Journal of Current Veterinary Research

ISSN: 2636-4026

Journal home page: http://www.jcvr.journals.ekb.eg

Microbiology and Immunology

Improvement of A Thermophilic Fungal Cellulase Production Upon Bioremediation of Wheat Bran

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ABSTRACT

Due to the considerable expense associated with cellulases and their extensive range of applications, numerous researchers and enterprises have been compelled to explore alternate avenues of production. These alternatives encompass the identification of novel sources and the development of innovative fermentation techniques, with the aim of discovering specific enzymes that exhibit enhanced stability and efficiency. Plackett-Burman design is primarily employed as a statistical method for the purpose of screening and selecting the most pertinent variables that contribute to the improvement of output. The study showed information on the optimal levels of each variable, their interactions with other variables, and their impact on product yield. This resulted in a reduction in the number of experiments required to optimize production for several parameters, as determined using statistical analysis. The results obtained from the submerged fermentation experiment indicated that the maximal values of cellulose activity, glucose release, and cellulose efficacy were 92.24, 84.9, and 31.41U/ml, respectively. In contrast, solid-state fermentation yielded maximum values of cellulose activity, glucose release, and cellulose efficacy at 106.36, 97.87, and 36.21, respectively. The study identified nine significant parameters that influenced cellulose production by *Aspergillus niger* NRLL3122. These factors included inoculum size, substrate concentration, incubation temperature, pH, shaking conditions, incubation time, peptone concentration, phosphate concentration, and urea concentration. Optimization of cellulase production was conducted using MINITAB 18.0 software, employing response optimization techniques to enhance the design properties. The experiment was conducted using the specified variables outlined in the Plackett-Burman design (PBD). The resulting enzyme activity reached 106.36 U ml $^{-1}$which closely approximated the anticipated value. Highest level of enzyme activity was achieved using a 5% inoculum size, a substrate concentration of 9.6%, an incubation temperature of 28 °C, a pH of 6, an incubation time of 96 hours, and a peptone concentration of 0.75 g/L.

Keywords: *Aspergillus niger*, Cellulase, Submerged fermentation, Solid state fermentation, Placket - Burman

INTRODUCTION

The significant demand for cellulase enzyme is attributed to its many applications across various sectors, particularly in the pursuit of clean and sustainable energy sources, such as biofuel production. According to (Koupaie *et al.*, 2019), cellulase is widely utilized as the predominant enzyme for the treatment of lignocellulosic biomass. This enzyme is widely used in various industries including food production, detergent manufacturing, beverage production (such as juices, wines, and beers), paper manufacturing, denim production, medicines, fine chemicals, and biofuel production (specifically ethanol and biogas). Recently, it is used in wastewater treatment and bioremediation efforts (Shah *et al*., 2019). Cellulase is a multi-enzyme complex consisting of endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91), and β-glycosidases (EC 3.2.1.21). Each of these enzymes has a crucial function in the process of cellulose hydrolysis (Zhou *et al*., 2021).

Enzymatic hydrolysis is the preferred method of waste treatment when compared to alternative chemical processes because of its ability to provide a highly efficient bioconversion process, while functioning under uncomplicated and mild circumstances. Additionally, enzymatic hydrolysis avoids the formation of harmful byproducts that might harm fermentative bacteria (Li *et al*., 2005). Fungi are recognized as the primary manufacturers of cellulases, with the *Aspergillus niger* species being widely employed owing to its notable proficiency in the secretion of cellulolytic enzymes (Zhang *et al*., 2017).

Cellulase production can be influenced by various factors, including the strain type, growth conditions, substrate characteristics, and nutrient availability within the medium (Sandhu *et al*., 2013). The activity of these enzymes is influenced by pH and moisture content. The charge and permeability of the cell membrane are influenced by the pH, which in turn impacts the release of cellulase by microorganisms. On the other hand, moisture is associated with the proliferation of microorganisms and their secondary metabolism (Xu *et al.*, 2018). From a commercial point of view, running researches are concerning with the tremendous costs that come with using commercial enzymes, as well as their poor stability and efficiency. According to (Liu *et al.*, 2016), a potential strategy for cost reduction could involve local production on a big scale or the optimization of enzyme loading. The industrial synthesis of these enzymes has the potential to yield long-term cost reductions by mitigating expenses associated with transportation and storage. The outcomes of enzyme production are also influenced by the growing method employed.

Microbial cellulase is often produced by solid-state fermentation (SSF) and submerged fermentation (SmF) methods (Cunha *et al*., 2012). Growth media volume requirements can be decreased by using suitable waste as a substrate in SSF (Shah *et al.*, 2019). According to (Chakraborty *et al.* 2019), the utilization of SmF offers advantages such as enhanced process monitoring and control, simplified scalability, and improved product recovery. Given the high cost of cellulases and their wide range of uses, several academics and businesses have made an effort to investigate alternative manufacturing routes. These endeavors primarily involve the identification of novel sources and the development of innovative fermentation techniques, with the aim of discovering specific enzymes that exhibit enhanced stability and efficiency. Placket-Burman design is primarily employed as a statistical methodology for the purpose of screening and selecting the most pertinent factors that

contribute to the improvement of output. (Sharma *et al.,* 2015) gave information on the optimal level of each variable, their interactions with other variables, and their impact on product yield. This knowledge allows for the reduction of the number of experiments required to statistically optimize production across several parameters. Hence, the primary aim of this study was to isolate *Aspergillus niger* from the soil and after identification to assess the utilization of wheat bran as a substrate to stimulate cellulase synthesis by *Aspergillus niger* by solid-state fermentation (SSF). Therefore, this methodology would be implemented through the assessment of the proliferation of fungal strains that possess a significant capacity for breaking down cellulose, utilizing wheat bran as a substrate. Additionally, several factors that impact and regulate the synthesis of cellulase enzymes would be examined by employing the Placket-Burman experimental design.

MATERIALS AND METHODS

Mandels enriched medium

Mandels enriched medium contained urea 0.3 gl^{-1}, $(NH_4)_2SO_4$ 1.4 gl^{-1}, $KH_2PO_4$2.0 gl^{-1}, $CaCl_2.7H_2O$ 0.4 gl^{-1}, $MgSO_4.7H_2O$ 0.3 gl^{-1}, Peptone 1.0 gl^{-1}, Tween 80 2.0 gl^{-1}, $FeSO_4.7H_2O$ 5.0 mgl^{-1}, $MnSO_4.7H_2O$ 1.6 mgl^{-1}, Zn $SO_4.7H_2O$ 1.4 mgl^{-1}, $CoCl_2.6H_2O$ 20.0 mgl^{-1}, Carbon source (cellulose) 10.0 gl^{-1}. The pH was adjusted to 5. Inoculum medium (Mandels and Weber., 1969) contained urea 0.3 gl^{-1}, $(NH_4)_2SO_4$ 1.4 gl^{-1}, KH_2PO_4 2.0 gl^{-1}, $CaCl_2.7H_2O$ 0.4 gl^{-1}, $MgSO_4.7H_2O$ 0.3 gl^{-1}, Peptone 0.75 gl^{-1}, Yeast extract 0.25 gl^{-1}, Maize steep liquor 10.0 gl^{-1}, (cellulose) 2.0 gl^{-1}, $FeSO_4.7H_2O$ 5.0 mgl^{-1}, $MnSO_4.7H_2O$ 1.6 mgl^{-1}, $ZnSO_4.7H_2O$ 1.4 mgl^{-1}, $CoCl_2.6H_2O$ 20.0 mgl^{-1}. The pH was adjusted to 5.5. Nutrient broth contained Peptones 10gl^{-1}, Beef extract 1gl^{-1}, Yeast extract 2gl^{-1}, Sodium chloride 5gl^{-1}. The pH was adjusted to 6.8 ± 0.2. (Daly & Stevenson, 1993)

Microbial isolation and identification

Soil samples were obtained from several locations inside Sadat City. A cellulose aqueous medium was created by introducing autoclaved fragments of Whatman no. 1 filter paper, which consists of approximately 98% cellulose, into a 250 ml volume of distilled water. An antibiotic was introduced into the medium in order to inhibit bacterial growth, hence increasing the selectivity of the medium towards fungus. 10 mL of the wastewater samples were introduced into the medium and subjected to incubation at a temperature of 30°C for a duration of 5 days, as stated by Sivaramanan in 2014. The study employed serial dilution techniques to extract the microbe from a selective cellulose aqueous medium. Subsequently, the spread plate technique was employed using Potato Dextrose Agar media (PDA). Purity of the isolates was examined under a microscope and then compared to the purity standards reported in reputable reference materials (Weststeijn & Okafor, 1971).

Isolation of Aspergillus niger

Aspergillus niger isolates were found to be positive on screening media (cellulose agar) *(Aspergillus niger* NRLL3122) microbe was identified through Scanning by MALDI-TOF MS- Bacterial Rapid Identification (Torres *et al.,* 2016).

Extracellular cellulase activity

The evaluation of fungal isolateto produce cellulose was done through cultivating fungal isolate in 50 ml of nutrient broth containing 1% cellulose in 250 ml Erlenmeyer flasks. The flasks were then incubated at 37±2°C for 5 days, with agitation at 120 rpm. Finally, the contents of the culture were centrifuged at 10,000 rpm for 15 minutes at a 4°C and then the liquid portion was collected. 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 (Casimir *et al*., 1996). The reaction was terminated by the introduction of 3 milliliters of 3, 5-dinitrosalicylic acid (DNS), after which the mixture was boiled for 5 minutes. The spectrophotometer was used to measure the color generated at a wavelength of 540 nm. The quantification of reducing sugar released was performed using glucose as a reference. The unit (U) of enzyme activity was established as the quantity of enzyme necessary to release 0.1 μM of glucose from CMC every minute, as per the specified assay conditions. The measurement of cellulase activity was quantified in units per milligram (Miller, 1959).

Determination of protein content

The determination of protein content in the culture filtrate was determined by using the Folin-Ciocalteu reagent, with the Bovine Serum Albumin (BSA) standard (Lowry *et al*. 1951).

Fungal fermentation

The medium utilized in this study consisted of Mandels mineral salts solution supplemented with 100 g l^{-1} of cellulose. The pH of the medium was adjusted to 4.8. In this experimental investigation, the unmodified cellulose substrate derived from a specific source is employed to ascertain the efficacy of the enzyme activity catalyzed by fungal Strain.

Fermentation experiment

Multiple fermentation experiments were conducted utilizing the shake-flask technique. A 5-milliliter of the inoculum was used to begin growth in 250-milliliter Erlenmeyer flasks, each containing 100 milliliters of Mandel's fermentation medium. Following sterilizing at 121°C for 15 minutes, and pH 4.8. The production of enzymes was investigated for 5 days at 30°C. Samples were then collected daily, followed by centrifugation at a force of 10,000 g for 10 minutes at 4°C. The subsequent supernatants were then examined to determine the cellulase activity and reducing sugar content. The resulting supernatants were then examined to determine the levels of cellulase activity and the content of reducing sugars.
The objective of this study is to identify the isolate that exhibits the highest enzyme productionproduction. The optimization of process parameters for the production of cellulase has been conducted using the standard 'one-variable-at-a-time' technique.

Optimization of Enzyme Production Parameters

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 procedure, a quantity of 100 grams of wheat bran was combined and subjected to incubation at ambient temperature for a duration of 12 hours. To neutralize, a water wash was applied to the previously indicated solution. The pretreated sawdust was stored at 4°C to be used as a carbon source.

Effect of substrate concentration

Various quantities of substrate (wheat bran) were utilized, specifically 4.8%, 7.2%, and 9.6% based on wet weight conditions. These concentrations were added to100 ml of fermentation media.

Effect of pH

pH values were adjusted to 4.0, 5.0, and 6.0 by 1 N hydrochloric acid (HCl).

Effect of temperature

The impact of temperature was assessed by subjecting the fermentation medium containing wheat bran to incubation at three

different temperatures: 23, 28, and 37°C. This process was carried out using an orbital shaker incubator set at a speed of 120 revolutions per minute. An enzyme assay was carried out at regular intervals.

Effect of inoculum size

The optimization of inoculum size was achieved through the preparation of the inoculum on a Potato Dextrose Agar (PDA) plate containing *Aspergillusniger*, utilizing a sterile cup borer with a diameter of 8 mm. In a sterile manner, the fermentation media containing wheat bran was inoculated with 5, 10, and 15 discs of *Aspergillusniger*. Following the inoculation process, the flasks were placed in an orbital shaker incubator set at a temperature of 28 ± 2°C and a rotational speed of 120 revolutions per minute. An enzyme assay was carried out at regular intervals.

Effect of fermentation time

The optimization of fermentation time involved placing wheat bran in various flasks within the fermentation medium. These flasks were subjected to a temperature of 28 ± 2°C and an orbital shaker incubator at a speed of 120 rpm for a duration ranging from 48 to 96 hours. The enzyme assay was carried out at regular intervals.

Effect of nitrogen source and its concentration

To enhance the optimization process, other nitrogen sources were employed, including peptone, $(NH_4)_2PO_4$, and urea. Various concentrations were employed when utilizing these sources. Peptone was employed in concentrations of 0.075%, 0.1%, and 0.125% within the experimental scope. Ammonium phosphate $(NH_4)_2PO_4$ was employed within the concentration range of 0.12, 0.14, and 0.16. Conversely, urea was utilized within the concentration range of 0.02, 0.03, and 0.04%.

Effect of static and agitated condition

Two experimental groups were created in order to investigate the impact of static and agitated condition on the activity of the enzyme. The experimental parameters, including pH, temperature, inoculum size, and substrate concentration, were maintained at consistent levels for both sets. One group of samples was subjected to agitation at a speed of 120 (rpm) using an orbital-shaker incubator, while the other group was maintained in static conditions. An enzyme assay was carried out at regular intervals.

Experimental designs

A total of nine variables were evaluated in the Plackett-Burman experimental design, encompassing factors such as substrate, pH, temperature, inoculum size, fermentation period, nitrogen source and its concentration, as well as the presence of static or agitated conditions.

Plackett-Burman design

In the experimental design known as PBD, a total of nine variables were considered, namely substrate, Hp (hydrogen peroxide), temperature, inoculum size, fermentation period, nitrogen source and its concentration, and static and agitated conditions. These variables were manipulated at two levels, denoted as -1 and +1, representing low and high levels, respectively. The consideration of interaction effects between variables was not included in the analysis conducted in the PBD study. Therefore, the use of this method involved the screening and evaluation of significant variables that affect cellulase activity. This was done by determining the impact percentage of the variables under study. The data obtained from the PBD experiment were subjected to analysis using JMP software (developed by SAS Institute Inc., located in Cary, North Carolina, USA). A total of 11 tests were conducted using various settings for the nine variables, as indicated in Table 1.

Table (1): Experimental levels of independent variables using the Plackett-Burman design.

Variable	Units	Low (-1)	High (+1)
Substrate concentration	%	**4.8**	**9.6**
pH	-	**4**	**6**
Temperature	°C	**23**	**28**
inoculum size	(v/v) %	**5**	**15**
Fermentation time	h	**24**	**72**
Peptone	G	**0.075**	**0.125**
(NH4)2SO4	g	**0.12**	**0.16**
Urea	g	**0.2**	**0.4**
Shaking conditions	rpm	**0**	**120**

RESULTS AND DISCUSSION

Waste bioremediation

The whole study was focused on utilization of wheat bran as a good and abundant lignocellulosic waste. The study aimed at not only bio remediate the environmental waste, but also production of very important industrial chemical analysis of the lignocellulasic waste under investigation is shown in table (2)

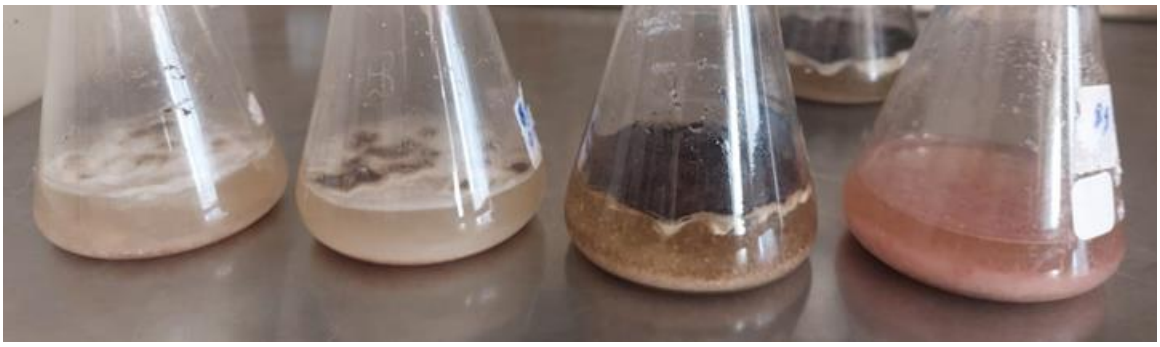

Figure (1): Standard Submerged fermentation by 7 days.

Table (2): Chemical characterization of in natural urban lignocellulosic waste.

Component	Content (%)
Moisture	7.99 ± 1.12
Ashes	9.93 ± 0.29
Extractives	60.47 ± 1.46
Cellulose	21.39 ± 3.68
Hemicellulose	5.98 ± 1.26
Lignin	29.95 ± 0.22

*Data are presented as mean ± standard deviation of duplicate experiments.

Comparison between SmF and SSF:

The current investigation yielded results indicating that the SF exhibited maximal values of cellulase activity, glucose release, and cellulase efficacy at 92.24, 84.9, and 31.41, respectively after three days of incubation (as presented in Table 3 and Figure 2). In contrast, the SSF demonstrated maximal values of cellulose activity, glucose release, and cellulose efficacy at 106.36, 97.87, and 36.21, respectively, as presented in Table 4 and Figure 3. Results shown here align with the research conducted by (Santos *et al*., 2022).

wherein they emphasized the significance of carefully choosing the fermentation technique due to its impact on both cellulase production and process efficiency. The findings indicate that solid-state fermentation (SSF) yielded comparable outcomes to submerged fermentation (Smf), albeit with a notably higher rate of enzyme activity in SSF. However, it is worth noting that the enzymes generated through the SmF-UB technique exhibited a higher degree of thermostability compared to those produced under alternative culture conditions on the same site. This finding suggests the necessity for more optimization in relation to the elimination of inhibitors. In general, the culture method of solid-state fermentation (SSF) for enzyme synthesis shown superiority over the standard submerged fermentation (SmF) technique, as evidenced by studies conducted by (Cunha *et al.* , 2012) and (Florencio *et al*., 2015).

Table (3): Cellulase activity and efficacy produced by *Aspergillus niger* in submerged fermentation.

Fungi Days	Enzyme Activity (U/ml)	glucose released (U/ml)	cellulase efficacy (U/ml)
1	**35.16**	**32.48**	**12.02**
2	**44.52**	**41.07**	**15.20**
3	**92.24**	**84.90**	**31.41**
4	**84.56**	**77.85**	**28.80**
5	**51.76**	**47.72**	**17.66**
6	**40.16**	**37.07**	**13.72**
7	**32.24**	**29.79**	**11.02**

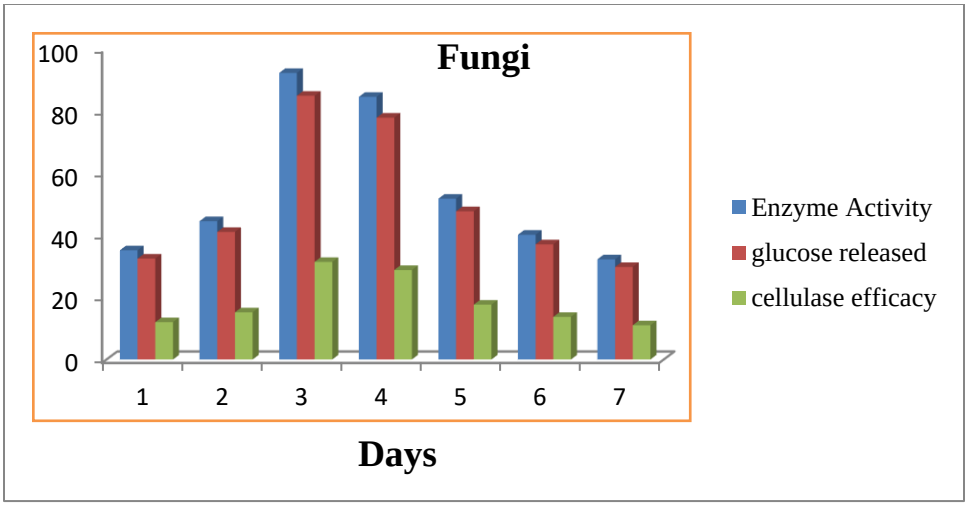

Figure (2): Cellulase activity and efficacy produced by *Aspergillusniger* in standard submerged fermentation

Table (4): Plackett-Burman experimental design applied on: (+1) high level, (-1) low levelin Solid state fermentation

No	pattern	Substrate	pH	temperature	inoculum size	fermentation time	peptone con.	$(NH_4)_2PO_4$	Urea	shaking conditions
1	−−−−++−−+	-1	-1	-1	-1	1	1	-1	-1	1
2	−−−++−−+−	-1	-1	-1	1	1	-1	-1	1	-1
3	−−+−−++++	-1	-1	1	-1	-1	1	1	1	1
4	−−++−−+−−	-1	-1	1	1	-1	-1	1	-1	-1
5	−−+++−+−+−	-1	-1	1	1	1	1	-1	1	-1
6	−+−−−−++−	-1	1	-1	-1	-1	-1	1	1	-1
7	−+−−++++−	-1	1	-1	-1	1	1	1	1	-1
8	−+−+−−−−+	-1	1	-1	1	-1	-1	-1	-1	1
9	−++−−+−−+	-1	1	1	-1	-1	1	-1	-1	1
10	−++++−+−+	-1	1	1	1	1	-1	1	-1	1
11	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0

13	+−−−−−++−−	**1**	**-1**	**-1**	**-1**	**-1**	**1**	**1**	**-1**	**-1**
14	+−−+−−+++	**1**	**-1**	**-1**	**1**	**-1**	**-1**	**1**	**1**	**1**
15	+−−++++−+	**1**	**-1**	**-1**	**1**	**1**	**1**	**1**	**-1**	**1**
16	+−+−−−−++	**1**	**-1**	**1**	**-1**	**-1**	**-1**	**-1**	**1**	**1**
17	+−+−+−−−−	**1**	**-1**	**1**	**-1**	**1**	**-1**	**-1**	**-1**	**-1**
18	++−−+−−++	**1**	**1**	**-1**	**-1**	**1**	**-1**	**-1**	**1**	**1**
19	++−+−+−−−	**1**	**1**	**-1**	**1**	**-1**	**1**	**-1**	**-1**	**-1**
20	+++−+−+−−	**1**	**1**	**1**	**-1**	**1**	**-1**	**1**	**-1**	**-1**
21	++++−+−+−	**1**	**1**	**1**	**1**	**-1**	**1**	**-1**	**1**	**-1**
22	+++++++++	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

Table (5): Cellulase activity and efficacy produced by *Aspergillusniger* in solid-state fermentation.

Treatment	Enzyme Activity (U/ml)	Glucose released (U/ml)	Cellulose efficacy (U/ml)	Specific Activity (U/mg protein)
1	**61.8**	**56.95**	**21.07**	**4.09**
2	**53**	**48.86**	**18.08**	**2.64**
3	**70.16**	**64.62**	**23.91**	**4.25**
4	**71.28**	**65.65**	**24.29**	**5.99**
5	**75.08**	**69.14**	**25.58**	**6.10**
6	**71.16**	**65.54**	**24.25**	**5.27**
7	**76.08**	**70.06**	**25.92**	**5.01**
8	**80.36**	**73.99**	**27.38**	**7.18**
9	**82.2**	**75.68**	**28.00**	**5.20**
10	**91.64**	**84.35**	**31.21**	**8.26**
11	**82.08**	**75.57**	**27.96**	**7.08**
12	**80.2**	**73.85**	**27.32**	**6.97**
13	**71.52**	**65.87**	**24.37**	**3.93**
14	**75.28**	**69.33**	**25.65**	**4.30**
15	**87.28**	**80.35**	**29.73**	**5.67**
16	**78.08**	**71.90**	**26.60**	**6.30**
17	**99.32**	**91.41**	**33.82**	**8.87**
18	**89.32**	**82.22**	**30.42**	**4.73**

19	**77.04**	**70.94**	**26.25**	**3.69**
20	**106.36**	**97.87**	**36.21**	**8.79**
21	**91.92**	**84.61**	**31.31**	**7.02**
22	**98.6**	**90.75**	**33.58**	**8.29**

ANOVA analysis for the linear model of variables factors effect on cellulase production:

According to the findings presented in Table 6, an ANOVA analysis was conducted to examine the impact of various conditions on cellulase production by *Aspergillus niger*. The statistical design's value is seen in the model's significance, as indicated by a p-value of 0.0001 in figure (4). In the examination of p-values, variables with values less than 0.01 were deemed to have a significant influence on cellulase productivity. However, when the p-value is more than 0.01, it indicates that the component being evaluated was not statistically significant, suggesting that it did not have a substantial impact on cellulase synthesis. Based on the obtained p-values, it can be observed that all factors, except for inoculum size, peptone, $(NH_4)_2PO_4$, Urea, and Shaking conditions, exhibited a positive effect on cellulase production, as displayed in Table 6. Several studies have indicated that certain parameters may not exhibit a significant impact on enzyme activity (Kumar & Satyanarayana, 2007). The present point's interpretation aligns with that of (El-Sesy & Aly, 2021), who employed cost-effective agronomic cellulosic wastes, such as cotton seed husks, barley straw, rice straw, and maize straw, as crude inducers to stimulate cellulose enzyme production. The adequacy of the goodness of fit model was assessed using the coefficient of determination (R^2), which demonstrated that the model had the capacity to account for a maximum of 90.0% of the observed variation in figure (3).

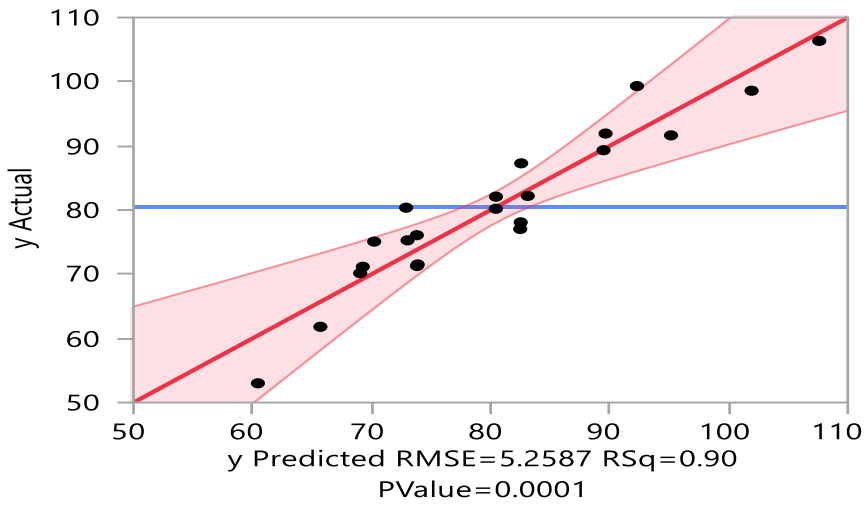

Figure (3): Experimental and theoretical values of cellulase activity by *Aspergillus niger* under nine variables using CMC as substrate through PBD.

Table (6): ANOVA analysis using Plackett-Burman design

Source	DF	Adj SS	Adj MS	F-Value	Prob> F	P-Value	Significant
Model	**9**	**2962**	**329**	**11.9**	**0.0001**	**0.0001**	**Significant**
Linear	**9**	**2962**	**329**	**11.9**	**0.0001**	**00001**	**Significant**
X1	**1**	**1007.6**	**1007.6**	**36.5**	**<.0001**	**0.00006**	**Significant**
X2	**1**	**742.7**	**742.7**	**26.9**	**0.0002**	**0.00023**	**Significant**
X3	**1**	**741.9**	**741.9**	**26.8**	**0.0002**	**0.00023**	**Significant**
X4	**1**	**1.02**	**1.02**	**0.04**	**0.8508**	**0.01205**	**Significant**
X5	**1**	**241.4**	**241.4**	**8.73**	**0.01**	**0.54440**	**Insignificant**
X6	**1**	**29.1**	**29.1**	**1.05**	**0.3253**	**0.20877**	**Insignificant**
X7	**1**	**48.7**	**48.7**	**1.76**	**0.2088**	**0.32531**	**Insignificant**
X8	**1**	**125.6**	**125.6**	**0.8719**	**0.3688**	**0.36883**	**Insignificant**
X9	**1**	**24.1**	**24.1**	**0.8719**	**0.3688**	**0.85080**	**Insignificant**
Error		**331.8**	**27.6**				
Total	**22**	**3293.97**					
R2	**0.899255**						
Adjusted R2	**0.823697**						
Predicted R2	**0.804436**						

*P<0.01 highly significant; 0.01<P<0.05 significant; P>0.05 not significant

Effects of process variables on the cellulase production Plackett-Burman design

The Plackett-Burman design was employed to evaluate the significant factors influencing cellulase production, focusing primarily on their primary impacts rather than their interaction effects. The primary impacts of the elements under analysis on cellulose production are displayed in Table 5 and Figure 4. The Pareto chart of process variables clearly demonstrates that the factors were ranked based on their importance (Figure 5). Nine factors, namely inoculum size, substrate concentration, incubation temperature, pH, shaking conditions, incubation time, peptone concentration, phosphate concentration, and urea concentration, were identified as significant for cellulose production by *Aspergillus niger*. The current study investigated the impact of the inoculum size of *Aspergillus niger* (5%) on the production, potentially resulting in an upregulation of cellulase yield. According to (Das *et al.*, 2011), prior research has indicated that smaller inoculum sizes (0.5%, 1%, and 2%) result in reduced cellulose synthesis. The impact of cultivation temperature on both the growth rate and cellulase production is significant. According to (Das *et al*., 2011), the fungal strains exhibited their highest level of activity at a temperature of 30 °C, however this activity dropped when the incubation temperature exceeded 37 °C. The growth rate of the fungus and enzyme synthesis are significantly influenced by the pH of the media. In the current investigation, it was shown that *Aspergillus niger* exhibited its highest level of activity at a pH of 6 in an acidic environment (see Table 6). This finding aligns with previous studies conducted by (Santos *et al*., 2022) and (Sivaramanan, 2014). According to the findings of (Priyanka *et al.*, 2017), it was shown that a pH of 7.0 exhibited the highest

level of cellulase enzyme activity. Furthermore, (Das *et al*., 2011) observed a reduction in fungal growth when the pH level was below 7. *A. niger* has been observed to exhibit activity throughout a broad range of pH levels. However, many studies indicate that the most favorable pH for the generation of cellulase by *A. niger* is within the range of pH 5.0-6.0, with the specific value depending on the cultivation factors (Saravanan *et al*., 2012). According to earlier research, the velocity of agitation plays a significant role in the formation of cellulase. The current investigation revealed a notable alteration in cellulase enzyme activity with the absence of agitation (0 rpm), followed by a decline in activity as the agitation speed increased from 60 to 120 rpm (Table 5). According to (Ma *et al.,* 2008), an increase in agitation speed resulted in the suppression of cellulase activity. The impact of the incubation period was assessed for a duration of 96 hours, revealing a statistically significant influence on the generation of cellulase. The activity of the enzyme exhibited a positive correlation with the duration of incubation, whereby an extended incubation time resulted in an increase in enzyme activity. Notably, the enzyme activity reached its maximum peak after 96 hours of incubation, after which it gradually declined. The present findings are consistent with prior research indicating that an extended incubation period may lead to a decline in enzymatic activity. This decline may be attributed to the utilization of nutrients present in the medium, which can induce stress in fungi and subsequently result in the inhibition of enzyme secretion (Azhar *et al*., 2017). According to a study conducted by (Leghlimi *et al*., 2019), it was determined that the most favorable duration for incubation in order to achieve optimal cellulase production using wheat bran was three days.

In the current investigation, it was observed that the concentration of peptone at 0.075 g coincided with the highest peak value of $(NH_4)_2PO_4$, which was measured at a level of 0.16. This level of $(NH_4)_2PO_4$ corresponded to the peak value of enzyme activity as determined in the study. The concentration of peptone in the growth medium plays a crucial role in the formation of cellulose. This concentration directly affects the manufacture of cellulase by various microorganisms (Deka *et al*., 2011). The inclusion of the definite article in this particular investigation. The cellulase activity was enhanced by the inclusion of ammonium sulfate due to the salting-out phenomenon. The phenomenon of salting out is characterized by a reduction in protein solubility, which can be attributed to the presence of large quantities of salt ions (Jamil *et al*., 2009) demonstrated that the precise concentration of salt facilitated the separation and precipitation of distinct proteins. Therefore, the inclusion of 80% ammonium sulfate in a mixed culture resulted in a substantial increase in cellulase activity ($P<0.05$). This might be attributed to the hydrophilic nature of the cellulase enzyme, which requires a high concentration of ammonium sulfate for precipitation, as reported by (Jamil *et al*., 2009) and (Septiani *et al*., 2019). The present study observed a notable increase in enzyme activity when urea was administered at a concentration of 0.04, which aligns with the findings published by (Abdullah *et al*. 2018).

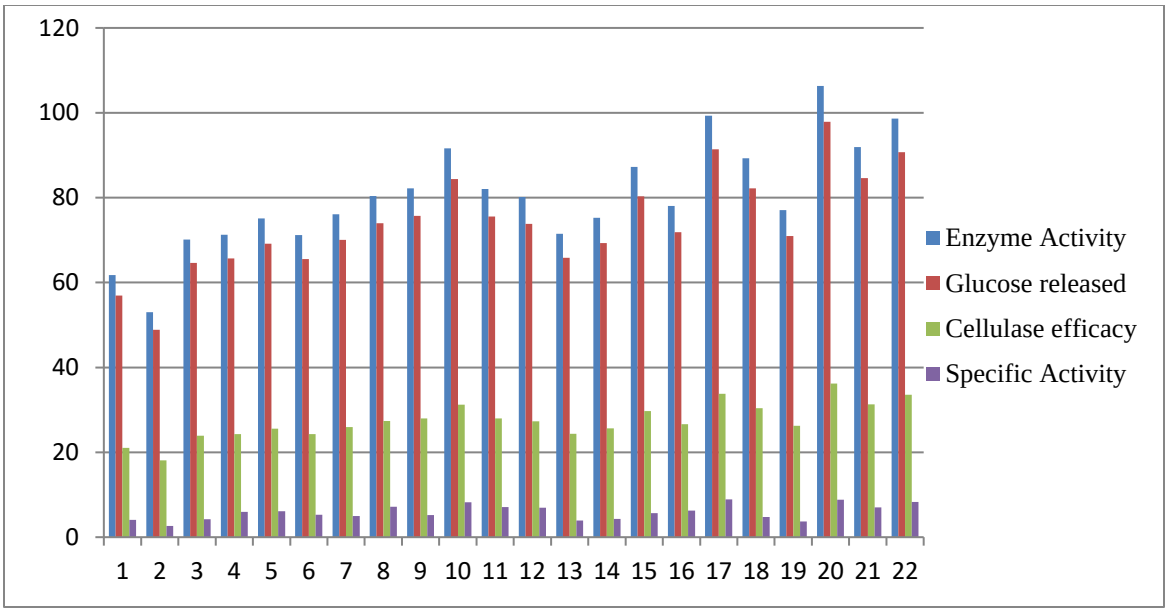

Figure (4): Cellulase activity and efficacy produced by *Aspergillus niger* in solid-state fermentation.

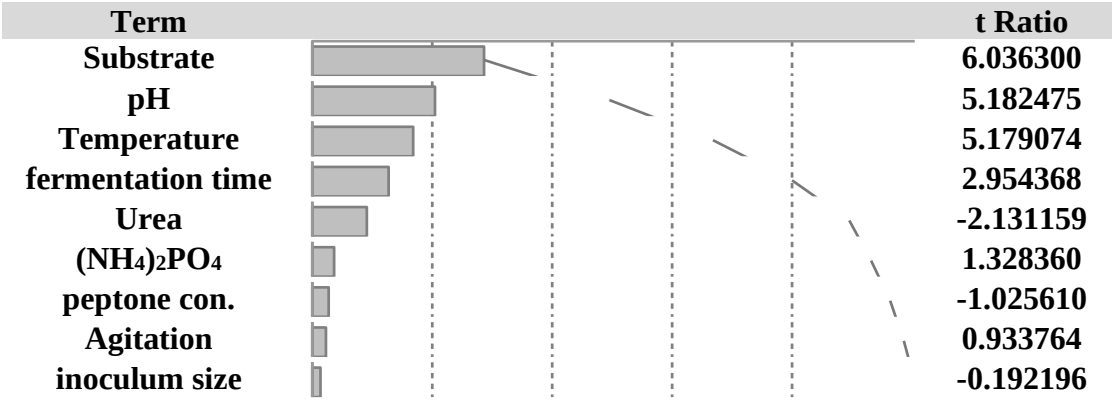

Figure (5): Pareto chart rationalizing the effect of nine variables by *Aspergillus niger* using CMC as substrate by PBD. The vertical line defines the 90% confidence interval.

Response optimization

The optimization of cellulase production was conducted using MINITAB 18.0 software through the application of response optimization techniques to enhance the design attributes. The experiment was conducted using the specified parameters outlined in the Plackett-Burman Design (PBD). The resulting enzyme activity was measured to be 106.36 U ml $^{-1}$, a number that closely approximated the expected value as indicated in Table 4. The highest level of enzyme activity was achieved when using a 5% inoculum size, 9.6% substrate concentration, incubation temperature of 37°C, pH of 6, no shaking (0 rpm), an incubation time of 96 hours, and a peptone concentration of 0.75 g L-1. The findings presented in this study are consistent with those published by (Hsu *et al.*, 2005), who observed that the greatest level of enzyme activity was achieved at the optimum circumstances of pH 6, a temperature of 37 °C, and an incubation period of 96 hours.

Table 7 :- Response prediction for cellulase activity.

Inoculum size	Substrate concentration	Incubation temperature	pH	Shaking conditions	Incubation time	$(NH_4)_2PO_4$	Peptone concentration	Cellulase activity (U ml1)	
								Experiment	Predict
5%	9.6%	28 C°	6	0 rpm	96	**0.16**	0.075 g	106.36	107.6

CONCLUSIONS

The strain of *Aspergillus niger*, which was isolated in the present investigation, exhibits cellulosic activity by successfully degrading carboxymethyl cellulose (CMC) and utilizing wheat bran as a carbon source for cellulase formation by fungal strains. The study demonstrated that the Plackett-Burman design is an effective and efficient method for identifying the elements that positively influence cellulase enzyme production. The design exhibited practicality, power, and convenience in accurately predicting the selected model, as evidenced by an R2 value of 0.996. The synthesis of cellulase enzymes was influenced by several key factors, including the size of the inoculum, the concentration of the substrate, the temperature and pH of the incubation environment, the shaking conditions, the duration of incubation, and the concentration of peptone. The results of this study indicate that cellulose generated by *Aspergillus niger* has potential applications in the field of waste management. An instance of this can be observed in the treatment of wastewater derived from cellulosic wastes, as well as in the fermentation process employed for the production of biogas.

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