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RESEARCH ARTICLE

Evaluation of Cellulases Production by Aspergillus niger Using Response Surface Methodology

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Abstract

The recent developments in bioconversion of agricultural and industrial wastes to chemical feedstock led to extensive studies on cellulolytic enzymes produced by fungi and bacteria. Aspergillus niger is well known for its ability to produce cellulases. This study aimed to produce cellulolytic enzymes from A. niger. Thirteen isolates of Aspergillus niger were screened for cellulases production. A. niger 270 was the best one which formed the highest inhibition zone (9cm) with hydrolysis capacity of 1.32. This isolate was used for cellulases production and then optimization of endoglucanase and exoglucanase activities, in addition to protein and biomass production in broth by using response surface methodology. The Box-Behnken Design method with 3-factors and 3-levels was used. The maximum endoglucanase and exoglucanase activities were 10.37 and 6.81 IU/ml respectively, while protein and biomass reached 137.33 mg/ml and 14.39 g/l at the optimal conditions which were 7 days incubation period, 3% cellulose mixture concentration and 0.35% ammonium sulfate concentration.

Keywords: Cellulases; Aspergillus niger; Optimization conditions; Response surface methodology; endoglucanase; exoglucanase.

Introduction

Cellulases are an important part of the global industrial enzymes market. The growing concerns about the depletion of crude oil and the emissions of greenhouse gases have motivated the production of bioethanol from lignocellulose (Bayer et al. 2004). The most promising technology for the conversion of lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulases (Ahmed and Vermette 2008). Cellulose consists of a linear polymer of β-1,4-linked glucose residues and is crystalline in nature (De vries and visser 2001). Cellulases divided to three types: endoglucanase (EC 3.2.1.4); exoglucanase (EC 2.3.1.91) and β-glucosidase (EC 3.2.1.21). Endoglucanase or CMCase, acts on the amorphous region of cellulose, Exoglucanase acts on the crystalline region resulting in the production of cellobiose units which are further attacked by β-glucosidase and ultimately production of glucose (Oliveira et al. 2019).

A large number of bacteria, actinomycetes and filamentous fungi have the ability to degrade cellulose. Filamentous fungi are preferred for commercial enzyme production because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria (Bakri, 2003).

Cellulase can be produced by two techniques: Solid state fermentation (SSF) or submerged fermentation (SmF). Submerged fermentation is used for industrial eenzyme production because of the ease handle and good control of environmental factors such as temperature and pH .

Aspergillus niger is known for its ability to produce a broad range of enzymes related to the degradation of polysaccharides of the plant cell, such as cellulose, xylan, xyloglucan, galactomannan and pectin (De vries and visser 2001). Jasani et al. (2016) and Imran et al. (2018) studied the production of cellulase produced from Aspergillus niger. Production of cellulase is affected by several factors like pH, temperature, carbon source, nitrogen source, incubation period and others. Optimization of these factors is very important to determine the optimum culture and environmental conditions for enzyme production from a certain strain. Thermal stability and substrate specificity may also vary with the origin (Bhat 2000).

Design Expert is a piece of software designed to help with the design and interpretation of multi-factor experiments. In this study, we use this program to optimize cellulase production conditions after the screening of some Aspergillus niger species. The finally produced cellulase was tested for its stability against different temperatures. The produced enzyme will be useful in further studies to assist lignocellulosic materials hydrolysis.

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Materials and methods

Microorganisms

Aspergillus niger isolates were collected from different sources in our Laboratories (Abdel-Sater et al. 2015 and Moharram et al. 2021). Cultures were grown on Czapek's agar medium for 7 days at 28˚C to be used in further experiments.

Screening for cellulases production

Thirteen isolates of Aspergillus niger were screened for their ability to produce cellulase according to Mandel's et al. (1974) method. The medium used in screening contains (g/l): urea,0.3; (NH4)2SO4,1.4; KH2PO4,2.0; MgSO4,0.3; yeast extract,0.25; peptone, 0.75; carboxy methyl cellulose (CMC),10 and agar, 17.5 per one liter of distilled water. The pH was adjusted at 5.0. Agar blocks from one week old fungal colony grown on Czapek's medium were inoculated in the center of plates containing Mandel's medium and incubated at 30˚C. After seven days, plates (3 for each isolate) were stained with 1% congo red for 20 min and then de-stained with 1 M NaCl solution for 20 min (Yoon et al., 2007). The ratio of the clear zone diameter to the colony diameter was calculated.

Enzyme production

The best isolate (A. niger 270) that produces cellulase was used for enzyme production and optimization of production conditions. The fermentation was carried out in 250 ml Erlenmeyer flask containing 50 ml of sterilized Mandel's medium. Flasks were inoculated with spore suspension of 6 days old culture of A. niger grown on Czapek's agar slants. Flasks were incubated at 30˚C at 150 rpm. After fermentation, media filtered through Whatman "No. 1" filter paper to separate mycelia and culture filtrates.

Optimization by using Response Surface Methodology

Response surface methodology (RSM) provided by Design Expert Software (2011) with a standard tool known as Box-Behnken design (BBD) (Box and Behnken, 1960) was used for studying the effect of independent variables and their interaction to get the optimum conditions of cellulases activity in addition to proteins and biomass production in medium.

The second-order quadratic model equation (Eq. 1) was developed based on the independent variables in terms of actual values and the experimental results to express endoglucanase and exoglucanase (activity), protein and biomass which are produced follows:

$$Y = b0 + b1A + b2B + b3C + b12AB + b13AC + b23BC + b11A2 + b22B2 + b33C2 \quad \text{(Eq. 1)}$$

Where Y is the dependent variable, A (incubation period), B (concentration of cellulose mixture), C (concentration of ammonium sulfate) are independent variables.

Effect of temperature on activity and stability of crude enzyme

The stability of endoglucanase and exoglucanase was determined by measuring their activities after incubation at different temperatures (40, 50, 60, 70˚C) at different time points (30, 60, 90 min). Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 26.0 software. All analyses were performed in triplicate, and results are expressed as mean ±standard deviations (SD). Differences among groups were assessed using Duncan's multiple range test, $p < 0.05$ was considered significant.

Analytical methods

- Determination of endoglucanase activity: the endoglucanase activity was determined by mixing 1ml of 1% CMC (dissolved in citrate buffer pH 5) with 1ml of culture supernatant and incubated at 50˚C for 30 min. This is followed by the addition of 4ml dinitrosalicylic acid reagent (Miller 1959) and boiling in a water bath for 10 min, cooled and absorbance was read at 540 nm. One unit of the enzyme is defined as the amount of enzyme that liberates 1 µmol of reducing sugar equivalent per minute (Ghose and Brisaria 1987).
- Determination of exoglucanase activity: Similar to endoglucanase activity but the buffer contains 1% MCC (microcrystalline cellulose) instead of CMC.
- Determination of biomass dry weight: dry weight of fungal mycelia was dried in an air oven at 70˚C for 24h.
- Determination of protein: soluble protein was determined in the filtrate by Lowry et al. (1951) method using bovine serum albumin as standard.

Table 1. Values of independent variables in the Box-Behnken design

Parameters (Independent variables)	Units	Coded levels		
		-1	0	+1
Incubation period (A)	Day	3	5	7
Concentration of cellulose mixture (B)	%	1	2	3
Concentration of ammonium sulfate (C)	%	0.2	0.35	5

Results and discussion

Cellulases are hydrolytic enzymes that are produced by microbes during the degradation process of cellulose or plant fibrous parts, bacteria and fungi are good producers of cellulases (Henriksson et al. 1999). Numerous studies have reported that Aspergillus species are capable of producing multi-enzymatic extracellular complex efficiently degrading lignocellulosic components in plant biomass (Ang et al. 2013; Ferreira et al. 2018).

Thirteen isolates of Aspergillus niger were tested for their ability to produce cellulases. Table (2) shows that all isolates have the ability to produce cellulases and the isolate Aspergillus niger 270 shows the highest inhibition zone was 9 cm with a hydrolysis capacity 1.32. Hydrolysis capacity when its value was less than 1 indicated that the substrate inhibited the growth of fungus whereas if its value was more than 1 revealed that the substrate enhanced the fungal growth (Imran et al. 2018).

Bakri et al. (2003) demonstrated that filamentous fungi are preferred for commercial enzyme production, due to the higher amount enzymes that produced by them compared to those produced by yeast and bacteria. Most of Aspergillus spp. synthesize cellulases; therefore, this genus has the potential to dominate the enzyme industry. Aspergillus and Trichoderma spp. are well-known efficient producers of cellulases (Peij et al. 1998). Many pieces of literature such as Perrone et al. (2008) and Soni et al. (2010) reported that filamentous fungi particularly Aspergillus and Trichoderma spp. are well-known efficient producers of cellulases. The current results produced by Aspergillus niger 270 were less than those produced by El –Nahrawy et al. (2017) which found that Aspergillus tubingensis KY615746 when grown on CMC produced a clear zone with 3.27±0.18 with 2.87 HC value.

Table 2. Screening for the cellulolytic activity of Aspergillus niger isolates.

Isolate number	Colony diameter (Cm)	Clear zone diameter (cm)	HC value*	Level of Cellulase production
A. niger 255	6.93±0.25	8.4±0.2	1.21±0.02	+++
A. niger 40	5.50 ± 0.17	6.13 ± 0.17	1.11±0.03	++
A. niger 13	5.43 ± 023	6.03±0.06	1.11± 0.03	++
A. niger 1531	6.80 ± 0.17	8.33 ±0.15	1.22±0.02	+++
A. niger 258	6.53±0.15	8.03±0.42	1.23±0.04	+++
A. niger 408	5.47±0.38	6.07±0.38	1.11±0.01	++
A. niger 4	7.23±0.23	8.83±0.35	1.22±0.01	+++
A. niger 1	5.83±0.40	7.03±0.21	1.21±0.07	+++
A. niger 252	7.13±0.12	8.33±0.15	1.17±0.03	+++
A. niger 27	7.03±0.15	8.33±0.21	1.19±0.03	+++
A. niger 270	6.83±0.06	9.00±0.01	1.32±0.01	++++
A. niger 44	5.30±0.26	6.10±0.36	1.15±0.04	++
A. niger 18	5.63±0.21	6.43±0.29	1.14±0.01	++

HC: hydrolysis capacity was calculated as the clear zone diameter / the colony diameter.

Response surface methodology is one of the statistics tools that is useful for developing, improving and optimizing processes and is used to determine the effects of several independent variables and the interaction between them on the system response, the main objective being the determination of optimum operational parameters within the operating design (Ravikumar et al. 2005). The results of BBD experimental design which include 17 runs of 3 independent of optimized variables (incubation period, concentration of cellulose mixture and concentration of ammonium sulfate) for the evolution of endoglucanase and exoglucanase activities as well as protein and biomass production are listed in Table 3.

Table 3. A Box-Behnken experimental design and the results of dependent variables (endoglucanase and exoglucanase (activity), produced protein and biomass) by A. niger 270

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Run	A: incubation period	B: Conc of cellulose mixture	C: Conc of ammonium sulfate	Endogluconase (activity)	Exogluconase (activity)	protein	Biomass
	day	%	%	IU/ml	IU/ml	mg/ml	g/l
1	5	3	0.2	10.09	6.04	120.17	14.95
2	7	2	0.2	7.59	3.88	106.17	9.23
3	7	1	0.35	4.84	2.51	68.67	4.92
4	3	3	0.35	6.17	4.32	64.5	12.8
5	5	2	0.35	7.03	5.36	85	10.71
6	5	1	0.5	3.31	3.38	55.83	5.33
7	5	2	0.35	6.34	5.14	87.83	10.56
8	3	2	0.2	4.07	2.71	55	10.22
9	3	1	0.35	2.93	2.44	42	10.97
10	3	2	0.5	4.37	3.33	55	9.32
11	5	2	0.35	7.48	5.32	89.5	10.75
12	7	3	0.35	10.92	7.22	142.83	15.29
13	5	1	0.2	4.74	2.84	45.33	5.55
14	5	2	0.35	6.99	5.51	85.33	10.75
15	7	2	0.5	6.88	5.54	100	9.42
16	5	2	0.35	7.43	4.95	88.67	10.42
17	5	3	0.5	8.19	5	85.33	15.63

The second-order quadratic model equation (Eq. 1) (coded units) was used to express endoglucanase and exoglucanase activities, protein and the produced biomass.

Y Endoglucanase (activity) = +7.05 +1.59A +2.44B - 0.4675 C + 0.7100AB - 0.2525 AC - 0.1175 BC - 0.8470 A^2 + 0.0080B^2 - 0.4795 C^2

Y Exoglucanase (activity) = + 5.26 + 0.7938A + 1.43B + 0.2225 C + 0.7075 AB + 0.2600 AC - 0.3950BC - 0.7918A^2 - 0.3418 B^2 - 0.5992C^2

Y Protein = + 87.27 + 25.15A + 25.13 B - 3.81C + 12.91AB - 1.54AC - 11.34 BC - 2.69 A^2 - 5.07B^2 - 5.53 C^2

Y Biomass = +10.64 + 1.06 A + 4.49 B + 0.0312C+ 1.13AB + 0.2725AC + 0.2250BC+ 0.2698 A^2 + 1.09B^2 -1.36C^2

Y Endoglucanase (activity) is the activity of produced endoglucanase IU/ml, Y Exoglucanase (activity) is the activity of produced exoglucanase IU/ml, Y Protein is the concentration of protein (mg/ml) and Y Biomass is the amount of biomass g/l.

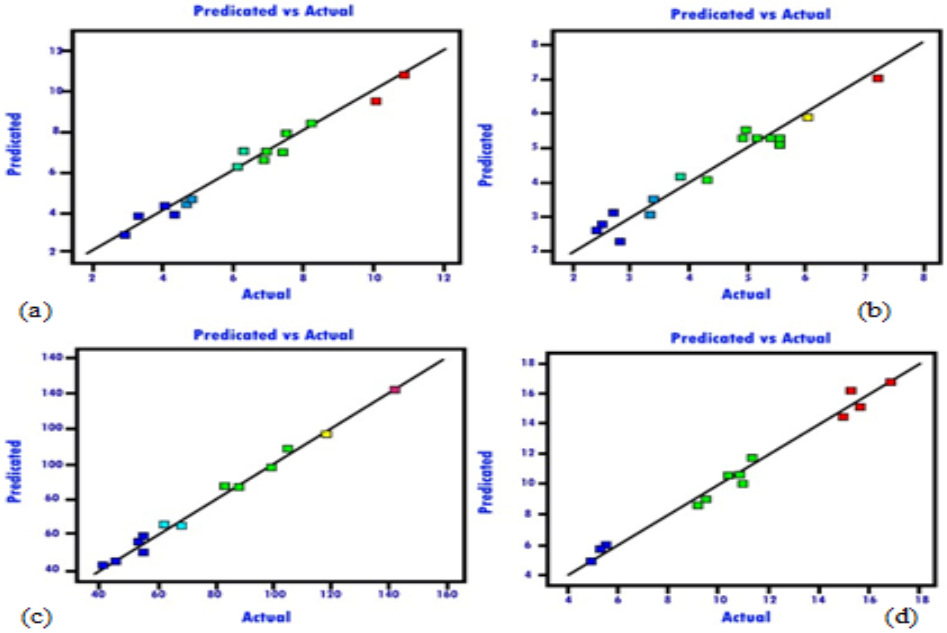

Figure 1. Predicted vs actual values where (a) for endogluconase activity, (b) for exogluconase activity, (c) for produced protein and (d) for produced biomass.

Table 4. Correlation coefficients that indicate the fitting of the mode of the dependent variables.

dependent variables	R^2	R^2%
Endogluconase (activity)	0.9737	97.37%
Exogluconase (activity)	0.9500	95%
protein	0.9937	99.37%
Biomass	0.9835	98.35%

The plots of the predicted versus actual results which indicate the model's performance (Fig, 1), that showed. high correlation coefficients table (4) indicating that the predicted and actual values were in reasonable agreement.

Table 5. ANOVA for the entire quadratic model of response (1) endoglucanase activity.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	76.18	9	8.46	28.81	0.0001*
A-incubation period	20.13	1	20.13	68.53	< 0.0001*
B-Conc of cellulose mixture	47.78	1	47.78	162.64	< 0.0001*
C-Conc of ammonium sulfate	1.75	1	1.75	5.95	0.0448*
AB	2.02	1	2.02	6.86	0.0344*
AC	0.2550	1	0.2550	0.8682	0.3825
BC	0.0552	1	0.0552	0.1880	0.6776
A^2	3.02	1	3.02	10.28	0.0149*
B^2	0.0003	1	0.0003	0.0009	0.9767
C^2	0.9681	1	0.9681	3.30	0.1123
Residual	2.06	7	0.2937		
Lack of Fit	1.22	3	0.4063	1.94	0.2648
Pure Error	0.8373	4	0.2093		
Cor Total	78.23	16			

* significant

Table 6. ANOVA for the entire quadratic model of response (2) exogluconase activity

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	29.72	9	3.30	14.77	0.0009*
A-incubation period	5.04	1	5.04	22.54	0.0021*
B-Conc of cellulose mixture	16.27	1	16.27	72.78	0.0001*
C-Conc of ammonium sulfate	0.3960	1	0.3960	1.77	0.2249
AB	2.00	1	2.00	8.95	0.0202*
AC	0.2704	1	0.2704	1.21	0.3078
BC	0.6241	1	0.6241	2.79	0.1387
A^2	2.64	1	2.64	11.80	0.0109*
B^2	0.4918	1	0.4918	2.20	0.1816
C^2	1.51	1	1.51	6.76	0.0354*
Residual	1.57	7	0.2236		
Lack of Fit	1.38	3	0.4596	9.86	0.0255
Pure Error	0.1865	4	0.0466		
Cor Total	31.29	16			

* significant

Table 7. ANOVA for the entire quadratic model of response (3) protein

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	11711.60	9	1301.29	122.19	< 0.0001*
A-incubation period	5058.67	1	5058.67	474.99	< 0.0001*
B-Conc of cellulose mixture	5050.13	1	5050.13	474.19	< 0.0001*
C-Conc of ammonium sulfate	116.36	1	116.36	10.93	0.0130*
AB	667.19	1	667.19	62.65	< 0.0001*
AC	9.52	1	9.52	0.8936	0.3760
BC	513.93	1	513.93	48.26	0.0002*
A^2	30.56	1	30.56	2.87	0.1341
B^2	108.31	1	108.31	10.17	0.0153*
C^2	128.73	1	128.73	12.09	0.0103*
Residual	74.55	7	10.65		
Lack of Fit	58.39	3	19.46	4.82	0.0815
Pure Error	16.16	4	4.04		
Cor Total	11786.15	16			

* significant

Table 8. ANOVA for the entire quadratic model of response (4) biomass.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	190.56	9	21.17	45.00	< 0.0001
A-incubation period	16.39	1	16.39	34.84	0.0006
B-Conc of cellulose mixture	161.08	1	161.08	342.37	< 0.0001
C-Conc of ammonium sulfate	0.0681	1	0.0681	0.1447	0.7149
AB	5.17	1	5.17	10.98	0.0129
AC	0.0022	1	0.0022	0.0047	0.9473
BC	0.2007	1	0.2007	0.4266	0.5345
A^2	1.76	1	1.76	3.75	0.0942
B^2	2.16	1	2.16	4.58	0.0696
C^2	4.10	1	4.10	8.71	0.0214
Residual	3.29	7	0.4705		
Lack of Fit	3.21	3	1.07	50.20	0.0012
Pure Error	0.0852	4	0.0213		
Cor Total	193.86	16			

* significant

The models considered as statically significant according to the F-test with 95% of confidence, as the F values are much higher than F (14, 14) = 2.48, the p-value of the model is (0.0001) which is less than 0.05 indicating model terms are significant. Sum of squares used as a mathematical way to find the function that best fits (varies least) from the data.

Response Surface Plots

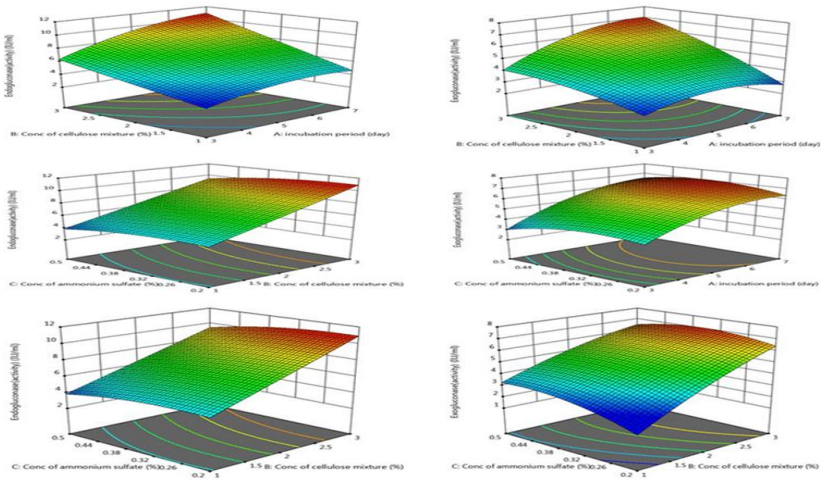

Figure 2. Response surface plots indicated the effect of the incubation period, the concentration of cellulose mixture and the concentration of nitrogen source for the evolution of endoglucanase and exoglucanase activities.

It was noticed from the RSM plots which describe the optimization of endoglucanase activity that figure (2) shows the interaction between the incubation period (3 – 7 days) and each of cellulose mixture concentration (1–3%) and ammonium sulfate concentration (0.2 – 0.5%), the maximum activity of endoglucansae was 10.92 IU/ml produced after 7 days incubation period with 3% cellulose mixture concentration and 0.2% ammonium sulfate. By increasing ammonium sulfate concentration, the endoglucanase activity slightly decreases.

In case of optimization of exoglucanase activity the interaction between cellulose mixture and ammonium sulfate with incubation period, where the enzyme activity increased by increasing fermentation period, the maximum activity was 7.22 IU/ml after 7 days at 3% cellulose mixture concentration and 0.35% ammonium sulfate.

Namnuch et al. (2021) determined the time course of enzyme production and found that the highest CMCase (1.27 U/ml), FPase (0.72 U/ml) and xylanase (376.81 U/ml) activities by Aspergillus flavus were observed at 14 days of fermentation when grown on sugar cane bagasse as substrate. Kanakaraju et al. (2020) reported that the activities of CMCase (endoglucanas) and FPase (exoglucanase) as observed by Aspergillus niger in SmF using wheat bran were 6.32 IU/ml and 3.1 IU/ml and using ground shell were 3.12 and 1.3 IU/ml, respectively after 4 days of fermentation period. A study conducted by Mrudula and Murugammal (2011) found that the maximum CMCase (endoglucanase) activity of Aspergillus niger was 3.29 (IU/ml) under submerged fermentation at 3% coir waste as substrate, pH 6, temperature 30˚C and after 72h incubation period. El-Nahrawy et al. (2017) indicated that the maximum cellulase (endoglucanase) activity was 0.063 IU/ml produced by A. tubingensis at 3% inoculum size, pH 4, temperature 30 °C, 1% carboxymethyl cellulose as carbon source and incubation period 6th day. Cellulase activities of Aspergillus niger ASP2 reached their maximum (4.47 U/ml) after 96h of fermentation at 25˚C according to Bellaouchi et al. (2021). Also, maximum activity of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass was reported after 96 hours (kang et al. 2004).Gori and Malana (2010) reported 72h as a best incubation time for cellulase production. They found that the maximum yield of exoglucanase (1.64 U/mL) and endoglucanase (1.84 U/mL) was obtained after 4 days.

Ammonium compounds are reported to be the most favorable nitrogen compounds for enzyme synthesis by Cellulomonas flavigena (Rajoka, 2004). Ja'afaru and Fagade (2010) reported that (NH4)2SO4 and NH4H2PO4 were good nitrogen sources for cellulase synthesis by A. niger YL128.The maximum CMCase activity (0.25 IU/ml) and exoglucanase (0.03 IU/ml) by Aspergillus niger were observed after 96 h, when ammonium sulfate was applied at 0.14 g (jasani et al. 2016).

Narasimha et al. (2006) found that carboxy methyl cellulose was the best source followed by cellulose for cellulase production by Aspergillus niger when they tested different carbon sources and reported that supplementation of cellulose at 1 % was optimal for cellulase production and a further increase in cellulose concentration did not result in proportionate in yields of fungal biomass and protein content. Also, Gautam et al. (2010) examined the effect of different carbon sources on production of cellulases by Aspergillus niger and reported that CMC served the best source followed by cellulose. Also, they found that supplementation of cellulose at 1% was optimal concentration for cellulase production.

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Ammonium compounds are reported to be the most favorable nitrogen compounds for enzyme synthesis by Cellulomonas flavigena (Rajoka, 2004). Ja'afaru and Fagade (2010) reported that (NH4)2SO4 and NH4H2PO4 were good nitrogen sources for cellulase synthesis by A. niger YL128.The maximum CMCase activity (0.25 IU/ml) and exoglucanase (0.03 IU/ml) by Aspergillus niger were observed after 96 h, when ammonium sulfate was applied at 0.14 g (jasani et al. 2016).

Narasimha et al. (2006) found that carboxy methyl cellulose was the best source followed by cellulose for cellulase production by Aspergillus niger when they tested different carbon sources and reported that supplementation of cellulose at 1 % was optimal for cellulase production and a further increase in cellulose concentration did not result in proportionate in yields of fungal biomass and protein content. Also, Gautam et al. (2010) examined the effect of different carbon sources on production of cellulases by Aspergillus niger and reported that CMC served the best source followed by cellulose. Also, they found that supplementation of cellulose at 1% was optimal concentration for cellulase production.

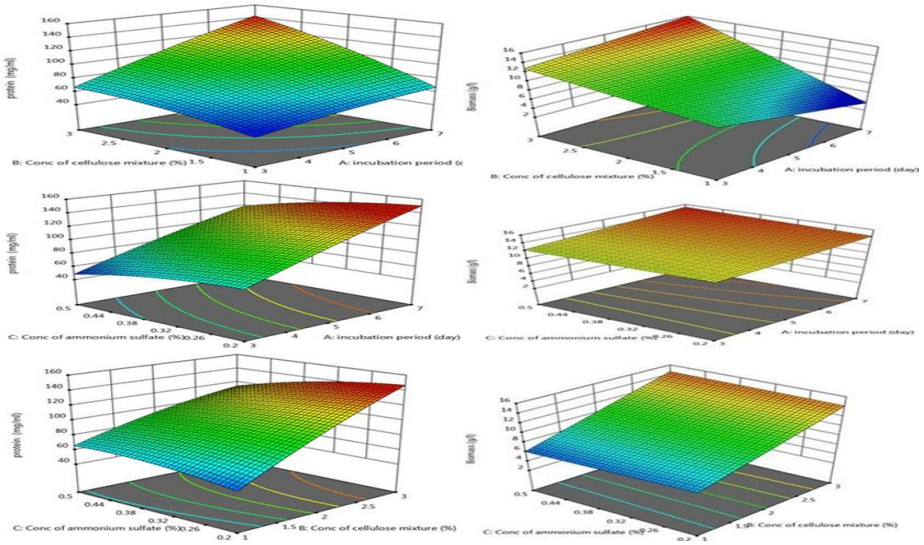

Figure 3. Response surface plots indicated the effect of incubation period, concentration of cellulose mixture and concentration of ammonium sulfate for evolution of protein (a) and biomass (b) production.

The amount of extracellular protein production was optimized due to the interaction of independent variables as shown in Fig (3a). The maximum amount of protein was 142.83 mg/ml which was produced at 7 days incubation period, 3% cellulose mixture concentration and 0.2% ammonium sulfate concentration.

Many studies reported that Aspergillus spp. produce relatively large quantities of endoglucanase and β-glucosidase with low levels of exoglucanase, together with high levels of protein (Duarte and Costa-Ferreira, 1994; De Vries and Visser, 2001).

Bijay et al. (2016) found that the maximum total protein content of Aspergillus niger NCFT 426310 during cellulase production was 869.27 ± 6.15 µg/ml when shaking fermentation was carried out at 100 rpm using wheat bran as a substrate. Reddy et al. (2015) reported that maximum protein secretion by A. niger was 1400 µg/ml using rice bran as a substrate and wheat bran was the second-best substrate that supported secretion of extracellular protein into the broth. Ardhia et al. (2016) reported that the amount of protein content and enzyme activity in broth medium were increased by increasing fermentation time. They found that the highest protein content produced by Aspergillus niger K3F4 was 0.740 g / ml at inoculum size 25% and 12 days incubation time.

Figure 3b shows the production of biomass which increased by increasing the incubation period, cellulose mixture concentration and ammonium sulfate concentration. The maximum amount of biomass was 14.39 g/l which was produced at 3% cellulose mixture concentration, 0.5% ammonium sulfate and 7 days fermentation period. Carbon and energy sources are the most important nutrient required for the growth of microorganisms and biomass formation (Nasseri et al. 2011; Suman et al., 2015).

In the present study, A. niger can transform cellulose mixture into reducing sugar during the fermentation process due to the presence of high amounts of cellulases, secreted by it. Therefore, sugar components of the substrate are metabolized by fungal strain resulting in enhanced production of biomass (Saheed et al. 2016; Yabaya and Ado 2008). Kamal et al. (2019) improved the production of biomass of Aspergillus niger from banana fruit peel by the use of response surface methodology. They found that the yield of biomass from A. niger was ranged from 7.84 to 24.68 g/l and by increasing in fermentation period (2–4 days) with a combination of substrate concentration (10–20%) resulted in an intensification of biomass yield.

Enzyme Activity Under Optimal Condition

Numerical optimization was performed using the suggested design for the endoglucanse and exoglucanase activities, as well as protein and biomass production. The independent variables were the incubation period (3-5 days), cellulose mixture concentration (1-3%) and ammonium sulfate concentration (0.2-0.5%). The design expert program was run for the optimum conditions and the solutions. The best solution was found with maximum desirability (99.9%) selected as the optimum conditions for enhanced the dependent variables. Therefore, the predicted optimum conditions were obtained as the incubation period 7 days, cellulose mixture concentration 3% and ammonium sulfate concentration 0.35%.

Table 9. Comparison of experimental values with predicted values at the optimized condition

	Predicated value	Actual value	Ratio Actual / Predicated %
Endogluconase units (IU/ml)	10.96	10.37±0.03	94.59±0.28
Exoglucnase units (IU/ml)	7.05	6.81± 0.02	96.59±0.25
Protein (mg/ml)	142.83	137.33±1.74	95.21± 1.29
Biomass (g/L)	15.97	14.39± 0.44	90.11±2.76

Verification of the models

To verify the established model, triplicate experiments were carried out under the recommended optimum conditions. It is important to compare between model and result from real fermentation under optimum condition. The comparison of results between model prediction and real experimental tests under optimum conditions is given in Table (9).

The obtained experimental values were adequate with the predicted values of the response surface model because the experimental values were very close to the predicted values which satisfy the predicted model.

The effect of temperature on enzyme activity and stability

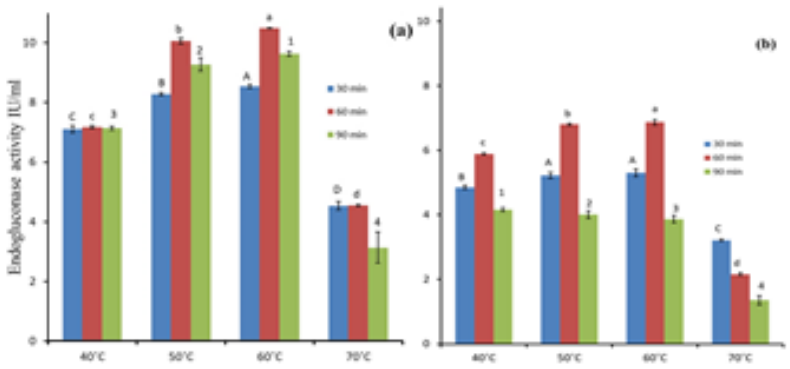

Figure 4. The effect of temperature (40˚, 50˚, 60˚, 70˚C) on the activity of endoglucanase enzymes (a) and exoglucanase enzymes (b) at 30, 60, 90 min. A,B,C and D; significant difference of endogluconase (a) and exogluconase (b) activities after 30 mins; a,b,c,d significant difference in endogluconase and exogluconase activities after 60 mins; 1,2,3,4 significant difference of endogluconase and exogluconase activities after 90 mins.

The effect of temperature on the activity and stability of endoglucanase and exoglucanase was determined on various temperature ranges from 40 - 70°C. The optimum temperatures for endoglucanase and exoglucanase activities were found at 60 °C. Thermostability studies revealed that endoglucanase and exoglucanase were highly stable in optimum temperature at 60°C for 60 minutes and it retained their activity for 90 minutes (Figure 4). While, Listyaningrum et al. (2018) found that the optimal cellulase temperature and thermostability which was obtained by Bacillus licheniformis C55 were 50°C for 30 min.

Conclusion

The results of this study indicate that Aspergilllus niger 270 is able to produce cellulases and that the Box Behnken (BBD) design was an effective method for optimizing endoglucanase and exoglucanase activities in addition to optimizing protein and biomass production. Thermostability studies indicated that the produced cellulases were highly stable which make it a good choice for using in hydrolysis of cellulosic materials on industrial scale. Accurate purification methods should be done to get the enzyme in pure form.

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