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Isolation and optimization of cellulase production by *Aspergillus penicillioides* 12 ASZ using experimental design

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ABSTRACT

A novel fungal cellulase producer namely: *Aspergillus penicillioides* 12ASZ (accession no.: MK713549) was isolated from Egyptian soil. The detected cellulase was reported as acidic which was highly active between 30-55°C. In a trial to test the environmental factors that lead to maximum cellulase production by the tested fungus, Plackett–Burman and BOX-Benken were tested, fifteen variables in Plackett–Burman were tested. Data revealed that soluble starch, $MnSO_4$ and $CaCl_2$ were the most significant variables that leads to 6 fold increase in cellulase production by *Aspergillus penicillioides* 12ASZ (MK713549) using Plackett–Burman design. Subsequently, BOX-Benken design was implemented to obtain the best process conditions among the selected variables vital for cultivating cellulase yield. Box-Behnken design level was pragmatic to generate a polynomial quadratic linking between the three variables and cellulase activity. The ideal preparations of the officer medium elements for cellulase production were estimated by algorithm of EXCEL-*solver* of non-linear optimization. The expected optimum cellulase activity was 195.57 U/mL/min, which was increased by 22.74 times of the activity using the basal medium where the enzyme activity with the basal screening medium was 8.6U/mL/min.

Key words: *Cellulase, Experimental design, Asperigillus penicillioides, Optimization, Activity*

Introduction

Enzymes are the natural material or natural macromolecules that are manufactured by an alive organism which perform as a catalyst to a specific biological reaction. Unlike most of the enzymes cellulase is complex enzymes that work synergistically to attack native cellulose (Gurung *et al*., 2013). Cellulase is a multi-enzyme system comprising of endoglucanase, exoglucanase, and β-glucosidase and the hydrolysis of cellulose (Estela and Luis, 2013). Cellulases accounted for approximately 20% of world enzyme market between 2005 and 2010 and its demand is thought to increase drastically due to its application in second generation bioethanol production. Cellulases are produced by bacteria, fungi, protozoan, plants and animals. Currently most of the commercial cellulases are obtained from fungi mainly *Trichoderma*, *Humicola*, *Aspergillus* and *Penicillium* (Agarwal *et al*., 2014).

Cellulase production is controlled by various parameters like media components such as (carbon source, nitrogen source, mineral salts, and micronutrients) and physical parameters (pH, aeration, temperature). Therefore it is required to improve the medium constituents for the superior assembly of

enzyme (Cherian *et al.,* 2016). The traditional one-variable-at-a-time (OVAT) method includes changing the concentration of one constituent and conserving the others, at an identified level. The shortcomings of this approach are overcome by the use of statistical technique like Plackett-Burman design (PBD) which used to identify optimum incubation temperature, moisture content and culture pH for cellulase activity. The ideal states enclosing three significant factors is resolute. Furthermore, response surface analysis and Box-Behnken design (BBD) are employed to find interactive effect between the three variables on the activity of cellulases (Rajeshkannan *et al*., 2009; Vimalashanmugam and Viruthagiri, 2012; Imran *et al.,* 2016; Shajahan *et al.,* 2017). The present investigation studied the optimization of cellulase production by fungal strain. The optimizations of environmental factor were studied using statistical design (Plackett–Burman and BOX-Benken).

Materials and Methods

Isolation and screening for cellulase decomposes

Qualitative cellulase detection

Soil samples (1g) were serially diluted and cultivated on PDA plates, purified and each isolate was cultivated on PDA medium supplemented with the 0.5% of carboxy methyl cellulose, incubated at 28°C for 72h. The appearance of a clear zone around the fungal growth after adding 0.2 % Congo red followed by washing with 1M sodium chloride points to cellulase producer.

Quantitative cellulase estimation

The basal medium pH 5.5 was prepared and used for cellulase screening. The pure cultures were cultivated in 250 mL Erlenmeyer flask that contained 50 mL of medium for 7 days 28 °C. Cellulase was dignified by checking the reducing sugar absorption obtainable as glucose by dinitrosalicylic acid (DNS) method (Miller, 1959). 1 mL of crude enzyme was mixed with 1 mL of 0.5% CMC (substrate) in citrate buffer (PH 5, 50 mM), incubated at 45°C for 30 min. The reaction mixture was ended by addition of 2 mL of DNS and boiler for 10 min. Read the absorbance at 540 nm. Cellulase unit was defined as the amount of 1 µg reducing sugar comparable to glucose per minute in standard check conditions.

Physiological and nutritional factors for *Aspergillus penicillioides* 12ASZ cellulase production

Plackett- Burman screening

Plackett-Burman investigational design was functional to examine the significance of several medium conformations for cellulase production. Fifteen variables were tried (Table 1). The Plackett-Burman matrix (Table 2) was constructed on a first-order model. The cellulase enzyme production statistics was exposed to statistical analysis, where Microsoft Excel 2016 was used for the analysis of data.

Box-Behnken design

To define the environment of the response surface in the investigational constituency and to find the optimal conditions for cellulase production, a Box-Behnken design was pragmatic in Table 3.

The matrix of design consist of 13 trials to educate the most significant variables Table 4. The acceptable excellence of the polynomial model equation was conveyed by determination of R^2 (Myers *et al.,* 2004).

Results and Discussion

Isolation and screening for cellulase decomposes

13 fungal isolates were achieved from different sources and tested for cellulase production. Qualitative and Quantitative approximation of cellulase by selected fungi demonstrated that isolate 12ASZ is favorable for cellulase production and produce 8.75 U/mL/min.

Phenotypic and Molecular characterization of the selected fungal isolate

Isolate12ASZ presented 89.97% similarity to *Aspergillus penicillioides* using 18S rDNA sequencing. A phylogenetic tree was created using Mega 5 program (Fig. 1), and indicated that the isolate 12ASZ is more related to *Aspergillus penicillioides* strain (ac: DQ985959.1) with 89.66 % identity. The accession no. at gene bank for the selected fungal isolate was MK713549.

Optimization of culture environments upsetting production of cellulase

The Plackett-Burman design is an approach com-

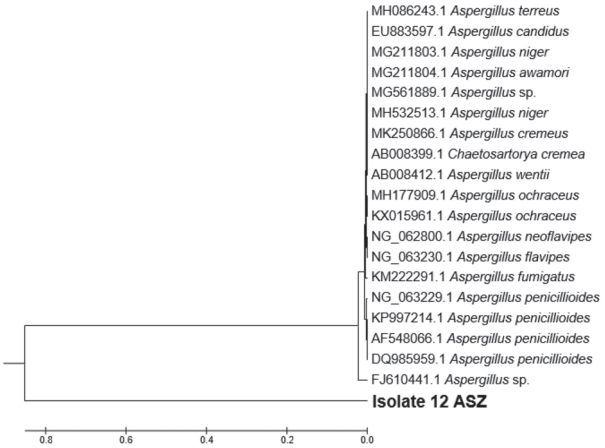

Fig. 1. Phylogenetic tree of isolate coded 12ASZ achieved display the location amid the selected *Asperigillus sp*. based on 18Sr RNA sequence assessments

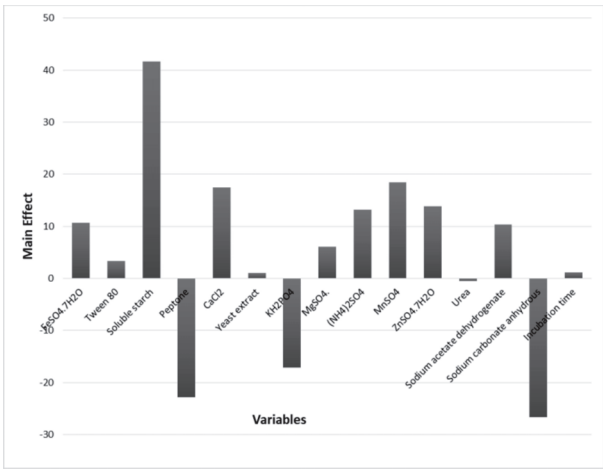

Fig. 2. Effect of culture environments on cellulase by *Aspergillus penicillioides* 12ASZ constructed using Plackett-Burman design.

bined with the result of cellulase activity in matrix (Table 2). The variation ranged (4.5- 52 U/mL/min) for cellulose activity through the altered trials. Effect of pH on cellulase production by fungi supports the findings of (Lee *et al.*, 2002) who reported that CMCase and FPase activities exhibit a pH optimum between 4-7. The main effects of the studied variables on cellulase activity were considered and explained clearly in Fig. 2. Soluble starch, $MnSO_4$ and $CaCl_2$ were the utmost significant variables increasing cellulase production. Both sodium carbonate anhydrous, peptone and KH_2PO_4 were the most significant variables decreasing cellulase production as presented in Table 5. The excellence of acceptable model equation was stated by determination of R^2, which was 0.9128. This is in reasonable agreement with the commonly used environmental factors that have been reported to produce cellulase, cellulose activity improved by some metal ions Mg^{2+} (Imran *et al.*, 2016; Sreeja *et al.*, 2013), Mn^{2+}(Imran *et al.*, 2016) and Ca^{2+} (Imran *et al.*, 2016; Sreeja *et al.*, 2013), $(NH_4)_2SO_4$(Estela and Luis, 2013; Cherian *et al.*,

Table 1. Levels of environmental Variables used in Plackett-Burman design upsetting cellulase production by isolate *Aspergillus penicillioides* 12 ASZ

Variable code.		Variable -1	Values % 1
X1	$FeSO_4.7H_2O$	-	0.0005
X2	Tween 80	-	0.1
X3	Soluble starch	-	0.004
X4	Peptone	0.4	2.00
X5	$CaCl_2$	0.001	0.01
X6	Yeast extract	0.1	1
X7	KH_2PO_4	0.0001	0.05
X8	$MgSO_4$.	0.001	0.05
X9	$(NH_4)2SO_4$	0.001	0.05
X10	$MnSO_4$	-	0.002
X11	$ZnSO_4.7H_2O$	0.0001	0.003
X12	Urea	0.001	0.1
X13	Sodium acetate dehydrogenate	-	0. 1
X14	Sodium carbonate anhydrous	-	0. 1
X15	Incubation time	5 days	7 days

Accession no of the isolated fungi:
(Accession no.: MK713549)

2016), yeast extract (1.0%) (Gautam *et al.*, 2011), soluble starch (Ibatsam *et al.*, 2011), manganese sulphate (Gurung *et al.*, 2013; Agarwal *et al.*, 2014), Tween 80 (Saha *et al.*, 2019) that using another isolate of fungi where our study is the 1st report for cellulase production by this strain.

Box–Behnken design used for description of the response surface as presented in Table 4. Correlation between independent variables and response of cellulase activity were proceeds for calculating the optimum condition. The significant independent variables (Soluble starch, $MnSO_4$ and $CaCl_2$) were supplementary discovered to method of optimum response region for cellulose activity. Table 4 represent the design matrix. Giving the results in the formula of surface plots, (Fig. 3). The optimum environments recognized from the design were confirmed and paralleled with the optimal activity predicted after the design. The predictable cellulase activity was 195.57 U/mL/min, where the actual activity after optimal condition were 192.4 U/mL/min. Therefore, accuracy grade (98.3%) established the strength of the ideal matrix in the succeeding optimum conditions: (%) CMC, 0.3; $FeSO_4.7H_2O$, 0.0005, Tween 80, 0.1; Soluble starch, 0.004279; $CaCl_2$, 0.010606; Yeast extract, 1; $MgSO_4$, 0.05;

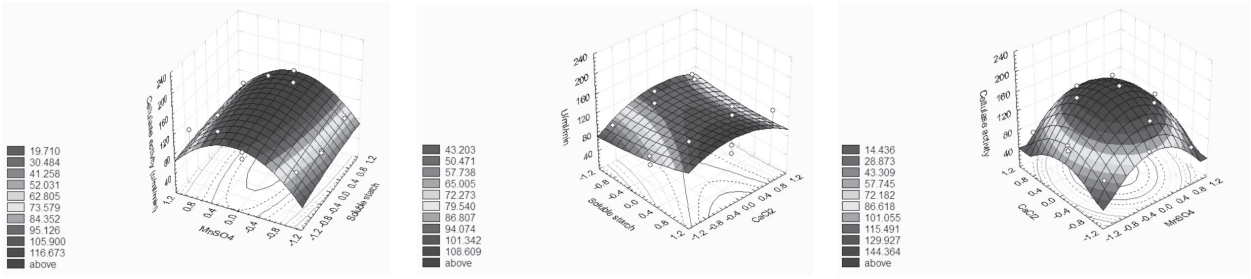

Fig. 3. Three dimensional response surfaces representing cellulase enzyme yield (U/mL/min) from *Aspergillus penicillioides* 12 ASZ as affected by culture conditions

Table 2. Plackett–Burman experimental design for estimating factors manipulating the cellulase production by *Aspergillus penicillioides* 12 ASZ

Trail No.	Variables															Cellulase activity (U/mL/min)
	x1	x2	x3	x4	x5	x6	x7	x8	x9	x10	x11	x12	x13	x14	x15	
1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	9
2	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	43.5
3	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	17
4	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	11.5
5	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	39
6	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	10.5
7	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	39.5
8	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	7
9	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	52
10	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	4.5
11	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	14.5
12	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	1	19
13	-1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	1	1	5.5
14	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	1	1	-1	15
15	-1	1	1	-1	-1	1	1	-1	-1	1	1	1	1	-1	1	39.5
16	1	1	-1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	35.5
17	1	-1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	41
18	-1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	5
19	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	44.5
20	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	28

* -1; low level, +1; high level.

$(NH_4)_2SO_4$, 0.05; $MnSO_4$, 0.002037; $ZnSO_4.7H_2O$, 0.003; Urea, 0.001 and Sodium acetate, 0. 1 at pH 5.5 with a cultivation temperature at 28 ºC and incubation time 7 days under shaking.

Characterization of cellulase produced by *Aspergillus penicillioides*

Effect of temperature

As described in materials and methods different temperatures (25-60 ºC) were tested for 30 min each to determine the optimal value. The optimal value for cellulase detected at 45 ºC. To determine the thermal stability for tested cellulase, the residual activities were plotted against time exposure for each temperature (30, 35, 40, 45, 50, 55 and 60 ºC) for cellulase (Heck *et al.*, 2005). The residual activity was measured under the following reaction conditions (45 ºC and pH 5 citrate buffer). The cellulase enzyme showed complete stability at 30, 35 and 40 ºC for 2h, at 45 and 50 ºC was stable for 1h and at 55 ºC stable for 30 minute then decreased to 80% after 1h. A noticeable drop of thermal stability for cellulase was recognized by raising the temperature to 60 ºC, where the cellulase enzyme lost 60 ºC after 10 min. The stability of cellulase were tested at -20 ºC for up to 2 months without thawing. The cellulase enzyme were stable under this condition without thawing. Thawing freezing the samples up to 10 times a week, it was recognized that cellulase lost 92% of its stability.

Effect of pH: The crude enzyme activity rate was studied as a function of pH ranging from 3.5 to 7.5. The reaction was performed under the following conditions: (citrate buffer for pH 3-5; phosphate buffer for pH 6-7 and tris-HCl for pH 7.5) and the temperature 45 ºC for cellulase. The enzyme has a great preference to work in acidic condition where the optimum was recorded at pH 5. The enzyme stability was monitored for 3h in an acidic condition (pH 3.5- 6) at room temperature where, it showed complete stability under these tested pHs.

Effect of different compounds on cellulase activity

The results showed that the presence of $CaCl_2$, NaCl, DMSO, $FeSO_4$, $MgCl_2$, $MgSO_4$, glycerol and ethanol did not decrease the activity, while on the other hand $MnSO_4$, $ZnCl_2$, $CuSO_4$, EDTA, isopropanol methanol and SDS caused a decrease in the activity (Fig. 5). Study by Imran *et al.*, 2016 reveled

Table 3. The intensities of selected variables used in Box–Behnken design for optimization of cellulase by *Aspergillus penicillioides* 12 ASZ

Variables	Variable code	-1	0	+1
Soluble starch	X1	0.002 %	0.004 %	0.006 %
$MnSO_4$	X2	0.001 %	0.002%	0.003%
$CaCl_2$	X3	0.002 %0.01 %	0.05 %	

Table 4. Box-Behnken factorial design representing response of cellulose enzyme activity as influenced by soluble starch, $MnSO_4$ and $CaCl_2$ by *Aspergillus penicillioides* 12 ASZ

Trail	Soluble starch	$MnSO_4$	$CaCl_2$	Cellulase activity (U/mL/min)	Measured Predicted
1	0	-1	-1	47.75	24.55
2	0	1	-1	24.55	43.25
3	0	-1	1	43.25	47.75
4	0	1	1	66.35	57.3
5	-1	-1	0	70.45	65.45
6	-1	1	0	100.65	66.35
7	1	-1	0	57.3	70.45
8	1	1	0	78.85	78.85
9	-1	0	-1	65.45	81.85
10	-1	0	1	81.85	100.65
11	1	0	-1	141.85	112.7
12	1	0	1	112.7	141.85
13	0	0	0	194.8	194.8

Table 5. Statistical analysis of Plackett-Burman design showing *P*-values, t stat, standard error and coefficient values for each variable on *Aspergillus penicillioides* 12 ASZ

	Coefficients	Standard Error	t Stat	P-value
Intercept	24.05	2.311851	10.40292	0.000482
x1	2.676959	3.519602	0.760586	0.48927
x2	0.824211	2.891835	0.285013	0.789782
x3	10.42697	2.863143	3.64179	0.02193
x4	-5.69568	2.839297	-2.00602	0.115321
x5	4.360172	2.874992	1.516585	0.203964
x6	0.250204	2.83175	0.088357	0.93384
x7	-4.28216	3.201245	-1.33765	0.251999
x8	1.526613	2.986993	0.511087	0.636208
x9	3.294253	3.201245	1.029053	0.361608
x10	4.603021	2.83175	1.625504	0.179381
x11	3.475312	2.874992	1.208808	0.293304
x12	-0.12649	2.839297	-0.04455	0.966601
x13	2.576261	2.863143	0.899802	0.419099
x14	-6.67139	2.891835	-2.30697	0.082306
x15	0.295621	3.519602	0.083993	0.937098

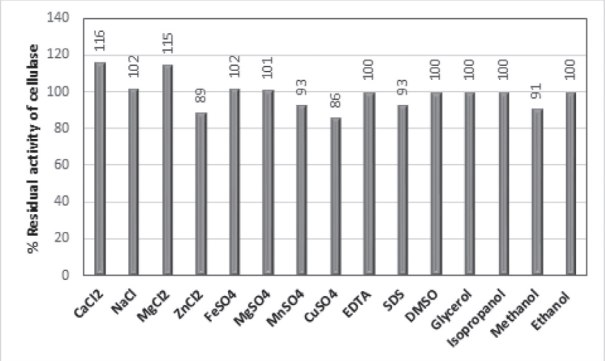

Fig. 5. Effect of different materials on cellulase activity by *Aspergillus penicillioides* 12 ASZ

that cellulase activity is enhanced by some metal ions K^{+}, Mg^{2+}, Ca^{2+} and Mn^{2+} while is inhibited by Zn^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, Ag^{+} and Fe^{3+}using *Aspergillus* and *Humicola* species.

Conclusion

In this work, the applied of Plackett-Burman design and Box-Behnken design Response surface methodology (RSM) proved to be efficient in improving functional factors for cellulase enzyme production. Using the optimized conditions the maximum cellulase production of 195.57 U/mL/min, which was nearby 22.74 times of the activity using the basal medium.

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