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Mutationsand Statistical Modeling for Cellulase Production by *Aspergillus flavus*

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Abstract

Cellulosic materials present in nature as environmental wastes, however, it is considered as a source of energy and sugars. Biological degradation of cellulosic materials using cellulose-degrading enzymes can solve these issues. For economic process, cellulase productivity should be at the highest yield and lowest cost. Therefore the improvement of cellulase productivity is a crucial step for beneficial process. The recent biotechnological methods such as mutagenesis and modeling provide effective tools for stimulation of microbial enzyme production. In the current work, Gamma and UV-radiation and Ethidium bromide have been used for improving cellulase production by a promising fungal isolate. In addition, statistical methods have been used to optimize nutritional and environmental conditions. The fungal isolate has been identified on molecular basis as *Aspergillus flavus* and given an accession number in NCBI. Mutagenesis resulted in 343.77% increase in cellulase activity compare with the wild-type, while statistical modelling resulted in 1.48% compared with the mutated cells productivity. The over all used methods resulted in 510.69% increase in cellulase production by the mutated *Aspergillus flavus* compared with the wild-type. Data showed that *Aspergillus flavus* is a promising cellulase fungal source for application in biotechnological industries. Mutagenesis and statistical modeling provide vital tools as driving force for evolution and improvement of metabolites productivity.

Keywords: *Aspergillus flavus;* Fungi; Cellulase; Gamma-radiation; UV-radiation; Design of Experiment; Central Composite Design.

Introduction

Cellulose; a linear biopolymer of β (1→4) D-glucose units linked together with glycosidic bonds, is a plant polysaccharide represents the most abundant polysaccharide on earth. It provides a mechanical support to the plant cells such as wood pulps [1] and can be found in the pure form i.e., cotton. Although, found in other organisms such as bacteria (e.g., *Acetobacter xylinum* [2]) and filamentous fungi [3], plants are the main sources of cellulose. It is bioavailability, utilization in many industries such as food-grade cellulose, fabrics, artificial fibers, papers [4], lacquers, yarns and ropes, glucose production, in some pharmaceuticals and cosmetic products [5] and biomedical applications [6], along with insolubility in water and most organic and weak acids, cellulose results in significant environmental issues and huge amounts of agricultural, industrial and municipal wastes. These issues urge the economic disposal of these cellulosic materials. The hydrolysis of cellulose and cellulosic materials imposes environmental, agricultural, economic and even strategic advantages such as: animal feed, waste management, biofuel production [7] …etc. In addition, cellulose hydrolysis is a core of many industries e.g., fodders and biotechnological applications. Group of enzymes; endo and exo-*β*-(1-4)-D-glucanases (cellulases) are responsible for complete hydrolysis of cellulose into glucose building blocks [8]. For industrial production of cellulases many microorganisms have been used i.e., *Aspergillus niger, Aspergillus Phoenicis, Trichoderma reesei, Phanerochaete chrysosporium, Trichoderma harzianum, Penicillium echinulatum, Pseudomonas sp.* and *Bacillus pumilus* [9, 10, 11, 12, 13, 14, 15]. For decades, cellulase(s) industries are based on its economic production using cheap components (e.g., agro-industrial wastes) for production media [16] and/or improving productivity of the microorganism using traditional [17] or statistical optimization methods [18]. Mutagens is a

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process of genetic information changing for organism with stable and consistent manner either naturally or laboratory by using physical (radiation) and chemicals mutagens [19]. Traditional genetic improvement depends upon random mutagenesis and protoplast fusion technique for producing strains with desirable qualities [20]. The progress in genetic engineering validated the concept of mutagenesis for improving and modification of the metabolic activities of microbial cells [21, 22]. However, mutagenesis may spontaneously take place, the induced mutation guarantees greater mutation with less mismatch repair [23].

Many mutagenic techniques have been developed for induction of the cell mutation including chemical and physical mutagens. Radiation (ionizing and non-ionizing) has been considered for a long time as a promising mutagen [24, 25]. The changes result from the induced mutagenesis are random and cannot be expected as it varies from one case to another depending on the work objectives, the biological cell composition, its resistance to irradiation and radiation conditions. Generally, it has been reported that microbes show more resistance to mutagenesis compared with plant and animal cells [26]. Many efforts were made to mutate the wild strain *H. insolens* TAS-13 for enhanced cellulase production through UV, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), Nitrous acid (HNO_2), Ethyl methyl sulphonate (EMS) or Ethidium bromide (EtBr) [27]. The using Ethidium bromide as a chemical mutagenic agent to mutagenesis of local isolates *Saccharomyces boulardii* was studied and the changes that happen in therapeutic and physiological characteristics was tested [28].

The current work aims to increase cellulase enzyme productivity by subjecting a fungal cellulase producer to multiple mutations (chemical and physical). The production process of the muted cells with the highest productivity is going to statistical modeled using Design of Experiment (DOE) and the variables affecting its production will be optimized for maximum production levels.

Materials and Methods:

Medium and Organism:

For deveolping microbial cells and screening for cellulase production, the following medium has been used (g/l): 20, cellulose (or carboxymethyl cellulose (CMC)); 1, K_2HPO_4; 1, $MgSO_4$; 1, NaCl; 1, NH_4Cl and 20, Agar-Agar (in case of solid medium). The medium pH was adjusted to 5 ± 0.2 and autoclaved at 121°C for 15 min. If not otherwise mentioned, cultures were incubated at 30°C and 150 rpm for 10 days.

Isolates from microbial biotechnology lab, national research centre, Egypt, have been screened for a promising organism capable of cellulosic materials degradation using CMC-plate method according to Kasana [29]. The hydrolysis area around the microbial growth indicates positive test for cellulase production.

Cellulase determination:

The enzyme activity was determined as CMCase activity according to Ghose [30] with few modifications. Breifly, 1.5 ml, of 1% carboxymethyl cellulose (CMC) in 0.05M citrate buffer (pH: 5) was added to 1.5 ml of the filtered culture media and incubated at 45°C for 90 min. At the end of incubation, reducing sugars were determined using 3, 5-dinitro salicylic acid (DNS) method [31] by adding 3 ml of DNS reagent solution to the reaction mixture, incubation in water-bath at 90°Cfor 15 min and finally adding 1 ml of Rochelle salt to stabilize the color. Absorbance was read at 575 nm and compared with glucose standard curve.

Cellulase Unit: was defined as the required amount of the enzyme to liberates (1 µmol) of glucose from carboxymethyl cellulose (CMC) per millilieter per minute under the assay conditions.

Mutagenic stimulation for cellulase production:

Three mutation methods (a chemical; Ethidium Bromide, and two physical; λ-ray & UV-radiation) have been used in different combinations for selecting a mutated fungal cells with the highest cellulase productivity compared with those subjected to a single mutation method. Gamma irradiation was performed using Cobalt-60 gamma cell (MC20, Russia) at a dose rate of 0.47 KGy h^{-1}. The γ-irradiated spores were incubated overnight at 7°C in dark room to avoid photo-reactivation.UV-irradiation was performed using UV-light source (254 nm, 70 µW cm^{-2}) [25]. Ethidium Bromide have been prepared at concentration of (100 µg/ml). This test has been accomplished at the Microbiology laboratory of Nuclear Research Center, Egyptian Atomic Energy Authority, Cairo, Egypt.

For designing of this experiment Quadratic Central Composite Design (CCD) has been selected for combination of the different mutation methods (γ-radiation (KGy), UV-radiation exposure time (min.) and Ethidium Bromide exposure time (min.)) at four levels each, as shown in table (1). Cellulase activity (U/ml) was represented as the design response. The design and analysis was performed using Design-Expert (version 7.0.0) software (Stat-Ease Inc., Minneapolis, MN, USA).

Identification of the fungal isolate using genotypic methods:

The most potent fungal isolate has been identified on molecular basis using 18s rDNA. All length identification process has been accomplished by Sigma Scientic Services Co. (http://sigmaeg-co.com/), Giza, Egypt, using forward and reverse primers and sequenced using ABI 3730xl DNA sequencer (SeqGen, Inc., Torrance, USA). The data were

submittedto the Genbank data base (https://www.ncbi.nlm.nih.gov/) and assigned accession number.

Table (1): Central Composite Design (CCD) used for mutation of the fungal cells:

Run	γ-ray (KGy)	UV-ray (min.)	EthidiumBromide (min.)
1	1	20	30
2	1	20	30
3	1.5	30	45
4	1	0	30
5	0.5	30	15
6	1	20	0
7	0.5	10	45
8	1	20	60
9	0	20	30
10	1.5	10	15
11	1	40	30
12	0.5	10	15
13	1.5	30	15
14	0.5	30	45
15	1	20	30
16	1	20	30
17	1	20	30
18	1.5	10	45
19	2	20	30
20	1	20	30

Optimization of Cellulase Production:

Two-steps optimization method have been validated for cellulase production. The most significant carbon and nitrogen source(s) affecting cellulase productivity have been screened using Plackett Burman design. Eleven variables were used in this study with three center points and overall fifteen runs. These factors along with their codes, levels and units were represented in table (2). Through this model two responses were recorded; enzyme activity (U/ml) and cell dry weight (g/l).

Table (2): Summary of Plackett Burman design used for screening of the significant factors for cellulase production:

Factor	Name	Units	Low level	High level
A	Cellulose	g/l	0	10
B	Carboxymethyl cellulose	g/l	5	15
C	Glucose	g/l	0	10
D	Sucrose	g/l	0	10
E	Yeast extract	g/l	0	5
F	Peptone	g/l	0	5
G	Urea	g/l	0	3
H	$(NH_4)_2SO_4$	g/l	0	10
J	$NaNO_3$	g/l	0	3
K	NH_4Cl	g/l	1	3
L	$CaCl_2$	g/l	0	2

The second step in optimization process was Box-Behnken design (a response surface method), in which four factors were involved; glucose and sucrose concentrations, pH and incubation temperature table (3). Two responses have been evaluated; cellulase productivity and cell dry weight.

Table (3): Summary of Box-Behnken design used for cellulase optimization:

Factor	Name	Units	Low level	High level
A	Glucose	g/l	10	50
B	Sucrose	g/l	10	50
C	pH		5	7
D	Temperature	°C	25	35

Plackett Burman and Box-Behnken designs were formulated and analyzed with the aid of Design-Expert (version 7.0.0) software (Stat-Ease Inc., Minneapolis, MN, USA).

Optimization of cellulase production and model validation:

The values of the model variables have been numerically optimized using Stat-Ease software for maximum cellulase production. The data obtained (theoretically) have been experimentally applied. Cellulase production was determined and compared with the theoretical value for calculation of the model validation.

Results:

Mutagenic stimulation for cellulase production:

According to the designed CCD model, the mutation of the most potent fungal isolate has resulted in the different cellulase productivities as shown in table: (4& 5). The data were compared with mutagen controls and the wild cells fig(1). Data showed that "run 3" revealed the maximum cellulase productivity (23.85 U/ml). The runs "2 & 4" showed comparative results with the mutagen controls Gamma ray at 0.5 KGy and exposure to Ethidium bromide for (15 min) which represent (≈ 3X) of the wild-type cellulase productivity (6.94 U/ml).

The actual (experimental) and predicted (theoretical) cellulase productivity of the current model have been shown in table (4). Figure (2) shows the relation between the actual and predicted results. The figure shows a significant correlation coeffecient between these results which is confirmed with ANOVA table (6).

Analysis of Variance (ANOVA) table (6) of the mutation CCD model showed that the model is significant along with the model terms: B, ABC and B^3. The results indicated insignificant model "lack of fit". Furthermore, R^2 of 0.85 and adequate precision of 8.24 which indicate adequate signal. The model is accurate and can be used for prediction of the model results

Table (4): Cellulase productivity of the mutated fungal cells according to CCD:

Run	Cellulase Production(U/ml)

	Actual[1]	Predicted	Residual
1	13.15	11.52	1.63
2	20.98	11.52	9.45
3	23.85	22.60	1.25
4	20.40	21.14	-0.74
5	16.03	15.79	0.24
6	9.12	10.79	-1.67
7	14.76	13.51	1.25
8	8.55	12.25	-3.70
9	16.26	17.00	-0.74
10	9.24	9.00	0.24
11	-6.87	-6.13	-0.74
12	7.51	7.28	0.24
13	6.13	5.90	0.24
14	7.28	6.03	1.25
15	7.40	11.52	-4.13
16	13.38	11.52	1.86
17	9.70	11.52	-1.82
18	NA	-1.25	1.25
19	NA	0.74	-0.74
20	6.94	11.52	-4.59

Table (5): Results of cellulase productivity of the mutagen controls:

Mutant	Unit	Dose	Cellulase (U/ml)
Gamma ray	**(KGy)**	0.5	19.48001
		1	6.477448
		1.5	7.628117
		2	7.397983
UV -ray	**Minutes**	0	6.132247
		10	6.477448
		20	6.822649
		30	6.707582
Ethidium bromide	**Minutes**	15	20.51561
		30	16.60334
		45	6.247314
		60	5.671979
Wild-type fungal cells (Blank)			6.937716

[1]: Runs 18 and 19 showed no growth.

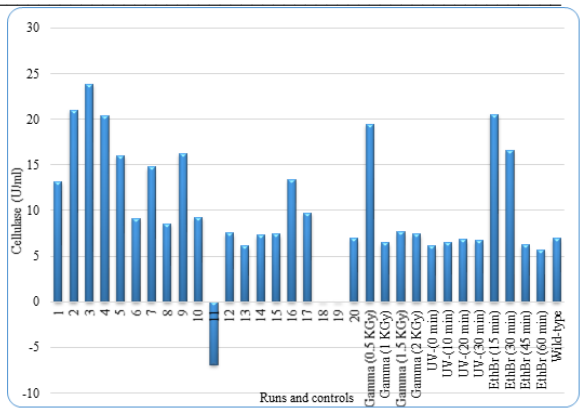


Figure (1): Comparison between cellulase productivities of CCD runs, controls and wild type.

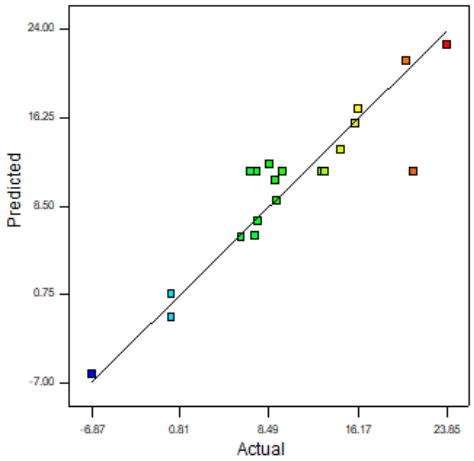


Figure (2): Relation between actual and predicted cellulase productivity according to CCD

Table (6): Analysis of variance (ANOVA) for CCD:

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-value Prob > F[2]
Model	910.131	11	82.73919	4.08469	0.0279
A-Gamma rays	0.367198	1	0.367198	0.018128	0.8962
B-UV	147.5704	1	147.5704	7.285295	0.0271
C-Ethidium Bromide	2.127744	1	2.127744	0.105043	0.7542
AB	48.56123	1	48.56123	2.397384	0.1601
AC	12.44288	1	12.44288	0.614283	0.4558
BC	15.02906	1	15.02906	0.741959	0.4141

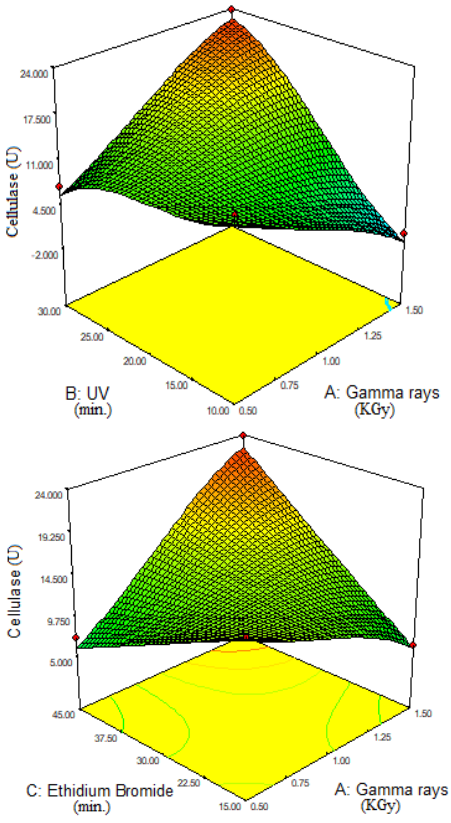
[2]: *p*-value of less than 0.05 indicates that the model term is significant.

A^2	11.57573	1	11.57573	0.571474	0.4713
B^2	26.55115	1	26.55115	1.310784	0.2853
ABC	230.6272	1	230.6272	11.38566	0.0097
A^3	42.75164	1	42.75164	2.110574	0.1844
B^3	364.0758	1	364.0758	17.97379	0.0028
Residual	162.0474	8	20.25593		
Lack of Fit	26.18327	3	8.727756	0.321194	0.8106
Pure Error	135.8642	5	27.17283		
Cor Total	1072.178	19			

The data extracted from the model, resulted in the generation of following model equation for prediction of the cellulase productivity on exposure to the studied mutagens. Figure (3) shows the interactions between the different variable and their effect on cellulase production.

Cellulase (U/ml)= 3.26554 + 7.84716 * A - 1.44357 *B + 1.10706 * C - 1.65493 * A * B - * 1.2655A * C- 0.062452 * B * C + 23.50283 * A^2 + 0.18077 * B^2 + 0.071589 * A * B * C + 8.71797 * A^3 - 0.00318013 * B^3

Where, A: Gamma radiation(KGy); B: UV-radiation exposure time; C: Ethidium bromide exposure time.



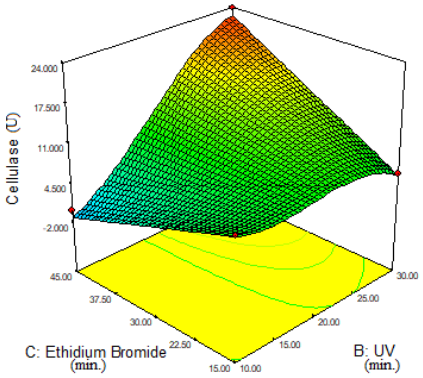


Figure (3):3D diagrams show the effect of interaction between different factors on cellulase productivity.

Identification of the fungal isolate using genotypic method:

According to molecular identification using 18s rDNA, the most potent fungal isolate was identified as *Aspergillus flavus* based on maximum and total scores (983) and query cover (97%). Data were submitted to the Genbank data base of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) and assigned the accession number of (MZ234077.1). The phylogenetic tree is represented in figure (4).

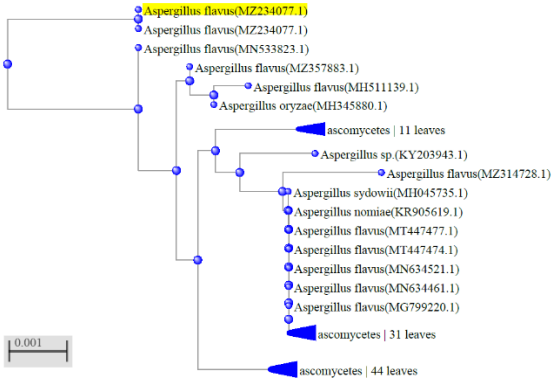


Figure (4): Phylogenetic dendrogram of the 16S rRNA sequence of the fungal species.

Selection of the significant nutritional substances:

Screening for the perfect carbon and nitrogen sources for cellulase production using Plackett Burman design showed the data (exprimental (actual) and theortical (predicted) results) along with the residual (the diffrenece between the actual and predicted results) represented in table (7). The relation between the actual and predicted cellulase productivity has been represented in figure (5) and showed a good correlation coefficient (R^2 = 0.9056) which has been confirmed with ANOVA as follows.

Table (7): Results of Plackett Burman design:

Run	Cellulase (U/ml)		
	Actual	**Predicted**	**Residual**
1	19.25	18.25	0.997247
2	-0.312	-3.303	2.99174
3	1.76	0.034	1.726004
4	3.60	7.053	-3.45201

5	11.31	10.198	1.112314
6	15.80	14.65	1.150669
7	3.025	0.0337	2.99174
8	26.15	23.20	2.953385
9	5.09	4.29	0.805469
10	7.74	10.66	-2.91503
11	3.37	2.105	1.265736
12	6.48	9.239	-2.76161
13	-4.68	0.0337	-4.71774
14	11.195	10.198	0.997247
15	9.699	12.84	-3.14516

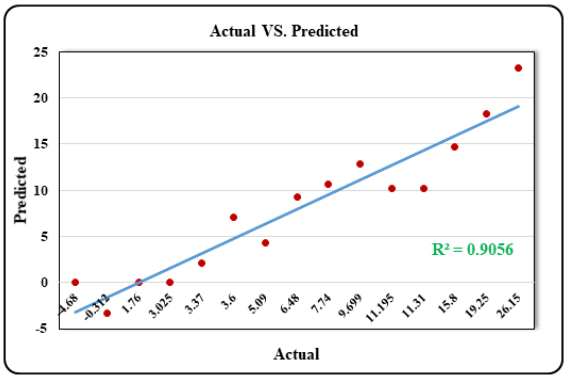


Figure (5): Relation between actual and predicted cellulase productivity according to Placket-Burman design.

Partial Analysis of Variance (sum of squares - type III) of the produced Placket-Burman model (Table: 8) showed that the model is significant along with the model terms C, D, G and H. Also, ANOVA showed significant curvature and insignificant model "Lack of Fit". The model R^2 of 0.85 indicated a good correlation and the Predicted-R^2 of 0.7063 is in reasonable agreement with the adjusted-R^2 of 0.7854. The model adequate precision (a ratio between signal and noise) of 12.79 indicated adequate signal.

Table (8): Analysis of variance (Partial sum of squares) of cellulase production according to Placket-Burman design:

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-value Prob > F[3]
Model	553.69	4	138.42	12.90	0.0009
C-Glucose	73.44	1	73.44	6.84	0.0280
D-Sucrose	173.03	1	173.03	16.12	0.0030
G-Urea	87.74	1	87.74	8.18	0.0188
H-$(NH_4)_2SO_4$	219.48	1	219.48	20.45	0.0014
Curvature	235.93	1	235.93	21.98	0.0011
Residual	96.59	9	10.73		
Lack of Fit	62.41	7	8.92	0.52	0.7832
Pure Error	34.19	2	17.09		
Cor Total	886.22	14			

According to data analysis, a final equation has been generated to represent cellulase productivity based on Placket-Burman model design as follows:

Cellulase (U/ml) = 10.6582135 + 0.494787851 * C + 0.759441817 * D - 1.802715424 * G - 0.855330935 * H

Where: C: Glucose, D: Sucrose, G: Urea, H: $(NH_4)_2SO_4$

According to the equation, glucose and sucrose have direct relations with cellulase production in contrarily with urea and $(NH_4)_2SO_4$ which have inverse relations. Therefore, only glucose and sucrose have been considered for the next step.

Modelling of cellulase production:

Statistical modelling of cellulase production by *Aspergillus flavus*has been performed using Box-Behnken design (Response Surface Method). Four significant variables have been used for this model and the experimental (actual) and theoretical (predicted) results along with residuals of the model have been shown in table (9).The results showed a good relation between the actual and predicted data which is represented in figure (6) with correlation coefficient of (R^2 = 0.9146) and confirmed with the ANOVA data analysis.

Table (9): Results of cellulase production model according to Box-Behnken design:

Run	Cellulase (U/ml)		
	Actual	Predicted	Residual
1	1.530	6.725	-5.196
2	30.642	29.106	1.536
3	7.743	7.876	-0.133
4	14.762	13.226	1.536
5	28.110	30.165	-2.055
6	3.256	2.506	0.750
7	18.099	21.717	-3.618
8	3.831	11.903	-8.072
9	21.436	21.717	-0.281
10	21.896	18.404	3.492
11	28.570	21.717	6.853
12	26.154	24.024	2.130
13	15.453	19.550	-4.098
14	1.415	-0.212	1.627
15	22.817	22.950	-0.133
16	1.530	0.780	0.750
17	17.869	14.377	3.492
18	26.154	23.031	3.123
19	0.609	6.375	-5.766
20	7.053	7.272	-0.219
21	35.014	35.233	-0.219
22	2.795	2.353	0.443
23	4.061	2.434	1.627
24	0.494	-1.636	2.130
25	1.415	-1.708	3.123
26	13.842	16.276	-2.434
27	5.097	5.483	-0.387

[3]: "Prob > F" Value < 0.05 indicates a significant model term.

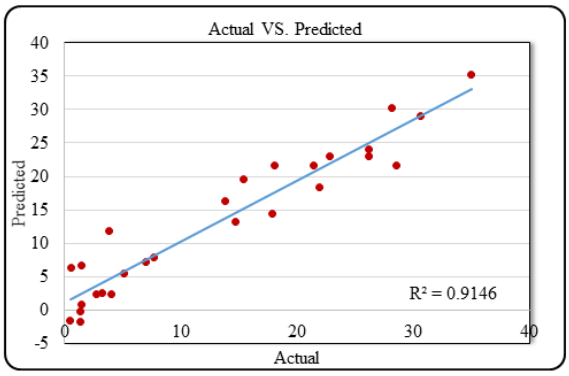


Figure (6): Relation between actual and predicted cellulase productivity according to Box-Behnken design.

Data obtained from Box-Behnken design have been analysed by ANOVA (Table: 10) where it showed a significant model *F*-value (6.695) along with the model terms: D, AB, BC, BD, D^2, A^2C and AD^2. The model shows insignificant "Lack of Fit".

The model R^2 of (0.914) indicates a very good correlations and the model adequate precision value of 8.845 indicates adequate signal. The model can be used to predict results at any point in the design space according to the following equation.

Table (10): ANOVA of cellulase production based on Box-Behnken design:

Source	Sum of Squares	df	Mean Square	*F*-Value	*p*-value Prob > F
Model	2967.67	16	185.48	6.695	0.0022
A-Glucose	66.20	1	66.20	2.389	0.1532
B-Sucrose	47.27	1	47.27	1.706	0.2207
C-pH	19.12	1	19.12	0.690	0.4255
D-Temperature	273.64	1	273.64	9.877	0.0105
AB	358.29	1	358.29	12.932	0.0049
AC	0.16	1	0.16	0.006	0.9405
AD	122.02	1	122.02	4.404	0.0622
BC	255.82	1	255.82	9.233	0.0125
BD	143.21	1	143.21	5.169	0.0463
A^2	70.51	1	70.51	2.545	0.1417
B^2	50.32	1	50.32	1.816	0.2075
D^2	923.04	1	923.04	33.316	0.0002
A^2C	196.99	1	196.99	7.110	0.0236
AD^2	252.11	1	252.11	9.100	0.0130
B^2C	28.84	1	28.84	1.041	0.3317
BD^2	52.00	1	52.00	1.877	0.2007
Residual	277.05	10	27.71		
Lack of Fit	219.83	8	27.48	0.960	0.6036
Pure Error	57.23	2	28.61		
Cor Total	3244.73	26			

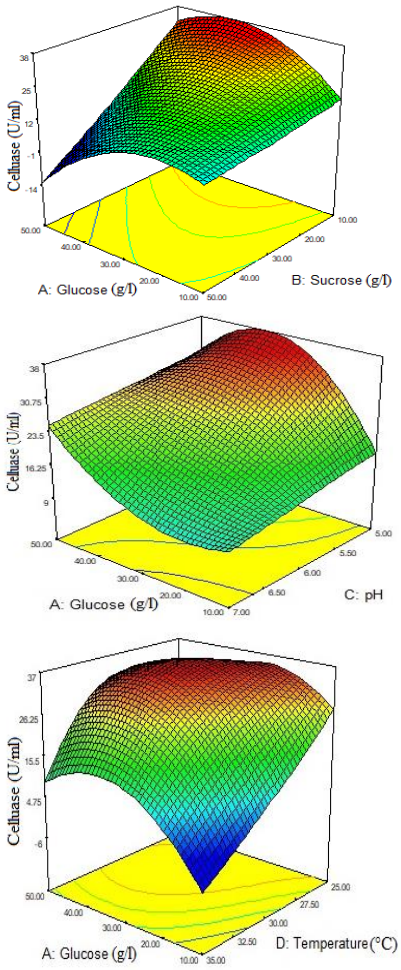
From the obtained data and data analysis, a final model equation of cellulase production by *Aspergillus flavus* could be concluded as follows:

Cellulase (U/ml)438.90341= - 8.91913 * A - 14.53727 * B - 0.69759 * C -25.53911 * D - 0.023661*A * B - 1.47861 *A*C + 1.22201*A*D + 0.96944 * B * C + 0.58972 * B * D - 0.15744 * A^2 + 0.049719 * B^2+ 0.35220 * D^2 + 0.024811 * A^2 * C - 0.019446 * A * D^2 - 0.009493023 * B^2 * C - 0.008831388 * B * D^2

Where, A: Glucose, B: Sucrose, C: pH, D: Temperature.

Optimization of cellulase production and model validation:

The values of the model variables have been numerically optimized for maximum cellulase production. Theoretically, the maximum cellulase production (36.3948 U/ml) at 36.34 g/l, glucose; 11.73 g/l, sucrose at pH (5.13) and incubation temperature (27.64°C). Application of these values resulted in 35.43 U/ml of cellulase which indicates 97.349% model validation. The following 3D figure (7) indicates the interactions between the different variables and their effect on cellulase productivity.



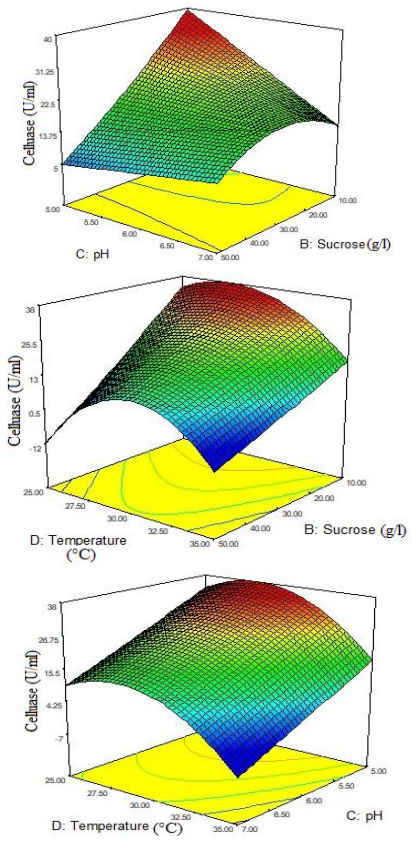


Figure (7): 3D diagrams plotted to show the interactions between the variables and their effect on cellulase productivity by *Aspergillus flavus.*

The overall improvement in cellulase productivity by *Aspergillus flavus* has been summarized in table (11). Data shows that cellulase production improved by 28.49 U/ml (510.69%). The multiple mutation modelling revealed 16.91 U/ml and 11.58 U/ml increase in cellulase productivity, respectively.

Table (11): Production improvement in cellulase according to the current research:

	Cellulase production (U/ml)	**Production improvement (%)**
Wild-type cells	6.94	100
Mutation	23.85	343.77
Plackett Burman	26.15	376.93
Box-Behnken	35.43	510.69

Discussion

Efforts were made to mutate the wild strain *Aspergillus flavus* MZ 234077.1 through UV, γ-rays, and Ethidium bromide (EtBr), in parallel with the most advanced statistical techniques (Design of Experiment (DOE)) for enhanced cellulase (s) production.

The solid cellulosic waste materials such as agricultural (i.e., sugar cane bagasse, rice and wheat bran, mango and citrus peels and corn cobs), municipal and industrial are, usually, dumped or discarded indiscriminately to be burnt or naturally decomposed causing serious environmental and health issues. The biotechnological hydrolysis of cellulosic wastes may serve to solve these problems and represent a source for microbial add-value products such as enzymes, organic acid, biofuels, food additives …etc [32].

Many companies produce cellulase using submerged fermentation (SmF) technology [33]. The cost of cellulase production is relatively high which restricted its use into special industries such as: laundry detergents, textile, and food processing. These industries can afford cellulase production cost. Although the difficulty of cellulase use in other industries, it stimulated continuous cost-reduction efforts. Cellulase production cost can be reduced using alternative and/or combined technologies which may include genetic manipulation, metabolic engineering, modeling and optimization of production process and stat of fermentation (solid or submerged stat). Despite this, these alternatives need to be studied in depth with special focus on the economic aspects and comparing with the current outputs.

Microorganisms represent the main source, most useful, economic, available, and non-seasonal source of cellulase enzymes and have been applied in many biotechnological and industrial applications. When fungi are grown with mutagens at sub-lethal concentrations, rate of enzyme production increased [34]. In this concern, much work has been conducted for improving microbial cellulase productivity [35, 36, 37, and 38]. Recently, genetic manipulations dominated the scope of most improvement processes [39]. Physical and chemical mutations represent simple methods for genetic manipulation. From the nonionizing radiations, UV- radiation was reported as the most simple and cheapest mutagen. According to Irum and Anjum [24], UV radiation is the mutagen of choice in most cases. The pyrimidine bases dimerization and DNA cross-linking are the main mechanisms by which UV radiation induces mutagenesis [40]. The γ-rays are ionizing electromagnetic radiations; when collides with an atom, it transfers some energy to electrons causing its ionization. The exposure of the biological cells to γ-rays may cause various changes in the DNA structure, free-radicals formation that may result in alternations in the molecular structure of the cell and finally phenotypic changes [41]. The changes result from the γ-rays are random and cannot be expected as it varies from one case to another depending on the work objectives, the biological cell composition, its resistance to irradiation and radiation conditions. The breakage of the sDNA and/or dDNA, DNA-protein cross linking, nucleotides removal and oxidation of the bases [42] are some of the mutations takes place, single or combined, in the living cells by γ-rays. The mutagenesis using UV irradiation depends on bond-formation between the adjacent pyrimidine residues (pyrimidine dimers) in the same polynucleotide strand which result in DNA distortion and inhibition of its

replication and transcription [21]. In industry, γ-rays are used for sterilization of medical and pharmaceutical products at (25 kGy) [43] which is the recommended dose to avoid any unfavourable biophysical changes [44]. Lower doses have been used to produce microbial mutants with advanced characters such as thermo- and halo-tolerance and stimulation of higher productivity of a certain metabolite(s).

The current study used different mutagenic methods to improve cellulase production by a selected fungal isolate. Data showed that a comined mutaion with 1.5 KGy gamma radiation, 30 min exposure to UV and 45 min exposure to ethidium bromide was the optimum mutagenic mix compared with the other combinations and mutagen controls. Mutagenesis resulted in (343.77%) incease in cellulase production.

One of the main applications of gamma irradiation is microbial mutagenesis for various fermentation industries. Due to their resistance, gamma irradiation is usually applied on fungi and spore-forming bacteria. The microbial response to gamma irradiation depends on various factors. Gamma and UV radiation and chemical mutagens were used for production of thermo-tolerant microbial mutants to be used in fermentation industries and for production of mutants with disease-controlling capabilities to be used in agriculture industries [45, 46, and 47]. Khaliq et al. [48] reported a higher tylosin productivity of *Streptomyces fradiae* by combination of UV and Gamma radiation. Mutagenesis has been used for maximizing cellulase enzyme productivity by *Aspergillus niger* [49] and by *Trichoderma reesei* [50]. Shahbazi and co-workers [50] reported increase in cellulase production by irradiation of *Trichoderma reesei* cells using γ-rays (250 Gy) compared with both wild type and UV irradiation. Moreover, Berger et al. [51] has also reported weak induction of frame shift mutations by UV irradiation. These radiations alter the genome by making pyrimidine dimmers, which are photo-chemically repairable. In the same way, Mostafa [49], reported increase of filter paper cellulase and carboxymethyl cellulase production by *Aspergillus niger* at 2 KGy of γ-ray. Javed et al., [27] studying many chemical mutagenesis in addition to UV for improvement cellulase production by *Humicola insolens* among of them EtBr. However, EtBr was unique among all the mutagens tested, by giving recovery of 11% survivals with a prolonged exposure time of 30 minutes. Hence, it is concluded that EtBr is less lethal to wild strain [52].

In addition to the increase of cellulase productivity, the used CCD design resulted in a significant model that can be used to detect cellulase production at different values of these mutagens which can save time and efforts.

The fungal isolate under investigation has been identified depending on molecular basis as *Aspergillus flavus* and given the accession number of (MZ234077.1) in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). *Aspergillus flavus* has been studied and evaluated for cellulase productivity in many researches [53, 54, and 55]. Through these researches *Aspergillus flavus* have been used for economic production of cellulase through different biotechnological strategies including optimization of culture and nutritional conditions.

Further improvement for cellulase production by *Aspergillus flavus* have been applied using two statistical optimization methods. The first design is Plackett Burman which has been used to select the most significant carbon and nitrogen sources that affect cellulase production. The data resulted in a significant model and good matching between the predicted and actual results. Glucose, sucrose, urea and $(NH_4)_2SO_4$ are signifcant model variables. From the model equation, it can be concluded that glucose and sucrose have a positive effect in contrarely with urea and $(NH_4)_2SO_4$ which have inverse effects. It has been noticed that cellulose and carboxymethyl cellulose are insignificant factors in this process which is translated as the cellulase production by the current *Aspergillus flavus* is "constitutive". Although this design caused a slight improvement in cellulase production (109.65%) compared with the value of mutagenesis, it gave valuable information about the significant factors.

The second design was Box-Behnken in which four variables have been used. The model showed compatible correlation between the actual and predicted results. Analysis of these data revealed significant model and model variables. The model can be used to predict results at any point in the design space. For optimization and validation of the variable values and results, Design-Expert software have been adjusted for maximum cellulase production. The predicted values of the model variables have been practically applied and the resulted cellulase units have been compared with the predicted units. It has revealed 97.349% data validity of the model which is a very promising for further investigations. This model resulted in (135.48% and 510.69%) increase in cellulase productivity compared with Plackett Burman and the wild-type cells, respectively.

Conclusions:

The statistical methods have provided highly useful and interactive tools in biological, expensive, and dangerous fields. These methods allow theoretical data before conducting them practically. In biology, the conventional optimization method (one variable at a time approach; OVAT) shows high difficulty and laboratory tools overconsumption especially in case of multiple variables. In addition, the conventional optimization method does not show the interactions between these variables and cannot predict the results at different values of the variables. These defects in conventional methods have been resolved in mathematical and statistical methods which also can calculate the significance of each variable and errors

and validate the result. Mathematical and statistical methods give meaningful data compared with the conventional methods.

The used of a combination of mutangic tools have revealed an effective method for increasing productivity of the microbial metabolites and therefore a promising tool in microbial applications and biotechnological industries. The use of statistical modeling and analysis saves a lot of time and resources and provides explainable data based on scientific basis. Testing of *Aspergillus flavus* in lot of works and ending in the current work proved a promising fungal strain for cellulase production.

Author contribution: Both authors shared justification of the idea, data collection, writing and revsion; **MMAE**: was responsible for organisms collection, statistical modeling, assays and results evaluation; **OEAA**: was responsible for mutation test and doses justification. Authors read and approved the manuscript.

Data availability: All data generated and/or analyzed during this study are included in this published article.

Declarations

Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest: The authors declare no competing interests.

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