0
44	1	0	0	0	0	0	0
45	1	1	-1	1	-1	1	1
46	1	-1	-1	-1	1	1	-1
47	1	0	0	0	0	0	2
48	1	0	0	0	-2	0	0
49	1	0	0	0	0	0	-2
50	1	0	0	0	0	0	0
51	1	0	0	0	0	0	0
52	1	1	-1	-1	-1	1	-1
53	1	1	1	1	1	1	1

The adequacy of the model was further confirmed by the "lack of fit" (F value) that was found to be 12.97 with 0.06 p values (Table 4a). Singh *et al*., (2014) reported the significance of the RSM model for CMCase production with F= 2.99 and p= 0.127 for lack of fit. The regression coefficient was determined through the student's t-test. The interaction, linear and square terms were found significant. Accordingly, CMC and incubation time are important for cell biomass (Table 4b) and CMCase production by *B. licheniformis* TLW-3. The significant square parameters were temperature and the concentration of yeast extract (relevant to the production of biomass) and incubation time, pH, CMC, and yeast extract (concentration) for enzyme synthesis. A clear interactive action for synthesis of biomass could be traced with pH*peptone, pH*CMC, temperature*CMC, and peptone*yeast extract, whereas hyperproduction of CMCase was found with the interaction of time and the CMC concentration.

The contour and surface plots provide quick and compatible source for obtaining the hyper response model (Fig 2a and 2b). Fifteen interactive graphs were plotted for each contour and surface plot (for both biomass and enzyme production). The circular contour helps clarify the absence of interaction, while an ellipse contour plot indicates an ideal connection among two independent parameters (Muralidhar *et al*., 2001). The given graphs help to interpret the optimization system to get the highest biomass, while also being effective in understanding the interaction and linear action of dual parameters simultaneously. Optimized figures regarding six parameters constituted 70ºC temperature, 9 pH, 5g/l peptone, 23.58g/l yeast extract and 25g/l concentration of the substrate after 49hrs of incubation for highest biomass.

Fig 3a and 3b demonstrate that the synthesis of CMCase was the best at 6.6 pH (nearly equal to the earlier pH value found in the analysis by OVAT). In contradiction to the data of the OVAT analysis (Kiran *et al*., 2018), RSM optimization led to the best enzyme output at 70ºC while the fermentation medium (with 6.6 pH) had incubation lasting 67.63hrs with 16.111g/l substrate value, 14.098g/l yeast extract and 18.13g/l peptone included in the medium. The deviation difference (therein) may be due to the lack of a specific interactive step during OVAT optimization.

Finally, the optimum conditions for maximum biomass and CMCase production obtained from the analysis are presented in (Table 5a and 5b). A value of 110mg/ml biomass was predicted was predicted by model, thereafter subjected to validation under optimized parameters. The experiment based synthesized biomass was 135.6mg/ml and that shows a significance relation to the predicted response. The predicted unit for enzyme synthesis came out to be 607.0349IU/ml/min, whereas 640IU/ml/min was observed, which is quite closer. During optimized maximum recovery production, *Bacillus* sp. and *Geobacillus* sp. with 0.12U/ml and 0.074U/ml in corresponding order were reported (Rastogi *et al*., 2010). As per another investigation *Bacillus pumilus* EB3 and *Bacillus megaterium* produced 0.076U/ml and 0.102U/ml units of cellulase enzyme after optimization through RSM (Beukes & Pletschke, 2006; Ariffin *et al*., 2008).

Table 3a. ANOVA of the model for enzyme production (IU/ml/min).

Source	Sum of squares	df	Mean square	F-value	P-value	*p*<0.01
Model	165062	27	62039	5.12	<0.000	Significant
A-pH	21217	1	21217	1.75	0.198	
B- Temperature	1022	1	1022	0.08	0.774	
C-Time	134474	1	134474	11.10	<0.003	Significant
D-Peptone	18342	1	18342	1.51	0.230	
E-Yeast Extract	13490	1	13490	1.11	0.301	
F-CMC	35704	1	35704	2.95	0.098	
AB	3452	1	3452	0.28	0.598	
AC	4437	1	4437	0.37	0.551	
AD	8914	1	8914	0.74	0.399	
AE	20949	1	20949	1.73	0.200	
AF	11464	1	11464	0.95	0.340	
BC	8563	1	8563	0.71	0.409	
BD	858	1	858	0.07	0.792	
BE	1294	1	1294	0.11	0.747	
BF	53	1	53	0.00	0.948	
CD	18940	1	18940	1.56	0223	
CE	13663	1	13663	1.13	0.298	
CF	14969	1	14969	1.24	<0.007	Significant
DE	27323	1	27323	2.25	0.146	
DF	7126	1	7126	0.59	0.450	
EF	2081	1	2081	0.17	0.682	
AA	357372	1	357372	29.49	<0.000	Significant
BB	93	1	93	0.01	0.931	
CC	353330	1	353330	29.16	<0.000	Significant
DD	42265	1	42265	3.49	0.074	
EE	167033	1	167033	13.79	<0.001	Significant
FF	168495	1	168495	13.91	<0.001	Significant
Residual	302920	25	12117			
Lack of Fit	292263	17	17192	12.91	<0.06	Insignificant
Pure Error	10657	8	1332			
Corrected Total	1977981	52				

** Significant at p<0.05; DF= Degree of freedom

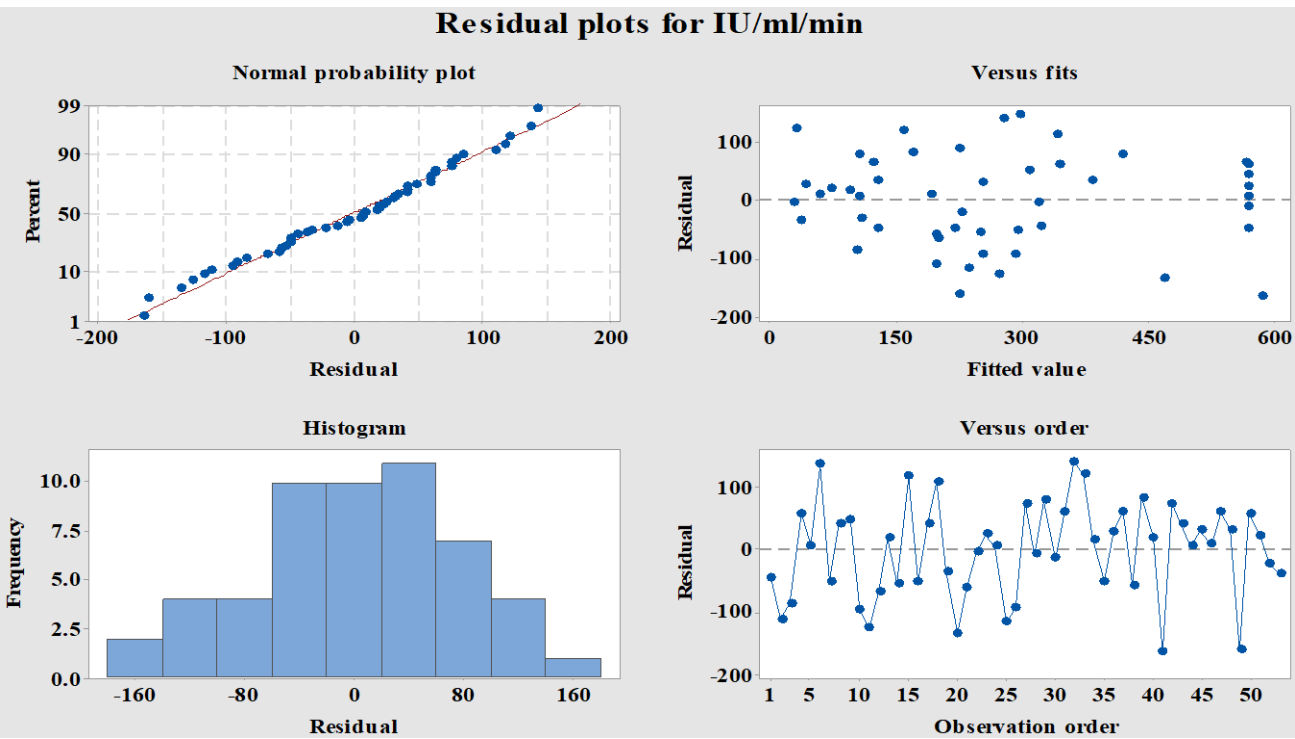

Fig. 1a. Residual plots of the response CMCase Production IU/ml/min.

Table 3b. ANOVA of the response surface model for *B. licheniformis* TLW-3 growth (biomass mg/ml).

Source	Sum of squares	df	Mean square	F-value	P-value	$p<0.01$
Model	18307.8	27	678.07	3.49	<0.001	Significant
A-pH	13.2	1	13.23	0.07	0.796	
B- Temperature	756.90	1	756.90	3.89	0.060	
C-Time	143.6	1	143.64	0.74	0.398	
D-Peptone	261.1	1	261.12	1.34	0.257	
E-Yeast Extract	1.5	1	1.52	0.01	0.930	
F-CMC	900.60	1	900.60	4.63	<0.041	Significant
AB	371.3	1	371.28	1.91	0.179	
AC	52.5	1	52.53	0.27	0.608	
AD	1439.2	1	143.92	7.40	<0.012	Significant
AE	19.5	1	19.53	0.10	0.754	
AF	2261.3	1	2261.28	11.63	<0.002	Significant
BC	10.4	1	10.35	0.05	0.819	
BD	3.0	1	3.00	0.02	0.902	
BE	31.6	1	31.60	0.16	0.690	
BF	1455.3	1	1455.30	7.48	<0.011	Significant
CD	12.3	1	12.25	0.06	0.804	
CE	562.8	1	562.80	2.89	0.101	
CF	27.8	1	27.75	0.10	0.709	
DE	892.5	1	892.53	4.59	0.042	Significant
DF	457.5	1	457.53	2.35	0.138	
EF	0.0	1	0.01	0.00	0.994	
AA	78.0	1	77.96	0.40	0.532	
BB	6562.8	1	6562.80	33.75	0.000	Significant
CC	668.3	1	668.33	3.44	0.076	
DD	706.2	1	706.20	3.63	0.068	
EE	955.4	1	955.43	4.91	0.036	Significant
FF	35.3	1	35.28	0.18	0.674	
Residual	4861.6	25	194.47			
Lack of Fit	3721.6	17	218.92	1.54	0.274	Insignificant
Pure Error	1140.0	8	142.50			
Corrected Total	23169.4	52				

** Significant at $p<0.05$; df= Degree of freedom

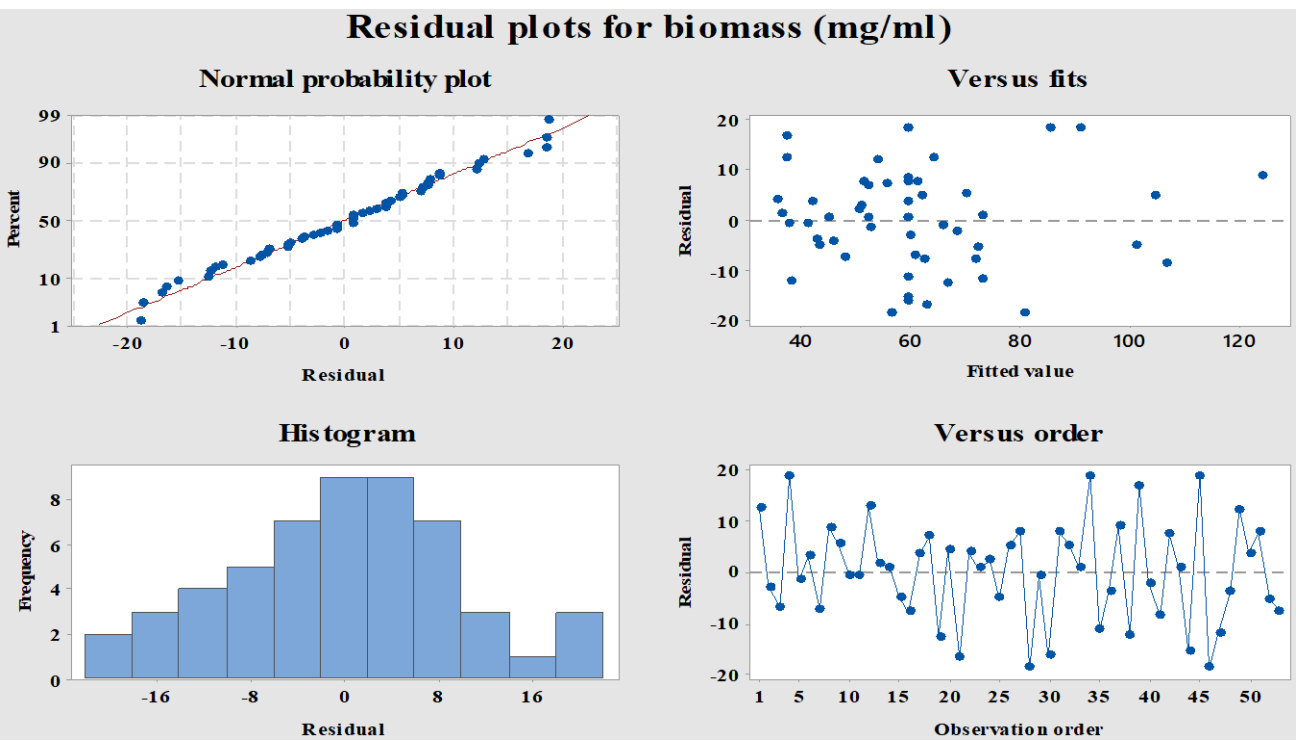

Fig. 1b. Residual plots of the response 'biomass mg/ml.

Table 4a. ANOVA for CMCase production.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	27	1675062	1675062	62039	5.12	0.000
Linear	6	224249	529654	88276	7.29	0.000
Square	6	1306728	1306728	217788	17.97	0.000
Interaction	15	144085	144085	9606	0.79	0.675
Residual error	25	302920	302920	12117		
Lack of Fit	17	292263	292263	17192	12.91	0.06
Pure error	8	10657	10657	1332		
Total	52	1977981				

*Statistically based significant at α=0.01

Table 4b. ANOVA for biomass (mg/ml).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	27	18308	18308	678.1	3.49	0.001
Linear	6	2077	12418	2069.7	10.64	0.000
Square	6	8634	8634	1439.0	7.40	0.000
Interaction	15	7597	7597	506.5	2.60	0.017
Residual error	25	4862	4862	194.5		
Lack of Fit	17	3722	3722	218.9	1.54	0.274
Pure error	8	1140	1140	142.5		
Total	52	23169				

*Statistical based significant at α=0.01

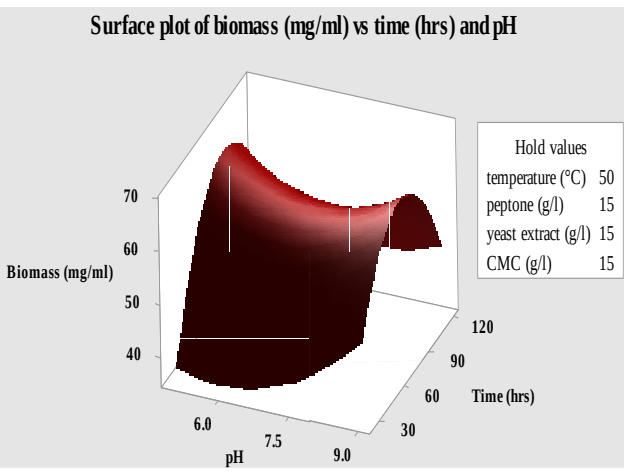

Fig. 2a. Response surface plot for time and pH interaction effect on biomass production.

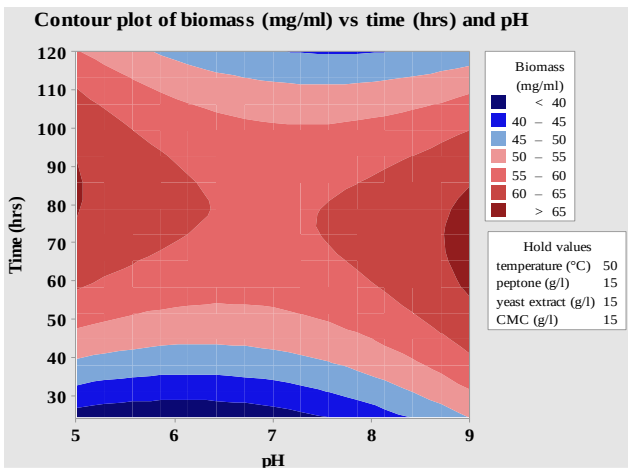

Fig. 2b. Contour plot for time and pH interaction effect on biomass production.

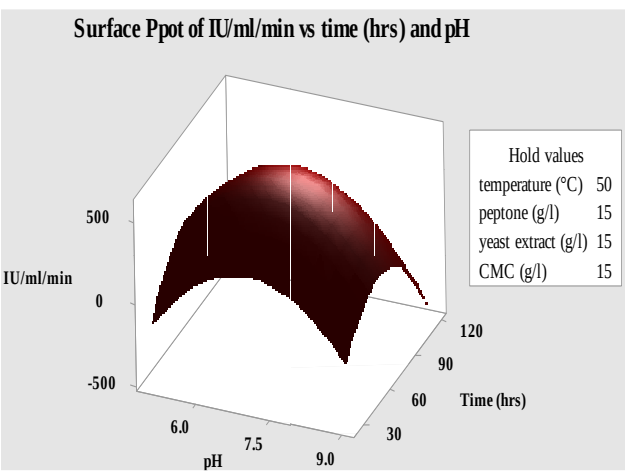

Fig. 3a. Response surface plot for time and pH interaction effect on enzyme yield.

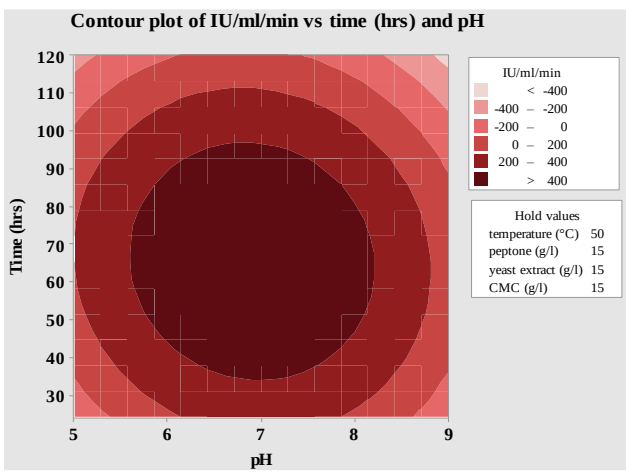

Fig. 3b. Contour plot for time and pH interaction effect on enzyme yield.

Table 5a. Predicted response for biomass (mg/ml).

pH	Temperature (˚C)	Time (hrs)	Peptone (g/l)	Yeast extract (g/l)	CMC (g/l)	Predicted response (mg/ml)	Actual response (mg/ml)
9	70	49.212	5.0	23.58	25.0	110	135.6

Table 5b. Predicted response for CMCase production (IU/ml/min).

pH	Temperature (˚C)	Time (hrs)	Peptone (g/l)	Yeast extract (g/l)	CMC (g/l)	Predicted response (IU/ml/min)	Actual response (IU/ml/min)
6.6	70	67.63	18.1313	14.090	16.11	607.0347	640.86

Conclusion

The high production cost of cellulase enzyme is limits its application in industrial level. In this study the newly isolated strain *B. licheniformis* TLW-3 found to have the tremendous ability to produce high level of thermostable cellulase enzyme at the conditions optimized through RSM. In future, the CMCase production will be tried to increase in liters (using the optimized conditions). Different biodegradation and industrial application of CMCase will be carried out. Purification, activity and stability characterization can also be performed.

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(Received for publication 22 February 2023)