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POTENTIAL OF *ASPERGILLUS NIGER* MT809753 FOR BIO-TREATMENT PAPER INDUSTRY WASTEWATER AFTER SCREENING EFFECTIVE FACTORS ON PRODUCTION OF CELLULASE ENZYME BY PLACKETT-BURMAN DESIGN

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ABSTRACT

Microorganisms capable of degrading cellulose present in rice straw was isolated from wastewater samples and identified as *Aspergillus niger* MT809753 by 18S rRNA. In the present study various cheap agronomic cellulosic wastes as (cotton seed husks, barley straw, rice straw and maize straw) were utilized as crude inducers for the cellulase enzyme production , cellulose activity was measured by dinitrosalicylic acid (DNS) method. The highest cellulase enzyme production was obtained by fungal isolate *Aspergillus niger* MT809753 within 24 h (0.532 IU ml $^{-1}$) using rice straw. Plackett-Burman design was used as conventional method for statistically screening of different variables. Seven variables from nine had influence on cellulase production with high confidence levels. Cellulase production became 1.08 IU ml $^{-1}$ after using response optimization. A bench scale study was performed to examine paper industry wastewater treatment efficiency by *Aspergillus Niger* MT809753. Results reveal that organisms have proved their bioremediation potency in treatment of paper industry effluent. Our goal is to obtain local isolates from fungi having a high ability to produce the cellulase enzyme, as well as developing an effective treatment processes to get rid of environmental cellulosic pollution and utilization of cellulosic wastes as cheap carbon sources.

Key Words: Cellulase enzyme, cellulosic wastes, fungi, paper industry effluents and Plackett-Burman.

1. INTRODUCTION

Municipal solid waste accumulating causes a serious problem in all developing countries and inadequate treatment of these municipal solid wastes can lead to a serious threat to the environment **(He , 2012).**

Cellulose is the most abundant renewable organic resource produced in the biosphere which built up from glucose unites then attached by β-1, 4 linkages and may be resulting from wastes of agricultural, industrial and sewage sludge. These waste products can potentially converted into value products through the action of enzymes.

Agricultural residues include leaves and stems from corn fiber, corn stover, sugarcane bagasse, rice hulls, woody crops and forest residues which considered a great source of lignocellulose biomass. Agricultural residue contains 40-50 % cellulose, it is renewable, unexploited and cheap. Whereas, these waste can used as an inexpensive feedstock for bioconversion to important products such as acetone and ethanol **(Yang and Wyman, 2008).**

Cellulase is an inducible enzyme that considered as one of the enzymes produced mostly by fungi, bacteria and protozoans that catalyze the decomposition of cellulose and of some polysaccharides. Recently, cellulase has more attention because of their diverse application in textile, detergent, leather, food, feed and paper industries. Also studies recorded that cellulase can be used in waste management. For example, cellulase can be used in the conversion of cellulosic municipal solid wastes to desirable chemicals, energy and able to degrading lignocellulosic materials that having wide range of applications **(Gautam et al., 2010).**

Already in the study by **Vimal et al. (2016)**, the cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. Several studies have been focused on the cellulase producing fungi as the most popular class for cellulase production as these cellulase have very high economic value. While a few bacteria have also been reported to yield cellulase activity. Fungi play an important role through nutrient cycling and humus formation in water bodies and soil because. They colonize the lignocelluloses matrix that other organisms are unable to decompose. Whereas, **Gupta et al. (2012)** reported that there are some fungi such as *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Trichoderma* sp., *Chaetomium* sp were able to produce cellulolytic enzymes.

Placket–Burman design is mainly used as statistical tools to screen out and selection of most relevant variables which enhancing production. The optimum level of each variable, their interaction with other variables and effect on the product yield were provided and thus minimized the number of experiments for large number of factors, by which the production has been statistical optimized **(Sharma et al., 2015).**

Paper industry is considered as an important industrial sector and one of the largest causer of industrial water pollution. The wastewater generated from paper industry having numerous toxic substances like high levels of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), turbidity, suspended solids and high concentration of lignin and cellulosic materials **(Singh and Sharma , 2013)**. Industrial effluents caused mainly an environmental problem so a quick need to

degrade these pollutants in an eco-friendly way is important. Many treatment technologies are already in practice, biological method degradation for wastewater effluent found to be efficient and cost effective method where cellulase enzymes were known as an eco-friendly process for hydrolysis cellulose components because it is accomplished without secondary polluted metabolites. This technique requires suitable microbial strains which can undergo various physico- chemical reactions in the polluted water and during the metabolism the pollutants are degraded and removed. Bioremediation studies for paper industry wastewater have reported using different bacterial and fungal strains for this proposal **(Kariman and Dabbagh, 2008)**.

Thus, this research was aimed to evaluate the growth of fungi strain with a high ability of cellulose degradation using agricultural wastes and screening different growth conditions factors influencing and controlling the production of cellulase enzyme according to Placket–Burman design. In the present work, the optimizing factors were applied *In Vitro* experiment to study the potential of fungal strain in bio treatment wastewater effluents from paper industry. The ultimate aim of this research is one of the most important solutions to get rid of cellulose environmental pollution through biodegradation of cellulosic wastes and converting them into useful important economical products and using the useful fungi in bio-treatment industrial wastewater.

2. MATERIALS AND METHODS

2.1 Study Area and Sampling Procedure

Ten wastewater samples were collected in duplicates from Bahr El Baqar drain near to Fakous city– Sharkia governate as represented at Fig. 1, noted that wastewater site appeared having maize straw and decaying leaf samples were collected into sterile containers and stored separately according to Standard Methods for Examination of Water and Wastewater **(APHA , 2017).**

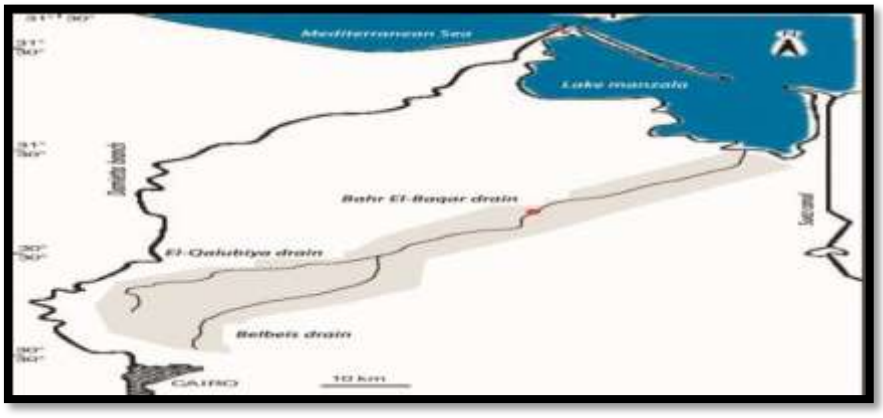

Fig. 1 Mapping of Bahr El Baqar drain near to Fakous city – Sharkia governate

2.2 Isolation of Testing Fungi

Cellulose water medium was prepared by adding autoclaved pieces of Whatman no. 1 filter paper of about 98 % cellulose as a sole carbon source into 250 ml of distilled water. To avoid bacterial growth, antibiotic was added and the medium become more selective to fungi. Ten ml of the collected wastewater samples was inoculated into the medium and incubated at 30° C for 5 day **(Sivaramanan, 2014).**

From the selective cellulose water medium serial dilution method were carried to isolate fungal isolates and then spread plate technique using Potato Dextrose Agar medium (PDA) were performed. The purity of isolates were examined microscopically and compared with those listed in standard reference books.

2.3 Screening for Cellulase Enzyme Production

Cellulose-degrading ability of fungi isolates was performed according to **Priyanka et al. (2017)** by plate assay method using agar plates containing 1 % Carboxyl Methyl Cellulose (CMC) agar media and after solidification, disk of the studied fungal colony at 5 mm in diameter a week old were loaded to plates then incubated at 30 $^{\circ}$C and cellulase activity was monitored daily until the fifth day. Plates were flooded with aqueous solution of 1 % Congo red for 15 min; followed by distaining with 1 M NaCl solution for 20 min and diameter of clear zones were then measured. This provides the basis for a rapid and sensitive screening test for cellulolytic fungi where appearance discolouration of Congo-red were taken as positive cellulose-degrading fungal isolates and only these were taken for further studies. Fungal colonies capable of utilizing cellulose as sole source of carbon were preserved for more studies.

2.4 Production of Cellulase Enzyme Using Agricultural Wastes

Several cheap agricultural residues like (cotton seed husks, barley straw, Rice straw and maize straw) were used as sole source of carbon with the best fungal strain during the study to estimate the best substrates for achieving the highest cellulase enzyme.

Agricultural residues (cotton seed husks, barley straw, rice straw and maize straw) were allowed to dry in the laboratory atmosphere then residue grind by grinder and each was used at a concentration of 2 %.

2.5 Preparing Basal Medium for Cellulase Enzymes Production

Set of 250 ml erlenmeyer flasks were prepared contain 100 ml of sterilized Cellulolytic basal medium (CBM), with the following constituents (g L^{-1}): $MgSO_4$ .7 H_2O, 0.1 g; KNO_3, 0.4 g; KH_2PO_4, 0.25 g; $FeSO_4$. 7 H_2O, 0.01 g; CaCl2. $2H_2O$, 0.02 g; Peptone, 1.0 g; pH 7.0

according to **(Li et al., 2003)** and different cheap agricultural substrates as (cotton seed husks, barley straw, Rice straw and maize straw) were added per flask at a concentration of 5 %, separately then flasks were sterilized. Each flask was inoculated with two plugs (5mm diameter) of fungal isolates showing high zone of cellulose break down on (CMC) agar media from 5 d old culture and incubated at 30 °C. After 5 d of cultivation the crude fungal enzymes were collected where the culture filtrates on each flask was filtered through normal filter paper then through Whatman No. 1 filter paper and the collected filtrate was transferred into falcon tube to centrifuge at 10,000 rpm for 15 min to remove cell debris where cellulase enzyme was recovered in cell free culture supernatant by centrifugation as reported by **Sethi et al. (2013).** The clear supernatants were used as fugal crude enzyme then subjected to cellulase assay and further purification.

2.6 Cellulase Activity Assay

The cellulase activity was measured by determining the amount of reducing sugars liberated using filter paper activity (FPase) assay which estimate total cellulolytic activity (exoglucanase, endoglucanase and β-glucosidase) quantitatively in the culture filtrate using a dinitrosalicylic acid (DNS) method, according to **Miller (1959).**

2.6.1 Measuring the Activity of Cellulolytic Enzymes

About 0.5 ml of fungal crude enzyme solution collected from filtrate of each flask was added separately to one milliliter of 0.05 M sodium citrate buffer of pH 5.0 and immersed with Whatman no. 1 filter paper strip (1 × 6 cm; weight 50 mg). Tubes were incubated at 50ºC for 1 h. Hence, the concentrations of the reducing sugars (products of enzyme activity) were measured by (DNS) method. The absorbance was measured using UV-Spectrophotometer at 540 nm as mentioned by **Miller (1960)**. One unit of filter paper (FPU) cellulolytic activity was defined as the amount of enzyme required for liberating 1 μmole reducing sugars as glucose from filter paper per ml per minute under standard assay condition and was expressed in term of international units IU ml^{-1}.

2.6.2 The Standard Glucose Curve

First, to estimate the effectiveness of cellulase enzymes the standard glucose curve was plotted. A standard solution of glucose was prepared by adding 1 g of D-glucose in 1 Liter of distilled water, then different concentrations were prepared 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 mg mL^{-1}. 1 ml of each concentration was taken into a test tube

containing 1ml of 0.05 M sodium nitrate solution at pH 4.8 and tubes incubated for 1 h at 50 °C then 3 ml of DNS reagent was added. The lids of tubes were tightly closed and placed in a water bath at 100 °C for 5 min. After this, the tubes were immediately transferred into an ice- cold bath and kept for few min to reach room temperature. Colour change in each tube was measured using UV spectrophotometer (HACH -DR/2010-Canada) 540 nm. Finally, the optical absorbance readings were compared and plotted with the standard glucose curve to find relation between the glucose concentrations and optical absorbance **(Bailey and Nevalainen, 1981).**

2.7 Identification of Cellulose Degrading Fungi by 18S rRNA Technique:

The fungal isolates were cultured on PDA medium and incubated at 30 °C. The DNA extraction, sequencing and analysis the Polymerase chain reaction (PCR) product of the isolate was carried out in the Faculty Agriculture, Cairo University, Egypt. The obtained sequences were compared with the other related sequences using BLAST search in Gen Bank of National Center for Biotechnology Information (NCBI). The sequence alignments were performed using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) according to **Edgar (2004)** web server with default settings and edited with Jalview **(Waterhouse et al., 2009)**. Maximum-likelihood phylogenetic trees was drawn among identified cellulose degrading fungi of study with international isolates registered in NCBI site by MEGA X **(Kumar et al., 2018)** using the best predicted substitution model for each group of aligned sequences, and 150 bootstrap replications.

2.8 Statistical Experimental Designs using Plackett Burman Design

Statistical Plackett-Burman Design (PBD) was used for screening and analyzing significant medium components and culture parameters that may significantly enhancement cellulase production. Nine independent factors (variables) were selected for this study and tested in two levels: -1 for low and +1 for high level represented in Table (1). The estimated mean of cellulase production were used as the experimental response (dependent variable).Experimental design is based on the first order model as given in Eq. (1).

$$Y = \beta 0 + \Sigma \beta i \, xi \quad \text{.....Eq. (1)}$$

Where, Y is the response of cellulase enzyme activity, β0 is the model intercept, βi is variable estimated coefficient, i is the variable

number and xi are independent variables. The variables were screened using design expert 13.0 software.

Amount of glucose produced was assayed by carrying out a DNS test. Using a standard curve, amount of glucose produced was calculated and values obtained used to determine specific enzyme activity. Finally, an experiment was carried out using the optimum conditions for 3 d. The cellulase enzyme activity was measured daily.

Table 1 Experimental levels of independent variables using Plackett-Burman design

Variable code	Variable	units	Low (-1)	High(+1)
X1	Shaking conditions	rpm	100	300
X2	peptone	g l^{-1}	0.5	5.0
X3	Substrate concentration	%	2	8
X4	incubation time	h	24	72
X5	temperature	°C	20	40
X6	pH	-	5	9
X7	inoculum size	(v/v) %	1	3
X8	KNO_3	g l^{-1}	2.0	5.0
X9	$MgSO_4 .7H_2O$	g l^{-1}	0.1	0.5

2.9 Experimental study for potential using fungal strains during the study in bio-treatment of industrial paper wastewater

Wastewater samples were collected from the outlet of effluent treatment plant from a Rio paper products factory, 10th of Ramadan city, Sharkia Governate, Egypt and stored at 40 °C. The manufacturing unit generates enormous quantity of wastewater which is having high levels of colour, high levels of BOD, COD, suspended solids and cellulosic components **(Selvam et al., 2011)**.

The collected samples were initially subjected to the physical chemical analysis as pH, BOD, COD, dissolved oxygen (DO) and cellulose on the basis of the standard methods given by the Examination of Water and Wastewater, **APHA (2017)**.The experiment was initiated using a set of triplicate batches of 5 L erlenmeyer flasks containing 250 ml of paper industry wastewater samples, inoculated with the best studied inoculum size of studied fungus. The three 5 L batches were incubated under the optimized conditions. The degradation studies were carried for two weeks and the post analysis were performed periodically at alternated periods.

3. RESULTS AND DISCUSSION

3.1 Selective Isolates

Injecting 10 ml of wastewater samples in the cellulose water medium contain pieces of filter paper Whatman no. 1 were the perfect for cultivation fungal isolates have an ability to degrade cellulose Fig 2. Total eight fungal isolates were isolated from selective cellulose water medium on PDA plates.

Fig. 2 Cellulose water medium contain pieces of filter paper.

Depending on the diameter of clear zone around the colony on agar plates containing 1 % CMC agar media, only three fungal isolates gave positive results among all eight fungal isolates, hence identified as cellulase producing fungi having the codes no (F1, F2 and F3) within 5 d of incubation and notice that hydrolysis zone around some fungal colony starting from the first day and the diameter continued to increase as the incubation period continued Fig. 3. The appearance of the clear zone around the colony after the congo red solution was added was strong evidence that the fungi produced cellulase enzyme in order to degrade cellulose. Out these three fungal isolates F1 was the greatest cellulase producing capability as it shows maximum zone about 4.2 cm of clear zone around the fungal culture Fig 4 while other two fungal isolates were weak enzyme production. Initial identification was done by morphological characters represented at Fig. 5 and fungal staining according to standards.

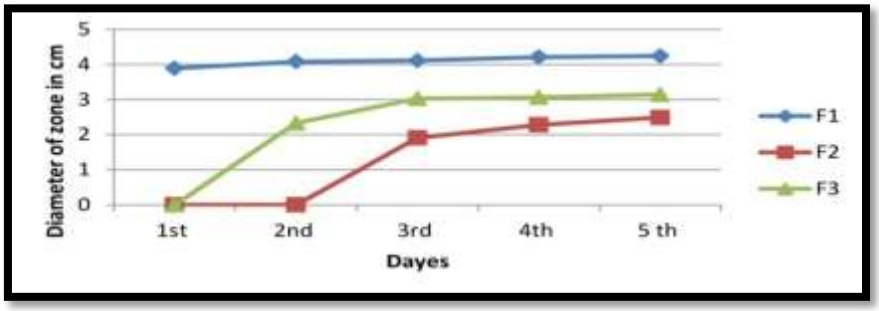

Fig. 3 Hydrolysis zone around three fungal colonies for 5d.

Fig. 4 Clear zone around the fungal colony (F1) on CMC agar plate.

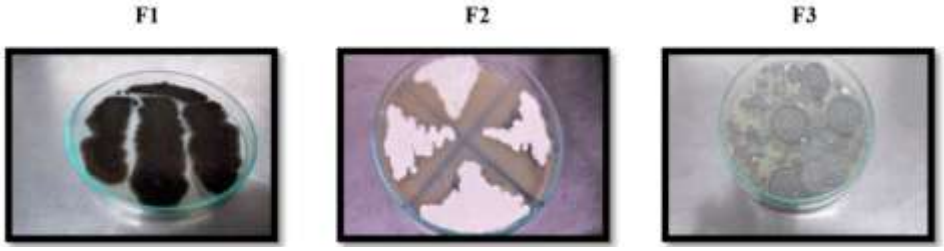

Fig. 5 Morphological characteristics of cellulase fungal isolates on PDA

3.2 The standard glucose curve

Different concentrations of glucose were prepared and measured the absorbance at wavelength (540 nm), then the relation between the glucose concentrations and optical absorbance were plotted and from the obtained standard glucose curve equation Y= 0.905 x the glucose released from fungal isolates in CMC solution were determined Fig. 6.

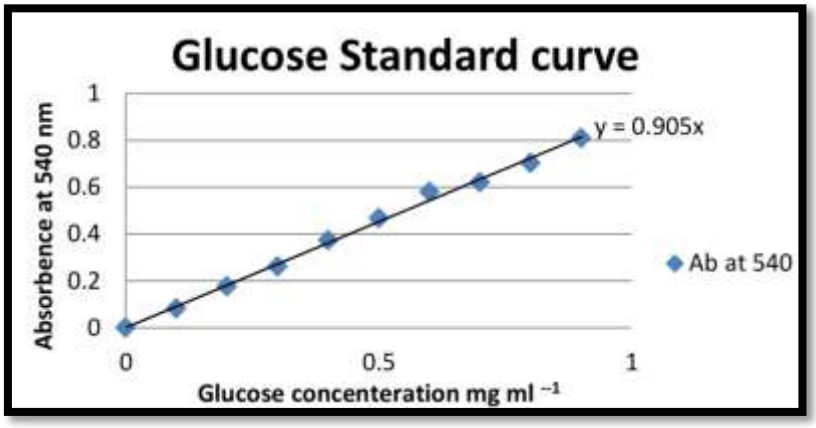

Fig. 6 Glucose standard curve

3.3 Using different agricultural wastes as carbon source

Kim et al. (2003) reported an increase economic interest for utilization of cellulosic wastes as cheap carbon sources. Where that raw agriculture cellulosic substrates can used as crude inducers and very effective in inducing cellulase production.

Cellulose activity of fungal isolates F1, F2 and F3 using different agricultural wastes substrate (cotton seed husks, barley straw, Rice straw and maize straw) as carbon source was analyzed by evaluating the cellulase liberated in CMC solution through DNS method. Among the various substrates used, maximum activity of cellulase was recorded from rice straw (0.532 IU ml $^{-1}$) using culture codes F1 followed by 0.441 , 0.429 and 0.501 IU ml $^{-1}$ of cellulase enzyme from maize straw, cotton

seed husks and barley straw respectively Fig. 7. Similar to the present study **Goyari et al. (2015)** indicated that rice straw showed the highest cellulase activity and sawdust showed the lowest activity. Also **Das et al. (2011)** recorded that fungal isolates during study giving the highest cellulose activity using rice straw.

The enzyme activity was calculated according to **Robson and Chambliss (1984):**

The enzyme efficacy (IU ml $^{-1}$ **) = 0.37 × glucose released**

A standard curve was used to find the unknown concentrations of reducing sugars in all samples

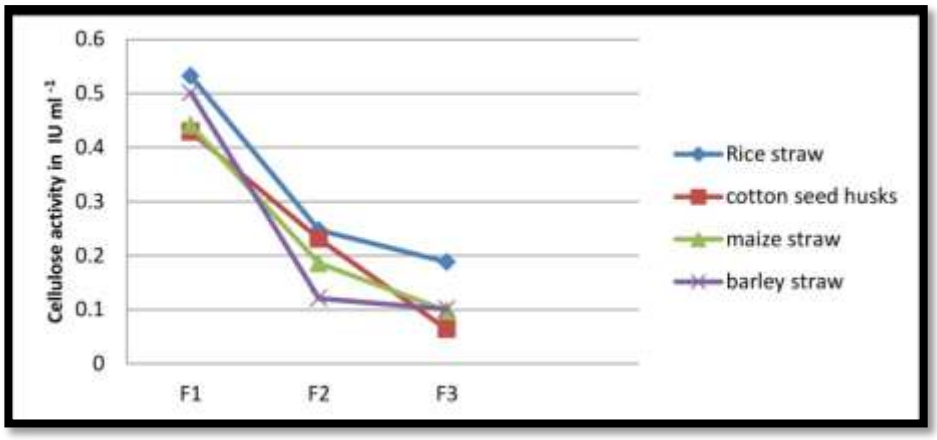

Fig. 7 Graph of enzyme activity using different agricultural wastes.

3.4 Identification of cellulose degrading fungi

Identification for the cellulolytic fungi isolate giving the highest hydrolysis zone of cellulose on CMC and giving the highest cellulase activity using different agriculture wastes by 18S rRNA then submitted to NCBI and accession number given from GenBank nucleotide sequence database was *Aspergillius niger* MT809753. Results obtained during this study indicated that cellulase activity of tested *Aspergillus niger* MT809753 were found relatively higher and comparable with some results of other investigators **(Kluczek-Turpeinen et al., 2005)** .Similarly, **Jahangeer et al. (2005)** indicated that *Aspergillus* species were the higher cellulase activity producer and amongst fungi capable of producing beneficial enzymes for industrial utilization. Also previous studies **(Gupta et al., 2012)** indicated that majority of *Aspergillus, Fusarium, penicillum and Trichoderma* isolates were found to possess cellulytic activity. A study of **Lakshmi and Narasimha (2012)** showed the potential of *Aspergillus* species with maximum zone of hydrolysis (42 mm). Also a study by **Bekele et al. (2015)** supported the cunrent

study and indicated the presence of four efficient isolates able to hydrolysis CMC confirming that *Aspergillus* species showed the greater hydrolysis zone. In agreement with the present study different species of genus *Aspergillus*, have been identified to possess all component of cellulase enzyme system **(de Vries and Visser , 2001).**

3.4.1 Phylogenetic tree

Tree represented the relationships among cellulose-degrading fungi (F1) *Aspergillius niger* MT809753 the promising strain of this study and recognized species of the genus *Aspergillus* Fig. 8.

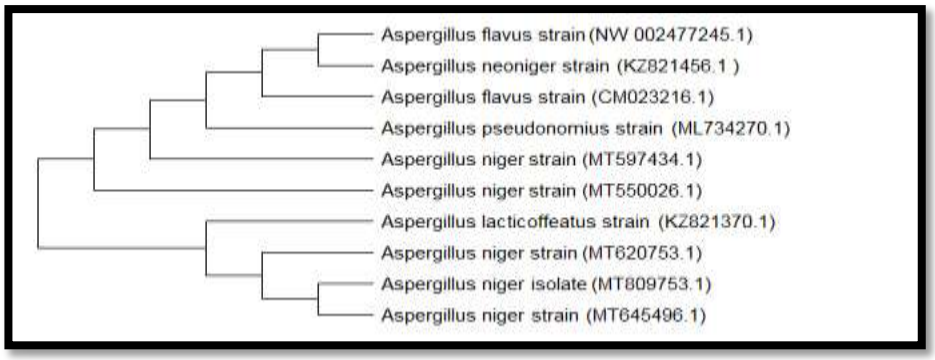

Fig. 8 Phylogenetic tree of *Aspergillus niger* MT809753 and other species of the genus *Aspergillus*.

3.5 Plackett Burman Design

Using an efficient approach as Plackett–Burman Design **(Plackett and Burman , 1946)** for screen and evaluate significant parameters that can influence enzyme yield was important as several studies have employed statistical methods for enzyme production. But this model does not explain the interaction among various variables. So the study then optimized, using a response surface methodology. This design has been successfully established for its efficacy in screening the important factors in few experimental runs **(Kammoun et al., 2008)**. Nine factors were investigated to determine the important factors suitable for cellulase production. Twelve experiments given by the model (Table 2) in which each column represents variables and each row represents an experiment. Variation in cellulase production from 0.237 to 0.864 IU ml $^{-1}$ by *Aspergillus niger* MT809753 is presented in (Table 2) where this variations revealed the importance of factors optimization. Maximum cellulase activity was obtained in run number 8th with (0.864 IU ml $^{-1}$) and 1st experimental run has minimum cellulase activity (0.237 IU ml $^{-1}$).The data in Table (2) based on the PBD was subjected to multiple linear regression analysis to estimate F- value and p -values of each component. the effect of independent variables on cellulase production is set by the first-order linear model and is given by Eq. (2).

$$Y = 0.39758 + 0.01625 X1 - 0.01008 X2 + 0.06375 X3 - 0.00608 X4 + 0.00975 X5 + 0.03242 X6 - 0.00442 X7 + 0.02792 X8 + 0.02392 X9 \quad \textbf{Eq. (2)}$$

Table 2 Plackett-Burman experimental design applied on: (+1) high level, (-1) low level.

Run No.	X1	X2	X3	X4	X5	X6	X7	X8	X9	Cellulase activity (IU ml $^{-1}$)
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.237
2	-1	1	1	-1	1	-1	-1	-1	1	0.426
3	1	-1	-1	-1	1	1	1	-1	1	0.396
4	-1	-1	1	1	1	-1	1	1	-1	0.422
5	-1	1	-1	-1	-1	1	1	1	-1	0.343
6	1	1	-1	1	-1	-1	-1	1	1	0.344
7	1	1	-1	1	1	-1	1	-1	-1	0.259
8	1	-1	1	1	-1	1	-1	-1	-1	0.864
9	-1	1	1	1	-1	1	1	-1	1	0.436
10	-1	-1	-1	1	1	1	-1	1	1	0.424
11	1	-1	1	-1	-1	-1	1	1	1	0.503
12	1	1	1	-1	1	1	-1	1	-1	0.517

Table (3) shows the ANOVA analysis for linear model of variables factors effect on cellulase production by *Aspergillus niger* MT809753. P-value itself of statistical design was clearly implied that model is significant with a p-value of 0.021. On analysis of p-value, variables whose was less than 0.05 was considered to have significant influence on the celluase productivity. However, whose p-value is larger than 0.05 which means that analyzed factor was not statistically significant; though not played varying role in celluase production. On basis of p-values, positive effect was appeared by all factors on cellulase production except (X8, X9) as indicated in Table (3). Some studies revealed that some factors may be not being significant on enzyme activity **(Kumar and Satyanarayana , 2007)**. The goodness of fit model was checked by the coefficient (R^2) which indicated that the model could explain up to 99.0 %.

Table 3 ANOVA analysis using Plackett-Burman design

Source	DF	Adj SS	Adj MS	F-Value	P-Value	Significant
Model	9	0.083803	0.009311	46.08	0.021	Significant
Linear	9	0.083803	0.009311	46.08	0.021	Significant
X1	1	0.003169	0.003169	15.68	0.058	Significant
X2	1	0.001220	0.001220	26.04	0.033	Significant
X3	1	0.048769	0.048769	241.33	0.004	Significant
X4	1	0.000444	0.000444	32.20	0.026	Significant
X5	1	0.001141	0.001141	25.64	0.041	Significant
X6	1	0.012610	0.012610	62.40	0.016	Significant
X7	1	0.006864	0.006864	33.97	0.028	Significant
X8	1	0.001141	0.001141	5.64	0.141	Insignificant
X9	1	0.000234	0.000234	1.16	0.394	Insignificant
Error	2	0.000404	0.000202			
Total	11	0.084207				
R^2		0.9952				
Adjusted R^2		0.9736				
Predicted R^2		0.8272				

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

3.6 Effects of process variables on the cellulase production

The Plackett-Burman design was chosen to screen the important factors for cellulase production with respect to their main effects and not their interaction effects. Based on the results of the Plackett-Burman design, the main effects of the analyzed factors on cellulose production are graphically plotted by Pareto chart Fig. 9. It is evident from the pareto chart of process variables ranking of the factors was done according to their importance where seven factors named inoculum size, substrate concentration, incubation temperature, pH, shaking conditions, incubation time and peptone concentration were found to be significant for cellulose production by *Aspergillus niger* MT809753. In the present study, the inoculum size of *Aspergillus niger* showed a highly significant effect on the production, and it possibly up regulated the yield of cellulase. Previous studies by **Das et al. (2011)** recorded that when the inoculum sizes were too small (0.5, 1 and 2 %), the amount of cellulase production was less. The cultivation temperature has a remarkable effect on the growth rate and also on the level of cellulase production. **Das et al. (2011)** recorded that maximum activity of fungal strains was at 30 °C and decreased when incubation temperature was above 37 °C. The pH medium highly affects the growth rate of the fungus also on the enzyme production. **Sivaramanan (2014)** reported that *Aspergillus niger* can give maximum activity at the acidic medium pH 6. Where **Priyanka et al. (2017)** recorded that pH 7.0 was the best suitable value for higher cellulase enzyme activity. Also **Das et al. (2011)** recorded that the growth of the fungus decreased when the pH values was less than 7. Agitation speed is an important factor in cellulase production as recorded in previous studies. A significant change in cellulase enzyme activity was observed when agitation speed increased from 100 to 200 rpm then decreased when agitation speed increased from 200 to 300 rpm. The cellulase activity inhibition occurred with higher agitation speed **(Ma et al., 2008)**. The effect of incubation period was estimated for 72 h and showing a significant effect on cellulase production. Enzyme activity increased with an increase in incubation time and the high peak value of enzyme activity was found after 48 h then it started declining in the 3^{th}day (72 h). The minimum enzyme activity was noted after 24 h. These finding are in agreement with some studies whose suggested that a decrease enzymatic activity with increasing incubation time may be due to using nutrients in the medium and this can cause fungal stress so causing an inactivation of enzyme secretion **(Azhar et al., 2017)**. The

level of the peptone source in the growth medium is an important factor in the production of cellulose which regulates the biosynthesis of cellulase from different microorganisms **(Deka et al., 2011)**. The $MgSO_4 \cdot 7H_2O$ has been reported as not essential mineral source for cellulase production.

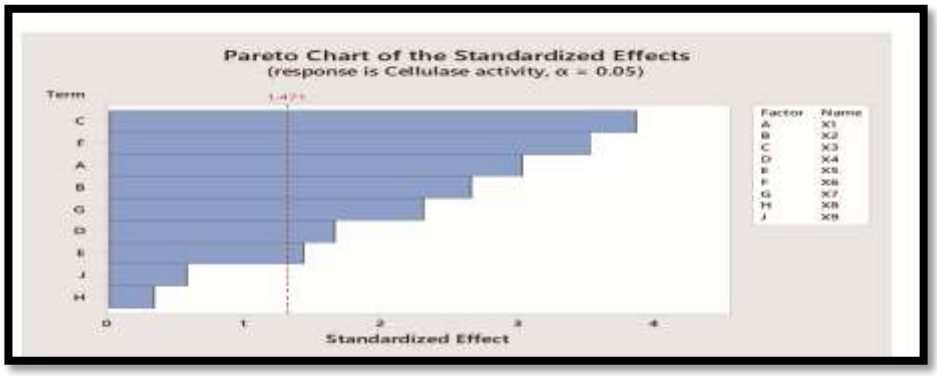

Fig. 9 Pareto chart to visualization the effect of nine variables by PBD.

3.7 Response optimization

Cellulase production was optimized in the MINITAB 18.0 through an application of response optimization to improve design characteristics. The experiment was performed with the given factors form PBD and the obtained enzyme activity was 1.08 IU ml $^{-1}$ where were near to the predicted value (Table 4). The maximum enzyme activity obtained with 3 % inoculum size; 8 % substrate concentration; 30 °C incubation temperature; pH 7 ; 200 rpm shaking conditions; 48 h incubation time and 5.0 g l^{-1} peptone concentration. **Hsu et al. (2005)** reported that the highest enzyme activity was obtained at the optimized conditions of pH 6.5, 37 °C and 30 h of incubation time.

Table 4 Response prediction for cellulase activity.

Inoculum size	Substrate concentration	Incubation temperature	pH	Shaking conditions	Incubation time	Peptone concentration	Cellulase activity (IU ml $^{-1}$)	
							Experiment	Predict
3 %	8 %	30 °C	7	200 rpm	48 h	5.0 g l^{-1}	1.08	0.99

3.8 Improvement for paper industrial wastewater in a bench scale study

The problems associated with wastewaters arising from paper processing industry are pH, colour, high levels of cellulosic components,

BOD, COD, etc., **(Singh and Sharma , 2013)**. There are several studies of potential ability of fungi for treatment of paper wastewater effluent. Recent studies using active enzymes from fungi as *Aspergillus* sp which reduce COD and other pollutants from the paper effluent **(Malaviya and Rathore , 2007)**.

The values of cellulose, BOD, COD, DO and pH in the collected samples was followed up in triplicates periodically at alternated periods and the mean was recorded. The results of the physico-chemical analyses of collected paper industry wastewater samples were characterized by a high content of BOD, COD and cellulose; where their values were 1000 ,5000 and 80.3 mg L^{-1}, respectively. Influence of fungus on the paper wastewater effluent was obvious comparing the characters of the mixture of wastewater before and after the test was done. The values of BOD, COD and cellulose shows slow reduction rates until the 6 th day in vitro conducted experiment whereas after this a fast degradation rate were observed (Fig. 10). These results are in accordance with those recorded by **Tricolici et al. (2014)** who studied the bio-treatment of industry wastewater rich in organic compounds in Romania. They found that some strains of fungi could remove 91 % of COD. Moreover, **Saritha et al. (2010)** applied the potential of two fungi *p. chrysosporium* and *T. hirsute* in the reduction of COD and cellulose content in the industrial paper wastewater with 78 %, 80 % and 89 %, 82 % respectively. The degradation of cellulose in samples was observed throughout the study until the 11^{th} day of experiment, after which the degradation were stabilized.

The initial DO concentration of paper processing wastewater was very low before starting aeration by shaking (1 mg L^{-1}) and increased to 7 mg L^{-1} at day 6, by the effect of shaking the metabolic activities of indigenous microorganisms gradually increased by the effect of excess oxygen diffused in wastewater, (Fig 10). Such findings are in accordance with those reported by **Abdel-Fatah et al. (2015)** who mentioned that shaking increasing the oxygen content in the reactor and elevating the biomass concentration lead to high biodegradation capacity. The pH was monitored during the batch experiment period; the results are clarified in Fig. 10e. It was slightly acidic through the first week and starts to be neutral, ranging from 7.0 to 7.2 through the second week. As the biodegradation products increased with time, the pH of the mixture increased **(Ayman, 2020)**.

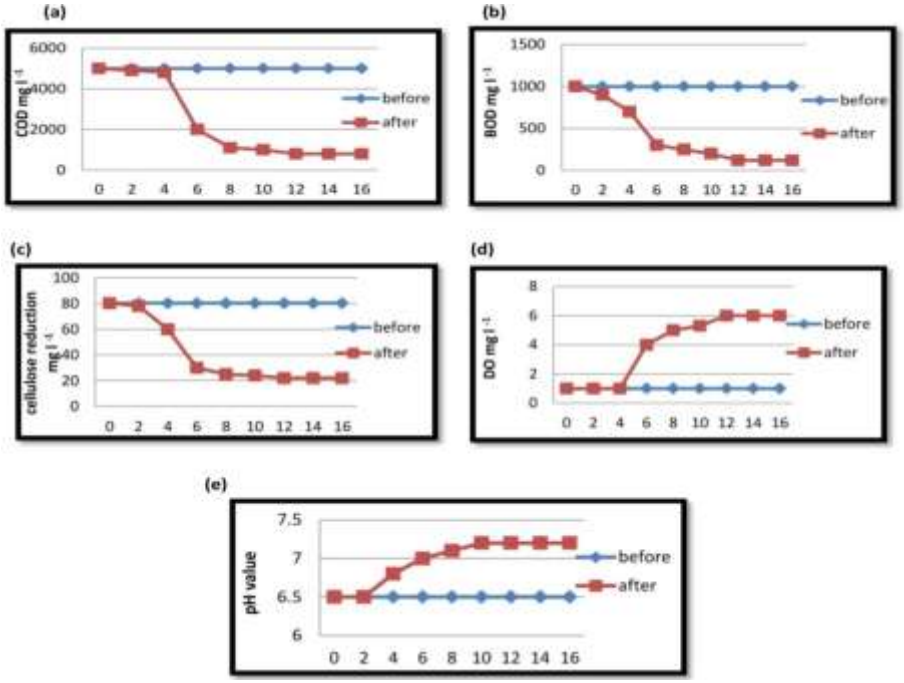

Fig. 10 Illustrations bio-treatment experiment (a) reduction in COD (b) reduction in BOD (c) reduction in cellulose (d) DO concentrations during the experiment (e) elevation in pH values.

CONCLUSIONS

The promising fungus *Aspergillius niger* MT809753 isolated in this study possess cellulosic activity that efficiently degrade CMC and having the ability to utilize different agronomic wastes such as (cotton seed husks, barley straw, Rice straw and maize straw) that represent the carbon sources for cellulase production from fungal strains. The ability of Plackett-Burman design proved in the presented study to be a practical, powerful and convenient tool for determining the factors that have a positive effect on cellulase enzyme production that have accuracy in the prediction of the selected model with an R^2 value of 0.996. From the foregoing, we found that the significant conditions for the production of cellulase enzymes were inoculum size, substrate concentration, incubation temperature, pH, shaking conditions , incubation time and peptone concentration. In the experimental bench study the bio-treatment of paper industry wastewater resulted in reduction of COD, cellulose and BOD in the order of 80 %, 72 % and 88 % in two weeks. A major part of reduction in these parameters was regarded after 6 d of treatment. Owing to these findings in this work, cellulase produced by *Aspergillus niger* MT809753 can be used in waste management. For example, in treatment wastewater from cellulosic wastes and either in fermentation process to produce biogas.

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إمكانات فطر الاسبراجيلز نيجر MT809753 فى المعالجة الحيوية لمياة الصرف الخاصة بصناعة الورق بعد فحص العوامل الفعالة فى انتاج انزيم السليلاز بواسطة التصميم الاحصائى بلاكيت بورمان

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تم عزل كائن حي دقيق قادر على تحطيم السليلوز الموجود في قش الأرز من عينات مياه الصرف الصحى وتم تعريفة بواسطة اختبار الحمض النووي الريبوزي للفطريات على انه اسبراجيلز نيجر.في هذه الدراسة تم استخدام العديد من المخلفات الزراعية السليلوزية الرخيصة مثل (قشور بذور القطن ، قش الشعير ، قش الأرز وقش الذرة) كمحفزات خام لإنتاج إنزيم السليلوزمن الفطريات المعزولة ، تم قياس نشاط السليلوز بطريقة حمض الساليسيليك. تم الحصول على أعلى إنتاج لإنزيم السليلاز بواسطة العزلة الفطرية خلال 24 ساعة (0.532 وحدة دولية مل $^{-1}$) باستخدام قش الأرز. تم استخدام تصميم بلاكيت بورمان للفحص الإحصائي للمتغيرات المختلفة. سبعة متغيرات من تسعة لها تأثير على إنتاج السليلاز بمستويات ثقة عالية. أصبح إنتاج السليلاز (1.08 وحدة دولية مل $^{-1}$) بعد استخدام تحسين الاستجابة فى البرنامج الاحصائى. تم إجراء دراسة على نطاق معملى لفحص كفاءة فطر الاسبارجليز نيجر المعزول خلال الدراسة فى معالجة مياه الصرف من صناعة الورق و تكشف النتائج أن فطر الاسبرجيلز المعزول خلال الدراسة قد أثبت فعاليته في المعالجة الحيوية للمياه الناتجة من صناعة الورق. هدفنا هو الحصول على عزلات محلية من الفطريات التي تتمتع بقدرة عالية على إنتاج إنزيم السليوليز ، بالإضافة إلى تطوير عمليات معالجة فعالة للتخلص من التلوث السليلوزي البيئي واستخدام النفايات السليلوزية كمصادر رخيصة للكربون.