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Comparative Study on Cellulase Production and Optimization by *Bacillus species* and *Aspergillus niger* Using Wheat Bran Under Solid-State Fermentation.

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ABSTRACT

Microbial cellulases are extremely adaptable catalysts that have a wide range of applications in the food and animal feed, brewing, paper and pulp, textile, laundry, biofuel, and agricultural industries. Because of the complexity of enzyme systems and their wide range of commercial applications, cellulases have garnered a great deal of interest from the scientific community. In this work, we examine how well *Aspergillus niger*, *Bacillus subtilis*, and *Bacillus cerious* can create and optimize cellulases following the use of Central Composite Design. The variables under investigation for optimum cellulase production in solid-state fermentation settings are substrate, pH, and incubation temperature. At pH 4.32, temperature 33, and substrate 12, *Aspergillus niger* produced the greatest cellulose activity of 124.48 (compared to the expected cellulase activity of 109.01 U/ml). On the other hand the lowest cellulose activity was 24.08 U/ml (predicted cellulase activity, 25.34 U/ml) obtained at pH 6, temperature 41 and substrate 12. The highest cellulose activity produced by *Bacillus subtilis* was 95.64 (predicted cellulase activity, 91.01 U/ml) obtained at pH 9, temperature 50 and substrate 7.96. On the other hand the lowest cellulose activity was 28.68 U/ml (predicted cellulase activity, 26.88 U/ml) obtained at pH 10.86, temperature 50 and substrate 12. The highest cellulose activity produced by *Bacillus cerious* was 88.08 U/ml (predicted cellulase activity, 91.61 U/ml) obtained at pH 9, temperature 50 and substrate 7.96. On the other hand the lowest cellulose activity was 33.96 U/ml (predicted cellulase activity, 39.01 U/ml) obtained at pH 7.32, temperature 50 and substrate 12. The application of CCD and the comparison study of this work revealed that the highest cellulose activity was produced by *Aspergillus niger* (124.48 U/ml) followed by *Bacillus subtilis* (95.64 U/ml) and finally *Bacillus cerious* (88.08 U/ml). *Aspergillus niger* is the most reliable for producing higher amount of cellulase than *Bacillus* species. The results of this work also offer a different method for making use of agricultural waste and a way to efficiently produce cellulase for the breakdown of lignocellulosic materials, both of which have positive consequences for sustainable waste management. In conclusion *Aspergillus niger* is the most reliable for

producing higher amount of cellulase than *Bacillus* species. Furthermore, this study's results offer a different way to use agricultural waste and a method for effectively producing cellulase, which is needed for the breakdown of lignocellulosic materials. These discoveries have a chance to improve sustainable waste management.

Keywords: *Aspergillus niger*, *Bacillus* species, Cellulase, and Wheat bran.

INTRODUCTION

In recent years, there has been a notable increase in interest about the use of efficient and sustainable operation. Biochemical catalysts replaced chemical catalysts in this evolution, and a number of enzymes have been discovered and used in industrial and biotechnological operations. As a result, every year there is a rise in both research and enzyme manufacturing. About 70% of them refer to the group of enzymes known as proteases and carbohydrases, which are where cellulases are classified (Arbige et al., 2019). Due to the wide range of industries in which these enzymes are used, particularly the need for clean, renewable energy sources like biofuel production, there is a significant demand for these enzymes. The most often used enzyme for treating lignocellulosic biomass is cellulase (Koupaie et al., 2019). Food, detergents, beverages (juices, wines, and beers), paper goods, jeans, medicines, fine chemicals, biofuels (ethanol, biogas), wastewater treatment, and bioremediation are among the industries that can use this enzyme (Shah et al., 2019).

Microorganisms, specifically fungus and bacteria, are capable of producing cellulase; in particular, the demand for bacterial cellulase is increasing due to its diverse range of environments and rapid development rate. Cellulase-producing bacteria have been isolated for a number of years from a variety of sources, including soils, plant debris, waste from the sugar industry, animal feces, and gut microbiota (Doi, 2008, Islam and Roy, 2018, Nandy et al., 2021). Furthermore, because the cellulose substrate is soluble and promotes the growth of microorganisms, submerged cultures have been used in a number of investigations on cellulolytic microbes. Nonetheless, there are numerous benefits to the solid-state bioprocess (SSF), including reduced costs, direct substrate-microorganism contact, and increased product yield (Bentil et al., 2018, Manan et al., 2018). Further, among microorganisms, *Bacillus* sp. was widely mentioned as a cellulose producing bacteria (Cross et al., 2019).

The majority of cellulases are produced by fungi, and companies have utilized the *Aspergillus niger* species because of its effectiveness in secreting cellulolytic enzymes (Zhang et al., 2017). Factors that affect the synthesis of cellulase include the kind of strain, the culture conditions, the substrate's composition, and the availability of nutrients in the medium (Sandhu et al., 2013). The activity of these enzymes is influenced by the pH and moisture content of the culture medium. While moisture is linked to microbial growth and secondary metabolism, pH impacts the charge and permeability of the cell membrane, preventing the microbe from secreting cellulose (Xu et al., 2018). At the moment, the primary challenges to be solved are the high cost of commercial enzymes and their limited stability and efficiency. According to Liu et al. (2016), reducing the amount of enzymatic loads used or producing locally (both inside the company and on a big scale) would be an alternative to raise costs. Long-term transportation and storage costs may be lowered by the

industry's own synthesis of these enzymes. The goal of the current investigation was to compare the maximum amounts of cellulase produced during SSF fermentation utilizing wheat bran as a substrate by *B. strains* and *Asperigillus niger*. Utilizing various factors, the central composite experimental design was carried out to maximize the production of cellulase and biomass.

MATERIAL AND METHODS

Isolation and identification of Bacillus strains and Asperigillus niger:

The isolation and identification of *Bacillus strains* and *Asperigillus niger* was previously done (Hanna Allam et al., 2024).

Fermentation experiment

Multiple fermentation experiments were conducted utilizing the shake-flask technique. A 10-milliliter of the inoculum was used to begin growth in 250-milliliter Erlenmeyer flasks, each containing 100 milliliters of Mandels fermentation media. Following sterilizing at 121°C for 15 minutes, and pH 4.8. The manufacture of enzymes was prepared for 5 days at 35°C. Samples were then collected daily, then came centrifugation at a force of 10,000 g for 10 minutes at 4°C. The subsequent supernatants were then examined to determine the cellulose activity and reducing sugar content. Multiple fermentation experiments were conducted utilizing the shake-flask technique. A 10-milliliter of the inoculum was used to begin growth in 250-milliliter Erlenmeyer flasks that were filled with 100 milliliters of Mandels fermentation media. Following sterilizing at 121°C for 15 minutes, and pH 4.8. The production of enzymes was sustained for a period of five consecutive days at 35°C. On a daily basis, samples were extracted, subjected to centrifugation at 51,000 g for 10 minutes at 4°C. The resulting supernatants were then examined to determine the levels of cellulase activity and the content of reducing sugars.

The cellulase assay involved the combination of 1 ml of a 1% Carboxymethyl cellulose solution, produced in a 50 mM sodium acetate buffer at pH 5.3, with 1 ml of a crude extracellular enzyme source. This mixture was then incubated at 50°C for 15 minutes as described by Casimir et al. (1996). Three milliliters of 3, 5-dinitrosalicylic acid (DNS) were added to stop the reaction, and the liquid was then brought to a boil for five minutes. The spectrophotometer was used to measure the color generated, namely at a wave length of 540 nm. Using glucose as a reference, the quantification of reduced sugar released was carried out. Under the given assay conditions, the amount of enzyme required to release 0.1 μM of glucose from CMC every minute was determined as the unit (U) of enzyme activity. According to Miller's 1959 description, cellulase activity was measured and expressed in units per milligram. Using the Folin-Ciocalteu reagent and the Bovine Serum Albumin (BSA) standard, the protein content of the culture filtrate was also ascertained. as described by Lowry et al. (1951).

Partial purification of enzymes

Partially purified enzymes (U/ml) were obtained by submerging the crude enzymes in solid ammonium sulfate at 60% saturation and stirring continuously at room temperature. Centrifugation was used to collect the precipitate for 20 minutes at 4°C at 12,000 g. After dissolving the precipitate in 10 mM sodium phosphate buffer (pH 7.5), the mixture was dialyzed for 24 hours, with three changes made every 8 hours. Following lyophilization, the dialyzed enzyme was concentrated and kept at -20°C (Shanmugapriya1 et al., 2012).

Central Composite Design (CCD)

The experiment was extended to a CCD based on the PBD's findings. Moisture

content (X1), starting pH (X2), and ammonium sulfate concentration (X3) were found to be significant parameters from PBD and were selected as the key variables. These factors were recommended at five different levels, with the values assigned the codes -2, -1, 0, +1, and +2. Twelve trials with three factors are part of the design (Tables 1 & 2). A second order polynomial function was fitted to link the relationship between the independent factors and the PG activity response in order to estimate the best location (ElMekawy et al., 2013; Granato and de AraújoCalado, 2014).

Table (1): Central composite design for *Asperigillus niger:*

CCD	Substrate concentration	X1	%	7.96	9.6	12	14.4	16.04	2	-1	0	1	2
	pH	X2	-	4.32	5	6	7	7.68	2	-1	0	1	2
	temperature	X3	°C	24.59	28	33	38	41.41	2	-1	0	1	2

Table (2): Central composite design for *Bacillus* species:

CCD	Substrate concentration	X1	%	7.96	9.6	12	14.4	16.04	-2	-1	0	1	2
	pH	X2	-	7.32	8	9	10	10.68	-2	-1	0	1	2
	temperature	X3	°C	33.18	40	50	60	66.41	-2	-1	0	1	2

Optimization of Enzyme Production Parameters: *(Acharya et al., 2008).*

Effect of pretreatment of wheat bran with NaOH: Wheat bran was subjected to alkaline pretreatment using varying concentrations of NaOH. A solution of NaOH with a concentration of 1 N was produced. In this experimental process, a quantity of 100 grams of wheat bran was combined and subjected to incubation at ambient temperature for 12 hours. The aforementioned solution was subjected to a water wash in order to achieve neutralization. The pretreated wheat bran was held at 4°C within a refrigeration unit and afterwards employed as a carbon source within the fermentation media.

Effect of substrate concentration: Various quantities of substrate (wheat bran) were utilized in this study, specifically 4.8%, 7.2%, and 9.6% based on wet weight conditions. These concentrations were added to 250 ml Erlenmeyer flasks, each holding 100 ml of fermentation media.

Effect of pH: The optimization process involved and adjusted the pH levels inside the wheat bran fermentation media, specifically targeting values of 5.0, 6.0, and 7.0. The pH of the media was modified by employing 1 N hydrochloric acid (HCl) or 1 N sodium hydroxide (NaOH).

Effect of temperature: By allowing the fermentation medium containing wheat bran to incubate at three distinct temperatures: 30, 35, and 40°C, the impact of temperature was assessed. An orbital shaker incubator running at 120 rotations per minute was used for this procedure. Periodically, an enzyme assay was carried out.

RESULTS AND DISCUSSION

The results of experimental and theoretical values of cellulase activity by *Aspergillus niger* produced under three variables using CMC as substrate through central composite experimental design is illustrated in table (3) and

figures (1,2 & 3). The Anova analysis for the results is showed in table (4). The highest cellulose activity was 124.48 (predicted cellulase activity, 109.01) obtained at pH 4.32, temperature 33 and substrate 12. On the other hand the lowest cellulose activity was 24.08 (predicted cellulase activity, 25.34) obtained at pH 6, temperature 41 and substrate 12.

The pH medium has a significant impact on both the fungus's rate of development and the synthesis of enzymes (U/ml). According to this study, *Aspergillus niger* is most active in an acidic medium with a pH of 6 which are similar to (Sivaramanan, 2014 & Santos et al., 2022). According to Priyanka et al. (2017), the ideal pH value for increased cellulase enzyme activity was 7.0. Additionally, Das et al. (2011) noted that when the pH value was less than 7, the fungus's development decreased. Despite the fact that *A. niger* works in a broad pH range, a number of research indicate that, depending on the growth conditions, pH 5.0–6.0 is the ideal range for cellulase production (Saravanan et al. 2012). The optimum temperature for maximum cellulose production recorded in this study is in agreement with El-Sesy and Aly (2021) also Das et al. (2011) who recorded that the fungal strains exhibited maximum activity at 30 °C and declined when the incubation temperature rose above 37 °C.

In the present study cellulase activity (U/ml) by *Bacillus subtilis* produced under three variables using CMC as substrate through central composite experimental design is illustrated in table (4) and figures (4, 5 & 6). The Anova analysis for the results is showed in table (5). The highest cellulose activity was 95.64 U/ml (predicted cellulase activity, 91.01 U/ml) obtained at pH 9, temperature 50 and substrate 7.96 U/ml. On the other hand the lowest cellulose activity was 28.68 U/ml (predicted cellulase activity, 26.88 U/ml) obtained at pH 10.86, temperature 50 and substrate 12.

Similarly cellulase activity by *Bacillus cerious* produced under three variables using CMC as substrate through central composite experimental design is illustrated in table (6) and figures (7, 8 & 9). The Anova analysis for the results is showed in table (7). The highest cellulose activity was 88.08 (predicted cellulase activity, 91.61 U/ml obtained at pH 9, temperature 50 and substrate 7.96. On the other hand the lowest cellulose activity was 33.96 (predicted cellulase activity, 39.01 U/ml) obtained at pH 7.32, temperature 50 and substrate 12.

The measurement of temperature is a critical factor that significantly influences the outcome of a fermentation process. Temperature is an essential environmental factor that manages the growth and synthesis of metabolites in microorganisms, exhibiting variability among different species (Abou-Taleb et al., 2008). The growth rate of microorganisms is hindered when exposed to temperatures below or above their optimal development temperature, resulting in a decreased rate of cellular synthesis. In the event that the growth temperature exceeds optimal levels without reaching fatal thresholds, it is possible for an untimely initiation of target protein expression. The optimal temperature for cellulose synthesis by *B. subtilis* CY5 and *B. circulans* TP3 was determined to be 40°C. The results of this study indicate that a temperature of 40°C was the ideal condition for the growth of

both *Bacillus cereus* and *Bacillus subtilis*. The findings align with the study conducted by Kumar et al. (2017). The commencement of a decrease in production was observed subsequent to a subsequent rise in temperature. In their study, Immanuel et al. (2006) observed that *Cellulomonas*, *Bacillus*, and *Micrococcus* sp. exhibited the highest levels of endoglucanase activity at a temperature of 40°C. In contrast, Bouzaiene et al. (2023) reported that the optimal temperature for cellulose production by *Bacillus amyloliquefacians* D1B3 was 36.99°C, whereas Islam et al. (2019) found it to be 35°C.

The highest level of enzyme synthesis was reported at a temperature of 35°C. The production of cellulase showed an important reduction as the incubation temperature exceeded 40°C (Islam et al., 2019). The optimal temperatures for cellulase synthesis by *Bacillus* spp. exhibit considerable variability. The optimal temperature range for *Bacillus alcalophilus* S34, as reported by Ray et al. (2007), is 30°C to 40°C. Similarly, *Bacillus subtilis*, *Bacillus circulans*, and other *Bacillus* isolates, as studied by Ariffin et al. (2006), recorded that the optimal temperature range of 40°C to 50°C. Lastly, *Bacillus amyloliquefaciens*, as investigated by Immanuel et al. (2006), has an optimal temperature range of 40°C to 50°C. Upon reaching a temperature of 430°C, There was a noticeable decrease in the activity of the enzyme. The pH level is a critical factor that exerts an influence on both enzyme activity and production, as highlighted by Odeniyi et al. (2009). The current investigation observed that the highest level of cellulase activity was achieved at a pH value of 7.0 for both *Bacillus cereus* and *Bacillus subtilis*. These findings are consistent with those reported by Ray et al. (2006). According to a study conducted by Immanuel et al. (2006), it was shown that the cellulolytic enzyme known as endogluconase, derived from *Cellulomonas*, *Bacillus*, and *Micrococcus* spp. obtained from estuarine coir netting effluents, exhibits substrate hydrolysis throughout the pH range of 4 to 9. The enzyme demonstrates its highest level of activity at a pH of 7. The enzyme exhibited stability throughout a broad pH spectrum (6 to 8), while demonstrating its highest level of activity at pH 7. In a study conducted by Bouzaiene et al. (2023), it was shown that a pH level of 6.2 resulted in the highest cellulose production by *Bacillus amyloliquefacians* D1B3. Similarly, Kumar et al. (2017) reported that a pH level of 6 was optimal for maximum cellulose production by *Bacillus subtilis*.

Using CCD, the comparison analysis of this work showed that *Aspergillus niger* (124.48 U/ml) produced the maximum cellulose activity, followed by *Bacillus subtilis* (95.64 U/ml) and *Bacillus cerious* (88.08 U/ml) as present in table (8). These results are confirmed by table (9) as a relation between the total volume specific activity and purification fold. Such results are parallel with Gislaine et al. (2022) and Antika Boondaeng et al., (2024) in respect that *Aspergillus niger* is the most common and efficient organism for higher cellulose production.

It can be concluded that *Aspergillus niger* is the most reliable for producing higher amount of cellulase than Bacillus species. In additionWith potential advantages for sustainable waste management, the study's conclusions offer a different method for making use of agricultural waste as well as a way to effectively create cellulase for the degradation of lignocellulosic materials.

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Table (3): Experimental and theoretical values of cellulase activity by *Aspergillus niger* produced under three variables using CMC as substrate through central composite experimental design

pattern	pH	Temperature	Substrate	cellulase activity	predicted cellulase activity
a00	4.32	33	12	124.48	109.01
−−−	5	28	9.6	65.48	64.15
−−+	5	28	14.4	71.04	69.54
−+−	5	38	9.6	53.16	51.09
−++	5	38	14.4	79.8	75.52
0a0	6	24.59	12	33.52	31.03
00a	6	33	7.96	43.32	41.01
0	6	33	12	111.56	112.35
0	6	33	12	111.76	112.35
00A	6	33	16.04	65.56	60.63
0A0	6	41.41	12	24.08	25.34
+−−	7	28	9.6	39.92	40.53
+−+	7	28	14.4	40.04	39.44
++−	7	38	9.6	29.96	27.79
+++	7	38	14.4	43.08	45.74
A00	7.68	33	12	65.88	66.88

Table (4): ANOVA for cellulase production by *Aspergillus niger* using CMC as substrate

Source	Degree of freedom	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	14213.21	1579.25	112.0827	<0.0001*
Error	6	84.54	14.09		
Total	15	14297.75			

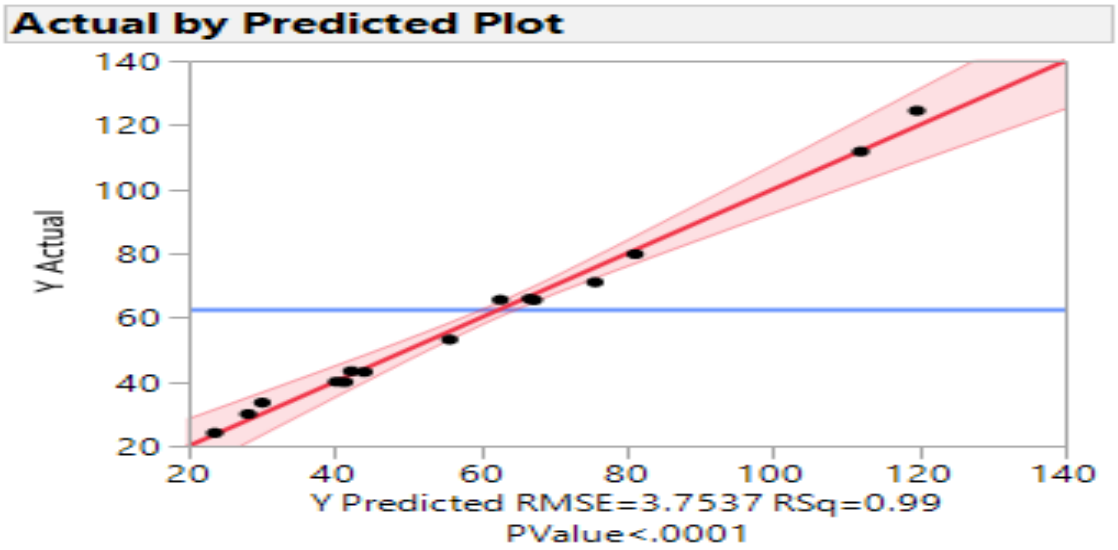


Figure (1): Experimental values versus predicted values for cellulase production by *Aspergillus niger* under three variables using CMC as substrate through central composite experimental design

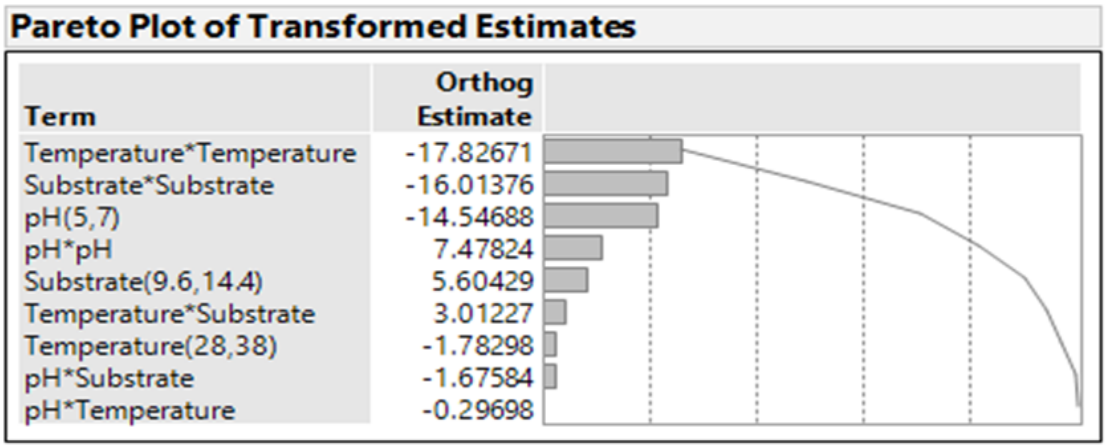
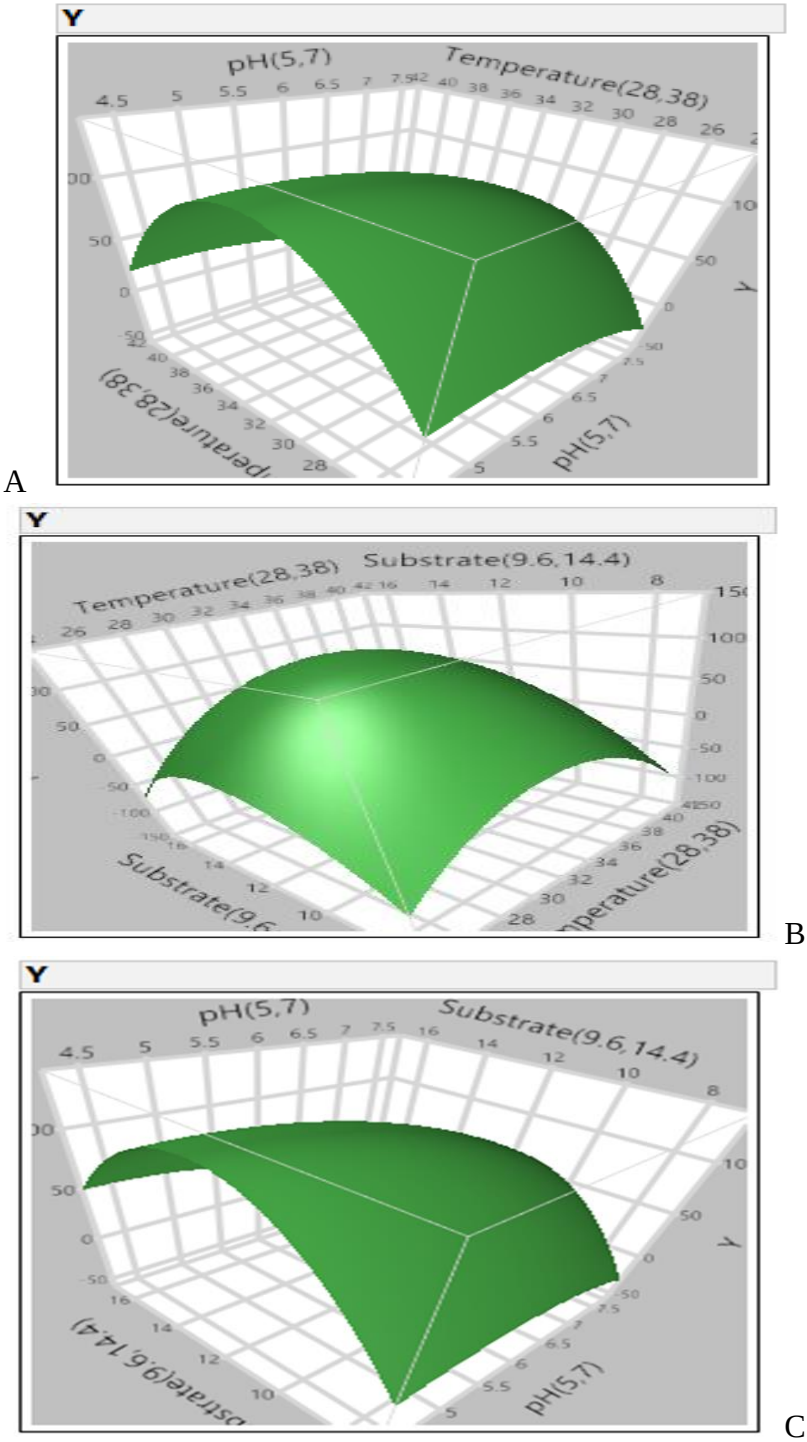


Figure (2): Pareto chart rationalizing the effect of each variable on cellulase production by *Aspergillus niger* under three variables using CMC as substrate through central composite experimental design. The vertical line defines the 98% confidence interval.



A

B

C

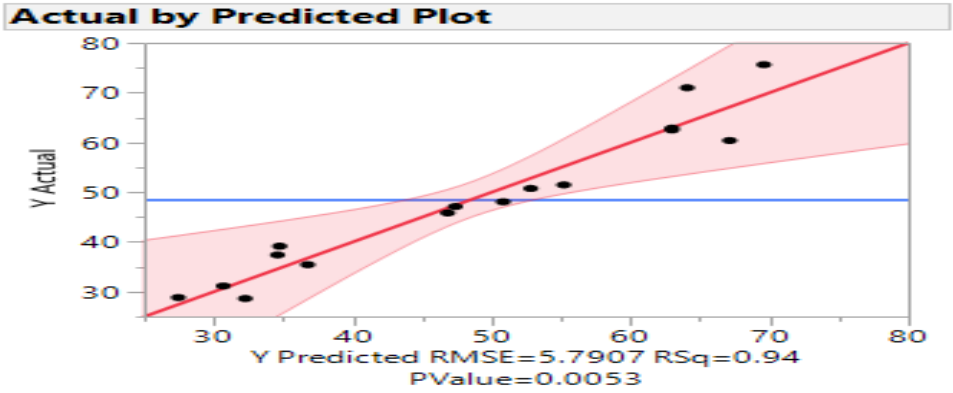
Figure (3): Respone surface plot showing the effect of pH and temperature (A), substrate and temperature (B), pH and substrate (C) on the production of cellulase by *Aspergillus niger* under three variables using CMC as substrate, other variables are held at zero level.

Table (4): Experimental and theoretical values of cellulase activity by *Bacillus subtilis* produced under three variables using CMC as substrate through central composite experimental design

pattern	pH	Temperature	Substrate	cellulase activity	predicted cellulase activity
a00	7.32	50	12	71	79.01
−−−	8	40	9.6	51.48	54.15
−−+	8	40	14.4	50.76	62.54
−+−	8	60	9.6	60.4	61.09
−++	8	60	14.4	45.84	45.52
0a0	9	33.18	12	31.16	31.03
00a	9	50	7.96	95.64	91.01
0	9	50	12	62.88	62.35
0	9	50	12	62.52	62.35
00A	9	50	16.04	48.08	46.63
0A0	9	66.82	12	37.4	35.34
+−−	10	40	9.6	35.44	40.53
+−+	10	40	14.4	39.16	39.44
++−	10	60	9.6	47.12	47.79
+++	10	60	14.4	28.84	25.74
A00	10.68	50	12	28.64	26.88

Table (5): ANOVA for cellulase production by *Bacillus subtilis* using CMC as substrate

Source	Degree of freedom	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	3072.35	341.372	10.1807	0.0053*
Error	6	201.19	33.32		
Total	15	3273.54			



Figure(4): Experimental values versus predicted values for cellulase production by *Bacillus subtilis* under three variables using CMC as substrate through central composite experimental design

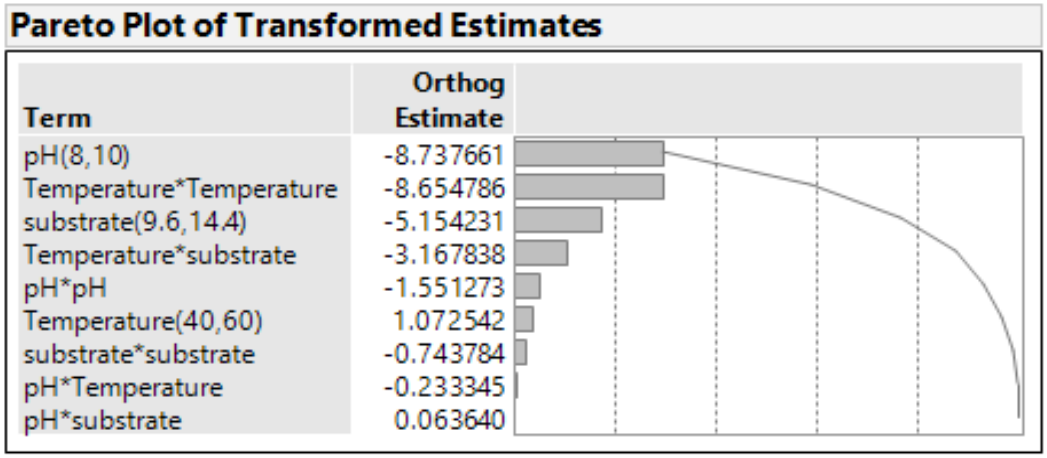


Figure (5): Pareto chart rationalizing the effect of each variable on cellulase production by Bacillus subtilis under three variables using CMC as substrate through central composite experimental design. The vertical line defines the 93% confidence interval.

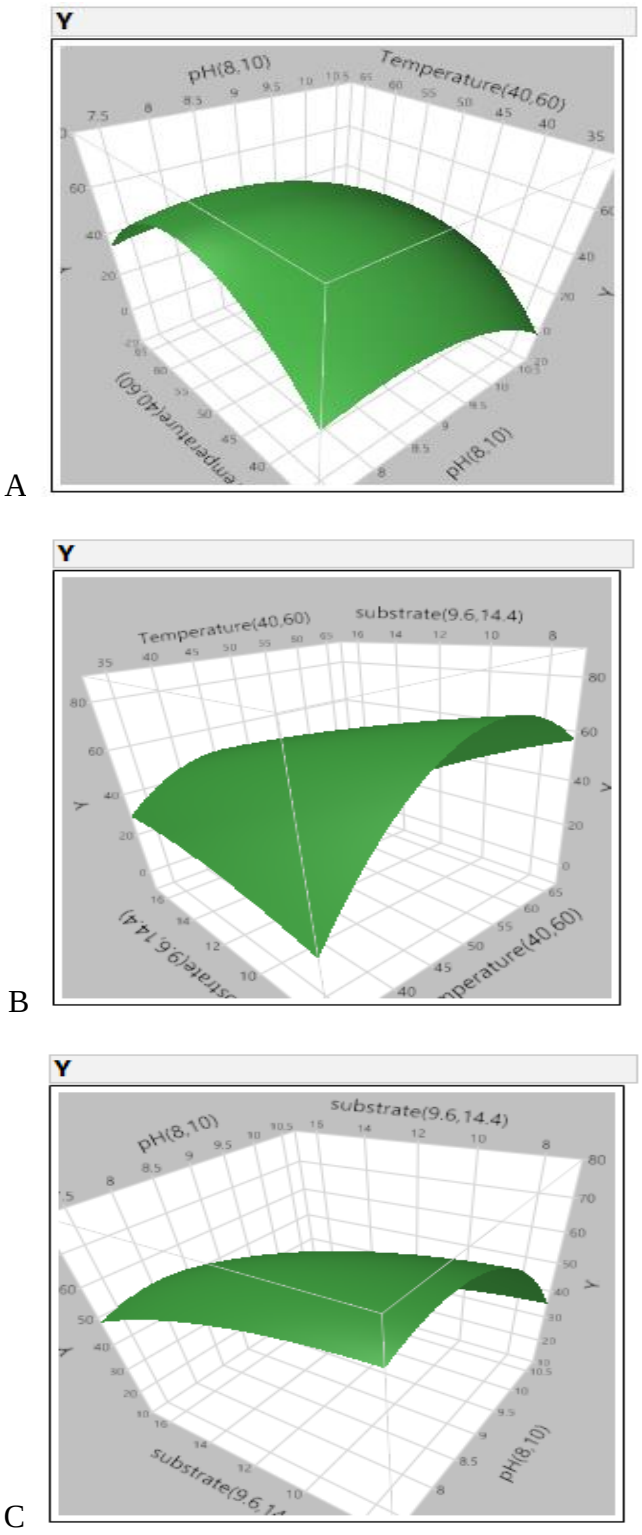


Figure (6): Respone surface plot showing the effect of pH and temperature (A), substrate and temperature (B), pH and substrate (C) on the production of cellulase by *Bacillus subtilis* under three variables using CMC as substrate, other variables are held at zero level.

Table (6): Experimental and theoretical values of cellulase activity by *Bacillus cerces* produced under three variables using CMC as substrate through central composite experimental design.

pattern	pH	Temperature	Substrate	cellulase activity	predicted cellulase activity
a00	7.32	50	12	33.96	39.01
−−−	8	40	9.6	56.12	54.15
−−+	8	40	14.4	31.56	32.54
−+−	8	60	9.6	47.68	51.09
−++	8	60	14.4	40.84	42.52
0a0	9	33.18	12	50.96	51.03
00a	9	50	7.96	88.08	91.61
0	9	50	12	49.96	52.35
0	9	50	12	48.88	53.35
00A	9	50	16.04	60.84	60.63
0A0	9	66.82	12	37	35.34
+−−	10	40	9.6	57.88	54.53
+−+	10	40	14.4	49.36	49.44
++−	10	60	9.6	50.2	47.79
+++	10	60	14.4	45.52	45.74
A00	10.68	50	12	39.52	40.88

Table (7): ANOVA for cellulase production by *Bacillus cerces* using CMC as substrate

Source	Degree of freedom	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	9	2501.68	277.964	11.4918	0.0038*
Error	6	145.13	24.188		
Total	15	2646.804			

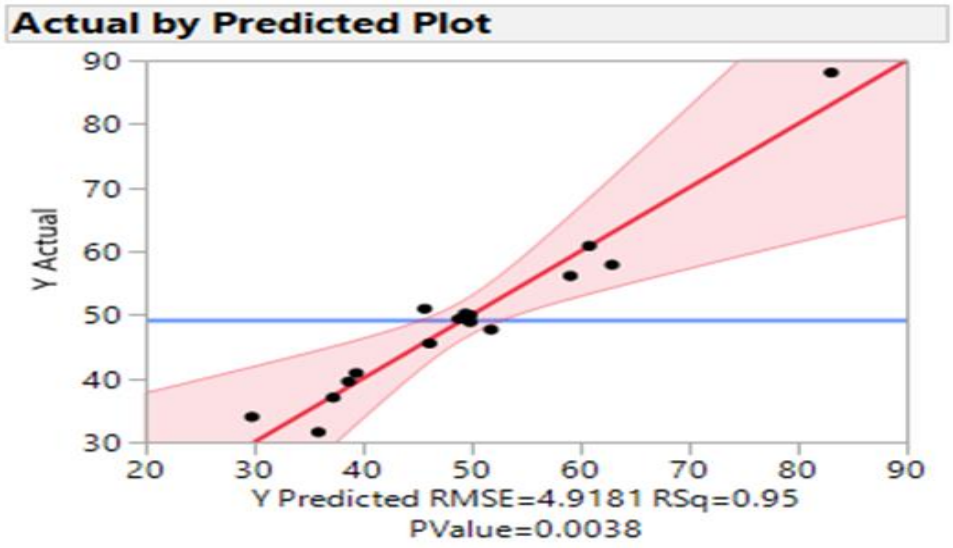


Figure (7): Experimental values versus predicted values for cellulase production by *Bacillus cerces* under three variables using CMC as substrate through central composite experimental design

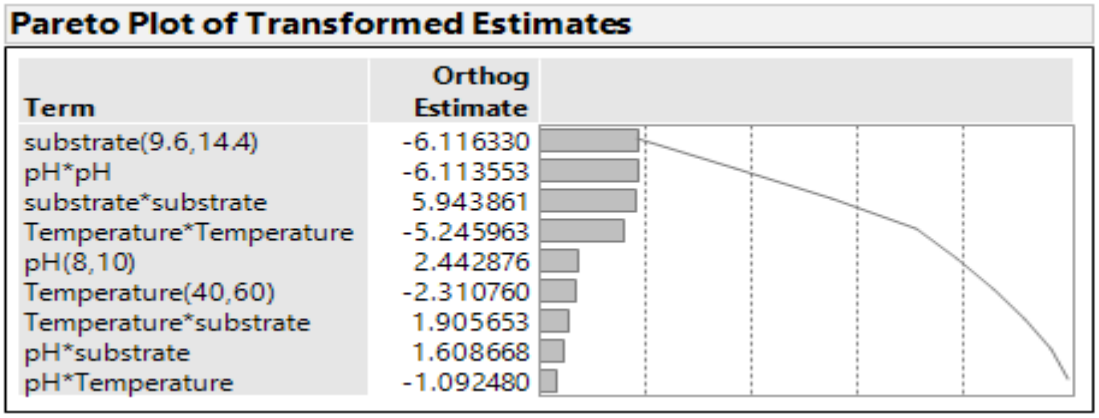
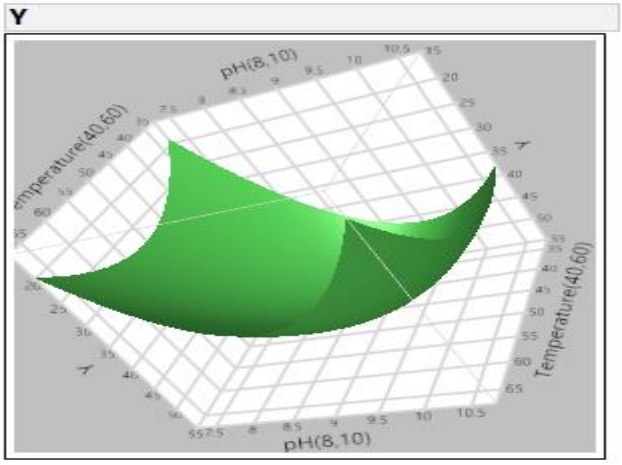
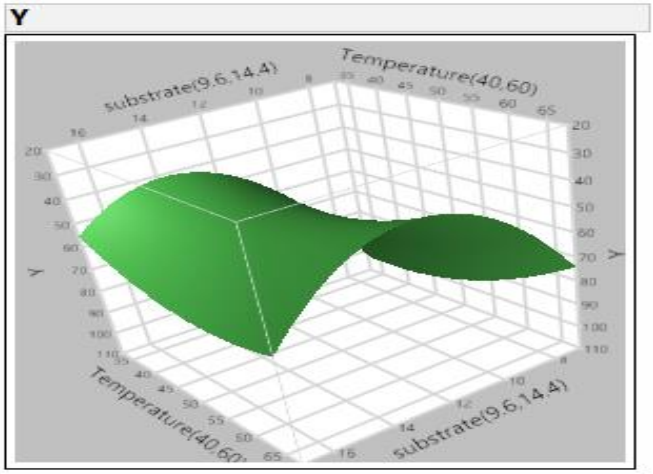


Figure (8): Pareto chart rationalizing the effect of each variable on cellulase production by *Bacillus cerces* under three variables using CMC as substrate through central composite experimental design. The vertical line defines the 94% confidence interval.

A



B



C

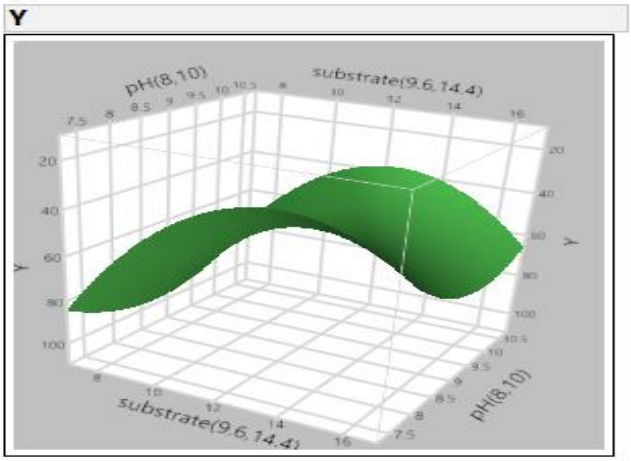


Figure (9): Respone surface plot showing the effect of pH and temperature (A), substrate and temperature (B), pH and substrate (C) on the production of cellulase by *Bacillus cerces* under three variables using CMC as substrate, other variables are held at zero level.

Table (8): Comparison of cellulose optimization by CCD produced by *Aspergillus niger, B. cereus* and *B. subtilis*

Microrganis	pH	TemPerature	Substrate	cellulase activity	predicted cellulase activity
Aspergillus niger	**4.32**	**33**	**12**	**124.48**	**109.01**
Bacillus subtilis	**9**	**50**	**7.96**	**95.64**	**91.01**
Bacillus cerces	**9**	**50**	**7.96**	**88.08**	**91.61**

Table (9): Specific activity of cellulose after optimization by CCD produced by *Aspergillus niger, B. cereus* and *B. subtilis*

Micro and Purification Steps	Total volume	Specific activity	Purification fold
Aspergillus niger			
Crude enzyme	**100**	**3.15**	**1**
Ammonium	**10**	**69.4**	**22.03**
sulfate	**10**	**99.63**	**31.63**
Dialysis			
B. cereus			
Crude enzyme	**100**	**2.28**	**1**
Ammonium	**10**	**42.2**	**18.5**
sulfate	**10**	**43.75**	**19.2**
Dialysis			
B. subtilis			
Crude enzyme	**100**	**2.56**	**1**
Ammonium	**10**	**53.4**	**20.86**
sulfate	**10**	**58.6**	**22.88**
Dialysis			

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