# Potential of Aspergilus 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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**Research**

**Keywords:** Cellulase enzyme, cellulosic wastes, fungi, paper industry effluents and Plackett-Burman

**Posted Date:** June 9th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-519880/v1

# Abstract

A microorganism capable of degrading cellulose present in rice straw was isolated from wastewater samples and identified as *Aspergilus niger* MT809753 by 18S rDNA. 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 and represent the carbon source for isolates where cellulose activity was measured by (DNS) method. The highest cellulases enzyme production was obtained by fungal isolate *Aspergilus niger MT809753* within 24 hours (0.532 IU/ml) using rice straw. Plackett-Burman design was used as conventional method for statistically screening of different variables. Nine variables of the production process were selected. The results illustrate those seven variables, namely as (inoculum size, substrate concentration, incubation temperature, pH, shaking conditions, and incubation time and peptone concentration) had influence with high confidence levels, while the remaining two variables did not show a significant effect on cellulase production. After using response optimization the experiment was performed and the obtained cellulase production was 1.08 IU/ml. 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. The importance of the research stems from the fact that it sheds light on the role of some fungi in the production of the cellulase enzyme. So 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.


# 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 [1, 2].

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, urban, industrial and sewage sludge these waste products can potentially be bio-converted into value-added products through the action of enzymes [3, 4]. 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 where 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 [5]. The agricultural residue contains 40–50% cellulose [6].

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 [7]. Also [8, 9] recorded that cellulase can be used in waste management. For example, cellulases 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 [10].

Vimal and co-workers [11] evaluated that 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 cellulases have very high economic value [12, 13, 14]. While a few bacteria have also been reported to yield cellulase activity [15]. 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 and co-workers [16] 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 cellulase 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 enzyme production has been statistical optimization of cellulose [17].

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 BOD, COD, turbidity, suspended solids and high concentration of lignin and cellulosic materials [18, 39]. 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 cellulases 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 [19].

Thus, this research aimed to evaluate the growth of fungi strain with a high ability of cellulose degradation using agricultural wastes 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 an in-vitro experiment was conducted to study the potential in remediate such industrial paper wastewater effluents. 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 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 [20].

## 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 [21].

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) plates were performed. The purity of isolates were examined microscopically and compared with those listed in standard reference books [22].

## 2.3. Screening for Cellulase Enzyme Production:

cellulose-degrading ability of fungi isolates was performed according to Priyanka *et al.* [23] 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° 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 1M NaCl solution for 20 min and diameter of clear zones were then measured [24]. This provides the basis for a rapid and

sensitive screening test for cellulolytic fungi where appearance discoloration 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 residue (cotton seed husks, barley straw, rice straw and maize straw) were allowed to dry in the laboratory atmosphere according to Eldin [25] then grind by grinder and each was used with concentrations (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 mediums (CBM), with the following constituents (g/L): $MgSO_4$ .$7H_2O$, 0.1 g; $KNO_3$, 0.4 g; $KH_2PO_4$, 0.25 g; $FeSO_4$. $7H_2O$, 0.01 g; CaCl2. $2H_2O$, 0.02 g; Peptone, 1.0 g; pH 7.0 according to *et al.* [26, 27] 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 were inoculated with two plugs (5mm diameter) of fungal isolates showing high zone of cellulose break down on (CMC) agar media from 5 days old culture and incubated at 30°C. After 5 days 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 minutes to remove cell debris where cellulase enzyme was recovered in cell free culture supernatant by centrifugation [28]. 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 (DNSA) method, according to Miller [29].

## 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 hour. Hence, the concentrations of the reducing sugars (products of enzyme activity) were measured by dinitrosalicylic acid (DNS) method. The absorbance was measured using UV-Spectrophotometer at 540 nm according to Miller [30]. 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. [24, 31].

## 2.6.2. The Standard Glucose Curve:

First, to estimate the effectiveness of cellulase enzymes the standard glucose curve was plotted. Prepare a standard solution of glucose by adding 1 g of D-glucose in 1 Liter of distilled water, then different concentration were prepared 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 mg / mL. Take 1 ml of each concentration into a test tube containing 1ml of 0.05M 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 minutes to reach room temperature. Color 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 [32].

## 2.7. Identification of Cellulose Degrading Fungi:

The fungal isolates were cultured on PDA medium and incubated at 30°C. The DNA extraction, sequencing and analysis the PCR product of the isolate was carried out by the Faculty Agriculture, Cairo University, Egypt. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI) [33] .

**Phylogenetic Analysis**:

The sequence alignments were performed using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) [34] web server with default settings and edited with Jalview [35]. Maximum-likelihood phylogenetic trees was drawn among identified cellulose degrading fungi of study with international isolates registered in NCBI site by MEGA X [36] 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) [37].

$Y = \beta 0 + \Sigma \beta i\ xi$……**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 DNase 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 three days. The cellulase enzyme activity was measured daily.

**Table (1): Experimental Levels of Independent variables using Plackett-Burman design**

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

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

Samples were collected from the outlet of wastewater 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 color, high levels of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), suspended solids and cellulosic components [38].

The collected samples were initially subjected to the physic chemical analysis as pH, BOD, COD, DO and cellulose on the basis of the standard methods given by the Examination of Water and Wastewater [20].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 days [39].

## 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 was 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 Potato dextrose agar medium (PDA) plates.

Depending on the diameter of clear zone around the colony on agar plates containing 1% Carboxyl Methyl Cellulose (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 five days 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 [40, 41].

# 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).

# 3.3. Using different agricultural wastes as carbon source:

Kim et al [42] reported an increase economic interest for utilization of cellulosic wastes as cheap carbon sources. Where [43, 44] explained 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) using culture codes F1 followed by 0.441, 0.429 and 0.501 IU/ml of cellulase enzyme from maize straw, cotton seed husks and barley straw respectively Fig. (7). Similar to our study [45] indicated that rice straw showed the highest cellulase activity and sawdust showed the lowest activity. Also [46] recorded that fungal isolates during study giving the highest cellulose activity using rice straw.

The enzyme activity was calculated according to Robson and Chambliss [47]:

**The enzyme efficacy (IU / ml) = 0.37 × glucose released**

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

# 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 RNA then submitted to NCBI and named under *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 as [48, 49] .Similarly, Jahangeer et al [50] indicated that *Aspergillus* species were the higher cellulase activity producer and amongst fungi capable of producing beneficial enzymes for industrial utilization. Also et al. [51, 52] reported that various studies indicated that majority of *Aspergillus, Fusarium, penicillum and Trichoderma* isolates were found to possess cellulytic activity. A study of Lakshmi and Narasimha [53] showed the potential of *Aspergillus* species with maximum zone of hydrolysis (42 mm). Also a study by Bekele et al. [54] supported our study and indicates 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 [55].

**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).

# 3.5. Plackett Burman Design:

Using an efficient approach as Plackett–Burman Design [56] for screen and evaluate significant parameters that can influence enzyme yield was important as several studies have employed statistical methods for enzyme production [57], but this model does not explain the interaction among various variables [58] so the study then optimized, using a response surface methodology [59]. This design has been successfully established for its efficacy in screening the important factors in few experimental runs [60]. 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 by *Aspergilus niger* MT809753 were presented in (Table 2) where this variations revealed the importance of factors optimization [61]. Maximum cellulase activity was obtained in run number 8th with (0.864 IU/ml) And 1st experimental run has minimum cellulase activity (0.237 IU/ml).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.

 = 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 **Eq. 2**

**Table (2): Plackett-Burman experimental design applied on trial runs: (+ 1) high level variables, (-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 | 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 *Aspergilus 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 were considered to have significant influence on the celluase productivity. However, whose p-value is larger than 0.05 which means that analyzed factor were 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 [61]. 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 | Siginficant |
|---|---|---|---|---|---|---|
| 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 is done according to their importance where seven factors were found to be significant for cellulose production by *Aspergilus niger* MT809753 were inoculum size, substrate concentration, incubation temperature, pH, shaking conditions, incubation time and peptone concentration. In the present study, the inoculum size of *Aspergillus niger* showed a highly significant effect on the production, and it possibly upregulated the yield of cellulase. Previous studies from Das and co-workers [45] 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. [45] recorded that maximum activity of fungal strains was maximum 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 [21] whose reported that A. niger can give maximum activity at the acidic medium pH 4.5. Where [23] recorded that pH 6.0 was the best suitable value for higher cellulase enzyme activity. Also Das et al. [45] recorded that the growth of the fungus decreased when the pH values was above 5. Agitation speed is an important factor in cellulase production as recorded in previous studies. We observed a significant change in cellulase enzyme activity 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 [62, 63]. The effect of incubation period was estimated for 72hrs 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 hrs then it

started declining in the 3thday (72hrs). The minimum enzyme activity was noted after 24 hrs. These finding in agreement with [64, 65] 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. 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 [66]. The $MgSO4{\cdot}7H_2O$ has been reported as not essential mineral source for cellulase production.

# 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 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; 7pH; 200 rpm shaking conditions; 48h incubation time and 5.0 g/l peptone concentration. Hsu and co-workers [67] 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 ) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Experiment | Predict |
| 3 % | 8 % | 30°C | 7 | 200 rpm | 48h | 5.0 g/l | 1.08 | 0.99 |

# 3.8. Bio-treatment 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, Suspended Solids (SS), etc., [68]. 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 [69, 70].

The values of cellulose, BOD, COD, DO and pH were followed up in triplicates periodically at alternated days 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, respectively. Influence of fungus on the paper wastewater effluent was obvious comparing the characters of the mixture of wastewater before and after the test is done.The BOD, COD and cellulose shows slow reduction rates until the 6th 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 and co-workers [71] 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 and co-workers [72] 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 11th 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 (1mg /l) and increased to 7 mg /L 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-Fatahand co-workers [73] 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 were 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 [74].

# Conclusion

The promising fungus *Aspergillius niger* MT809753 isolated in this study possess cellulolylic activity that efficiently degrade carboxyl methyl cellulose and having the ability to utilize different agronomic wastes such as (cotton seed husks, barley straw, Rice straw and maize straw) as 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 days of treatment. Owing to these findings in this work, cellulase produced by *Aspergilus niger* MT809753 can be used in waste management. For example, in treatment wastewater from cellulosic wastes and either in fermentation process to produce biogas. Further studies will focus on the development of methods for utilization of this enzyme in industrial processes.

# Declarations


### Acknowledgements

Not applicable.

### Funding

Not applicable.


### Availability of data and materials

All data generated or analyzed during this study are of my own work and it is my pleasure to be available publically.

### Author’s contributions

The author read and approved the final manuscript.

### Competing interests

The author declares that he has no competing interests.

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# Figures

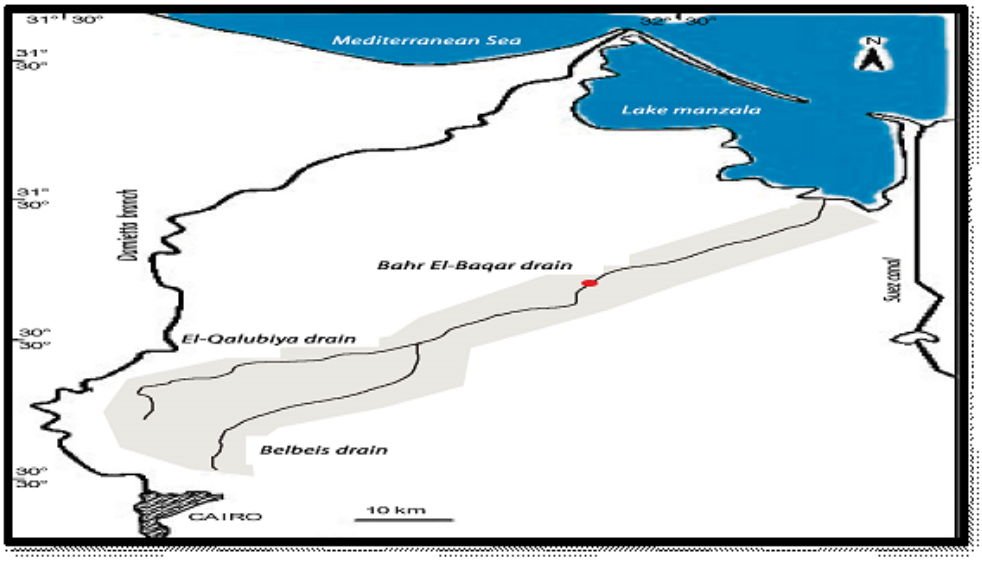



**Figure 1**

Mapping of Bahr El Baqar Drain near to Fakous city – Sharkia governate. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

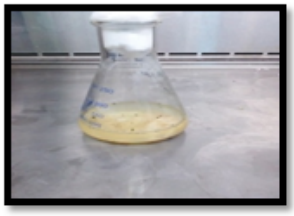


**Figure 2**

Cellulose water medium contain pieces of filter paper.

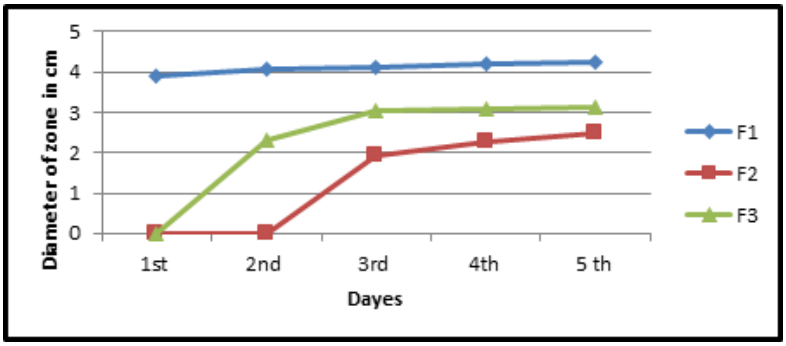



**Figure 3**

Hydrolysis zone around three fungal colonies for five days

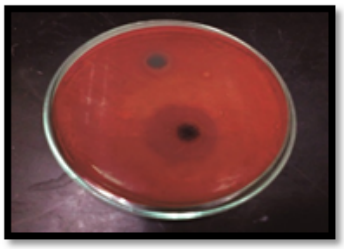


Figure 4

Clear zone around the fungal colony (F1) on carboxyl methyl cellulose agar plate.


Figure 5

Morphological characteristics of cellulase fungal isolates on PDA

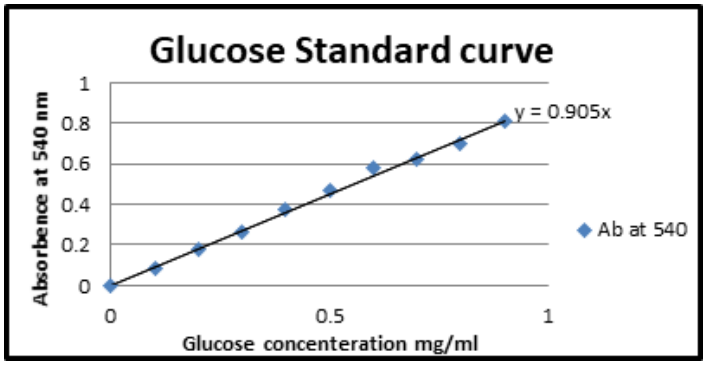



Figure 6

Glucose standard curve

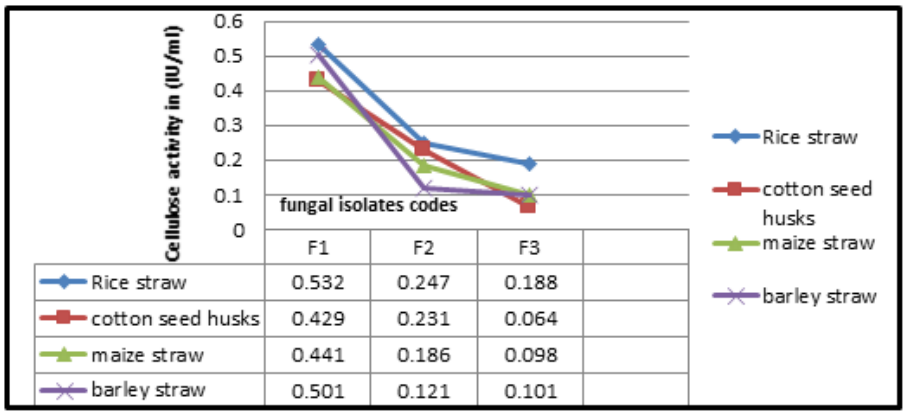



Figure 7

Graph of enzyme activity using different agricultural wastes.

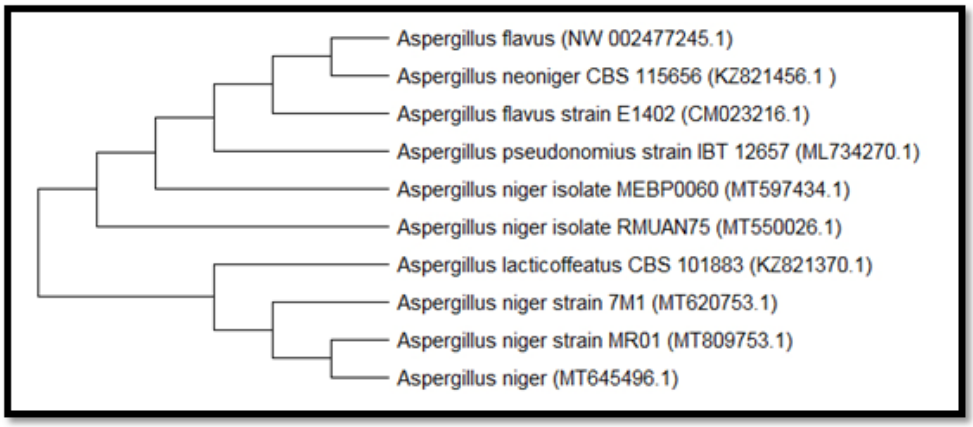



**Figure 8**

Phylogenetic tree of the cellulose-degrading fungi Aspergilus niger MT809753 the promising strain of this study and other species of the genus Aspergillus.

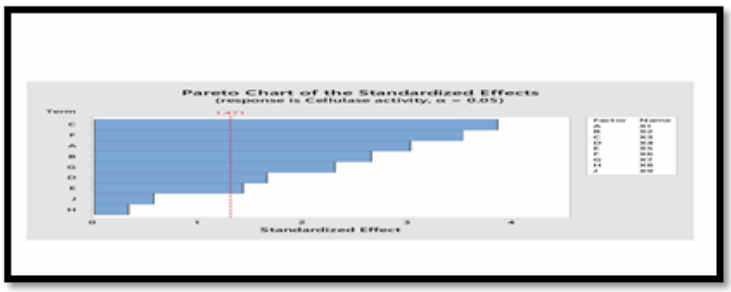
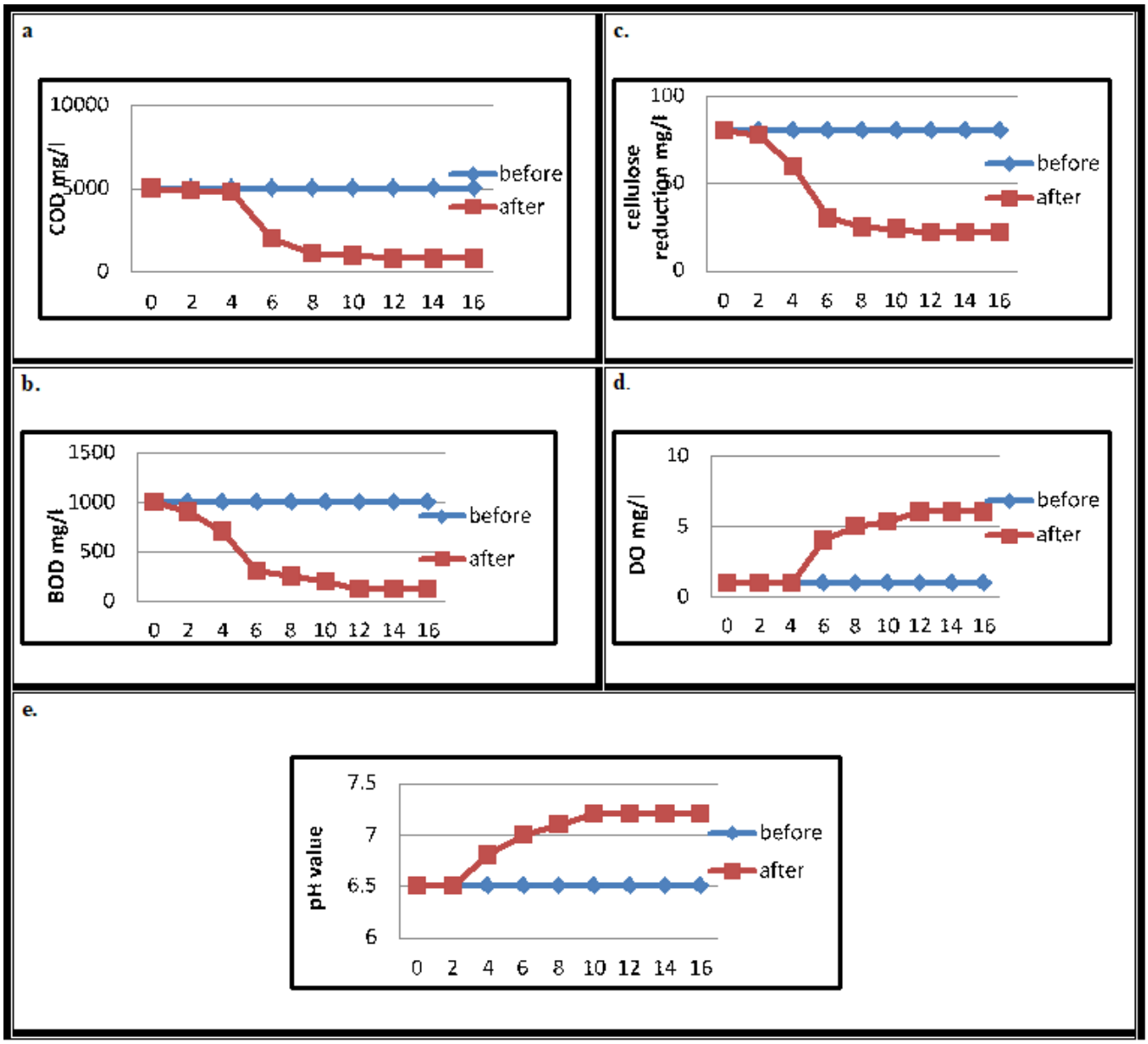



**Figure 9**

Pareto chart to visualization the effects of nine variables by PBD for cellulase produced from Aspergilus niger MT809753.




## Figure 10

Illustrations concerning bio-treatment of paper industry wastewater by Aspergilus niger MT809753 (a) reduction in COD (b) reduction in BOD (c) reduction in cellulose (d) DO concentrations during the experiment (e) elevation in pH values