Solid-state fermentation and optimization of cellulase production using local fungal isolate

Maha A. Basha[a], Yousseria M. Shetaia[a], Fathy M. Mehaya[b], Fatma H. Abdelzaher[c]

[a]Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt, [b]Food Technology Department, Food industries and nutrition institute, National Research Centre, Dokki, Giza, [c]Department of Agricultural Microbiology, Agricultural and Biology Research Institute, National Research Centre, Dokki, Giza

Correspondence to Fatma H. Abdelzaher, Professor, Department of Agricultural Microbiology, Agricultural and Biology Research Institute, National Research Centre, El-Behowth Street, PO Box 12126, Dokki, Cairo, Egypt. Tel: +20 122 357 1676/ +20 112 517 4718; fax: +20 233 370 931; e-mail: fatmahelmyabdelzaher@gmail.com

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Background
Cellulase is the most employed industrial enzyme in biological conversion of many cellulosic wastes. In this work, economic cellulase production by fungi in solid-state fermentation (SSF) by using solid wastes of medicinal plants was studied. Optimization of growth conditions for production of cellulase was the main target of this study.
Objective
The current study aimed to isolate and screen fungal isolates that have the ability to produce enzymes to degrade solid wastes of medicinal plant process and optimization of growth factors that affect cellulase production.
Materials and methods
Thirty-five fungal isolates were isolated from different sources by plating and screened for their cellulase activities using Czapek–Dox broth medium amended with 1% cellulose. Cellulase production by tested fungal isolates was carried out through utilization of olive (*Olea europaea*), black seeds (*Nigella sativa*), and castor bean (*Ricinus communis*) cakes in SSF. Optimization of the cellulase productivity was performed by Plackett–Burman design (PBD) and Box–Behnken design.
Results and conclusion
Out of the isolated 35 fungi, only 12 (34%) produced cellulase in SSF using olive, black seeds (Nigella), and castor bean cakes. Out of these fungal isolates, only 4, that is, no. 1, 7, 10, and 17 were superior in reducing sugar production from olive cakes (13.04, 15.61, 17.03, and 12.85 mg/ml), respectively. While four fungal isolates no. (1, 7, 7, and 10) were active producers of reducing sugars from black seeds (15.45, 18.96, 20, and 18.08 mg/ml), respectively. Only a fungal isolate no. 7 gave high reducing sugars (15.34 mg/ml) in castor cake SSF. The most potent fungal isolate (no. 10) produced 20 mg/ml of reducing sugars using black seed cakes as substrate for SSF. The potential fungal isolate was identified as *Aspergillus terreus* (OQ085169) based on the extracted fungal DNA that was amplified by PCR using specific internal-transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared with the other related sequences in GenBank (NCBI).
The screening of seven factors using PBD showed that only three variables: pH, incubation time, and aeration rate (rpm) affected significantly cellulase production. Box–Behnken design was used to estimate the optimal level of the selected variables based on the results of the PBD. All variables increased significantly cellulase using *A. terreus* (OQ085169). The *P* value was very low (0.0207) that indicated the significant, high correlation between the predicted and actual values (R^2=0.98), this indicating 98% of the variation in the cellulase activity was owing to the selected independent variables.

Keywords:
black seed waste, castor bean waste, cellulase, carboxymethyl cellulose, filter paper cellulase, fungi, olive cake waste

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Introduction

Egypt is a country based on agriculture, with many agroindustrials by products and wastes. Some of the most often-used food industry wastes include wheat bran, corn cobs, and rice bran. These waste items may cause environmental problems if poorly handled [1].

Medicinal and aromatic plant wastes represent a serious problem: the poor management of these wastes causes risks to public health and the environment [2]. The residues left behind after steam or the hydro-distillation method that is used to extract the valuable essential oil are deemed useless and treated as waste materials, these wastes are either burned by growers or left in the field, which pollutes the environment, and their disposal is problematic [3].

Egypt ranked second in the world for olive production, with around 450 000 tons produced in 2018/2019, according to Food and Agriculture Organization. Olive oil extraction by traditional systems generates alongside the olive oil (20%) solid (30%) and liquid waste (50%), which represents a serious environmental problem [4]. High quantities of organic matter and mineral nutrients, primarily phosphorus, nitrogen, magnesium, and potassium, can be found in olive-mill solid wastes. Following a composting process, it is applied as organic soil fertilizer or used as fuel in a cogeneration system.

Castor bean cake that remains after the extraction of the oil from castor beans (*Ricinus communis*) represents around half of their weight [5]. The residue contains 34–36% protein. Castor bean waste is used as an organic fertilizer rather than a protein complement because of its toxicity [6].

Castor bean cake must be free of harmful substances in order to be used as fertilizer, animal feed, or pretreatment of wastewater (as solid enzymatic preparation) [7]. Castor and other contaminants have been detoxified using solid-state fermentation (SSF) by filamentous fungi, with promising results [8]. Castor bean cake under SSF offers an intriguing and affordable alternative for producing valuable enzymes, such as cellulases, in addition to promoting residue cleansing [9,10].

Nigella sativa (black seeds), a plant native to North Africa, Southwest Asia, and Southern Europe [11], that has been utilized for thousands of years in Egypt and Greece for both medicinal and food uses [12]. Oil is the primary by-product of cold-pressing the seed; nevertheless, seed cake has not yet found much use [13]. According to some research, oil residues even include bioactive chemicals that can be employed as antioxidants [14].

These wastes may damage both terrestrial and aquatic environments if they are directly disposed of because of their high organic and polyphenol content. When disposed of in the soil, the phenolic content of these wastes produces phytotoxic effects that degrade soil quality and are harmful to plants and soil microflora.

Wastes from aromatic and medicinal plants have been treated using a variety of management techniques. Physical processes, physicochemical processes, thermal processes, and biological processes are a few of them. Microorganisms are used in biological processes to break down the biodegradable chemical components in these wastes [15]. For the management of solid waste, some soil fungi are useful. Some saprophytic fungi secrete enzymes that cause the complex substances (cellulose and lignin) present in dead plant remnants and their waste to degrade and decompose [16]. Other contaminants have been detoxified biologically utilizing SSF with filamentous fungi, with good results [8].

One of the predominant groups found in soil, fungi have a significant impact on ecosystem structure and functioning and are therefore essential to many ecological services [17].

A preferred source for the manufacture of commercial enzymes is filamentous fungus because of their higher yield level versus yeasts and bacteria [18,19].

Almost all *Aspergillus* species produce cellulase, they have the potential to monopolize the enzyme sector [20]. *Aspergillus aculeatus* is frequently isolated from rotting fruit and soil. *A. aculeatus* isolates have been utilized to manufacture a number of significant industrial enzymes, including cellulases, hemicellulases, and proteases that are used commercially in the food and feed sectors. These enzymes are produced because *A. aculeatus* can quickly break down plant cell walls [21]. Agricultural wastes are turned into value-added products by *Aspergillus fumigatus* employing inexpensive substrates for xylanase synthesis during submerged fermentation [1].

This study aims to isolate and screen fungal isolates that have the ability to produce cellulase enzyme to degrade solid wastes with ecosystem-friendliness. In this search, two statistical steps were applied for studying the effect of different variables on the production of cellulase using *A. terreus* (OQ085169). The first was the Placket–Burman design (PBD) technique, which was used for optimization and selection of the majority of factors that improve cellulase production. By using this model, we may test a big number of variables with a small number of experiments [22]. The second step design was Box–Behnken (BBD) [23] was that used to estimate the optimum level of the selected variables based on the results of the PBD.

Materials and methods

Waste materials

Cakes from oil extraction from olive, black seeds, and castor bean were collected from the local Egyptian

industries, olive cake collected from Agricultural Research Center – El Giza governorate, and black seeds and castor bean cakes collected from Elcaptain cappharm–ElAzhar Cairo governorate. pH was determined for all cakes and it was 6.44 for olive, 6.18 for black seeds, and 6.42 for castor bean. After drying, they were cut into pieces and grounded. They were used without any processing.

Chemicals

The chemicals used during this study were obtained from Sigma Aldrich Chemical Co. (St Louis, Missouri, USA).

Isolation and purification of fungi

Fungal isolates were isolated from soil collected from different locations (Giza, Sharqia, Gharbia, and Elmenia) and from animal wastes collected from Giza, by serial dilution technique [24,25] on potato dextrose agar (PDA) at 28°C for 3–5 days. And isolated from rabbit, sheep, and cow wastes that were added to saline solution (0.5% NaCl) and $10\ddot{E}^{1}$, $10\ddot{E}^{2}$ dilutions were prepared, 1 ml of each dilution was inoculated into both basal salt media for enrichment of fungal isolates from animal wastes, and Martin media to get separate pure fungal colonies. Also, fungi were isolated from rotted grape. Pure fungal colonies were picked, streaked on PDA slants, and kept at 4°C.

Preparation of spore suspension: 7-day-old PDA slant cultures of the fungal isolates were used for preparing fungal spore inocula. Spores were suspended by gentle agitation in sterile 0.85% saline solution containing 0.01% Tween-80.

Primary screening

All fungal isolates were prepared for primary screening by growing at 28°C for 7 days on Mendel's mineral salt agar medium (g/l) that contains urea 0.3; $(NH_4)_2SO_4$ 1.4; KH_2PO_4 2; $CaCl_2$ 0.3; $MgSO_4$ 0.3; yeast extract 0.25; proteose peptone 0.75; carboxymethyl cellulose (CMC) 10; and agar 17.5 [26]. For observations, plates were stained with 1% Congo red dye (30 min), followed by distaining with 1 M NaCl solution for 20 min, cellulase activity was visually observed regarding the zone around the microorganism colonies [27]. The fungal isolates were inoculated into Czapek–Dox broth medium for cellulase production. A volume of 100 ml of Czapek–Dox broth medium amended with 1% cellulose was distributed into separate 250-ml conical flasks, the pH of the medium was adjusted to 5. After autoclaving at 121°C, the fungal spore suspensions were inoculated into the conical flasks. The flasks were incubated at 28°C on a rotary shaker at 120 rpm. After 7 days of incubation, the contents of the flasks were aseptically passed through Whatman No. 1 filter paper to separate mycelial mat from culture filtrates and centrifuged at 5000 rpm for 10 min. The obtained clear supernatant was utilized as the source of crude enzymes [28].

The cellulolytic enzyme assay was carried out using dinitro-salicylic (DNS) acid reagent, for estimation of reducing sugar produced by each fungal isolate. The reading was taken in triplicates, and the density of the color was recorded using a spectrophotometer at wavelength 540 nm. The production of cellulase was estimated using glucose standard curve.

Enzyme activity (U/ml)=reducing sugars (mg/ml)×dilution factor×1000/incubation time×volume of enzyme×molecular weight of glucose.

Enzyme assay: filter paper cellulase (FPase) and CMCase (carboxymethylcellulase) activities were determined using DNS [29].

For cellulase activity

Cellulase activity in the culture filtrate was determined by carboxymethyl cellulase method [29]. The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was preincubated at 50°C in a water bath for 20 min. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50°C in a water bath for 1 h. Appropriate control without enzyme was simultaneously run. The reducing sugars produced in the reaction mixture were determined by DNS methods [30]. DNS reagent was added to aliquots of the reaction mixture and the color developed was read at a wavelength of 540 nm.

For measuring FPase activity, 50 mg of Whatman no. 1 filter paper was used as a substrate. Crude culture filtrate was used as an enzyme sample diluted in 0.05 M sodium citrate buffer (pH 4.8). The reaction mixture contained 0.5 ml of culture filtrate, 1.0 ml of 0.05 M sodium citrate buffer (pH 4.8), and the substrate was incubated at 50°C for 60 min for enzymatic reaction. After incubation, 3 ml of DNS was added and heated for 5 min in boiling water to obtain a colored reaction mixture. Absorbance of the solution was measured at 540 nm using a spectrophotometer.

For measuring CMCase activity, 2% w/v CMC was used as a substrate prepared in 0.05 M sodium citrate

buffer (pH 4.8). The reaction mixture contained 0.5 ml of culture filtrate and 0.5 ml of substrate was incubated at 50°C for 30 min for enzymatic reaction. After incubation, 3 ml of DNS was added and heated for 5 min in boiling water to obtain a colored reaction mixture. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the appropriate substrate per ml per min under the assay conditions [31].

Solid-state fermentation on plant wastes

Solid wastes of three medicinal plants were used for production of cellulolytic enzymes (olive cake, black seeds, and castor bean). Three culture tubes are used for each 12 fungal isolates and were incubated for 7, 18, 21, and 28 days. Noninoculated solid waste was incubated in the same conditions and used as a control (without spores). The maximum cellulase activity was determined. Ten grams from the examined three types of wastes were placed in 36 culture tubes for each type, and moisture was adjusted to 80%. Then, the tubes were autoclaved at 121°C for 20 min. Each tube was inoculated with 1 ml (1×10^7 spores/ ml) and incubated at 28°C for 28 days. Samples were taken after the 7-, 18-, 21-, and 28-day intervals for cellulase analysis [32].

The concentration of reducing sugars was determined by the modified DNS methods [30]. CMC, at 1% w/v, was used as substrates within 0.5 l of 50 Mm citrate buffer at pH 4.8 for cellulase activity determination, then measuring FPase and CMCase by the same above technique.

Molecular identification of the selected cellulose-decomposing fungal isolate

The most potent fungal isolate identified molecular by DNA extraction, PCR; the primer sequences were TCCGTAGGTGAACCTGCGG (ITS1: forward primer) and TCCTCCGCTTATTGATATGC (ITS4: reverse primer), those were designed for amplification of the ITS1–5.8S–ITS2 genomic region. Sequencing and analysis of the product were provided by Sigma Scientific Service Company. Sequencing was performed by GATC Company (Germany). The obtained sequences were compared with the other related sequences using BLAST search in GenBank of National Center for Biotechnology Information (NCBI) [33].

Statistical analysis

Statistical analysis for the previous experiments was performed by IBM SPSS Statistics, version 20, USA. All the experiments were carried out in triplicates. The results were expressed as mean values, using one-way analysis of variance followed by Duncan 's test [34]. Differences at *P* value less than 0.05 were considered to be significant.

Optimization of culture conditions for fungal cellulase production

Two steps of statistical design were used to study the most important variables that enhance cellulase production by the fungal isolate *A. terreus* (OQ085169). Seven variables, that is, temperature, pH, incubation period, inoculum density, aeration rate, surfactant concentration (%), and ammonium sulfate, were evaluated using this design and the levels of each variable were represented as high (+1) and low (−1) levels. All trials were conducted in triplicates and the mean values of cellulase activity were taken as response. The average value of enzyme activity (U/ml) in each trial was taken as an independent response [35].

The experimental runs were calculated as n+1, where n is the number of the selected variables. The variable levels are shown in Table 1. The design resulted in eight trials in which the seven variables were estimated (Table 2).

Each response for each variable was calculated according to equation (1)

$$Y = B0 + \Sigma Bi\ Xi \tag{1}$$

where Y is cellulase production response, B0 is the model intercept, Bi is the linear coefficient, and Xi is the level of the independent variable.

The main effect of each variable was calculated by the following equation (2) and shown graphically in Fig. 1:

$$E_{(xi)} = 2\left(\Sigma M\ I_{+M\ I-}\right)/N \tag{2}$$

where $E_{(Xi)}$ is the effect of the tested variable. Mi+ and Mi− represent cellulase production high and low values from the experimental runs, and N is the number of runs.

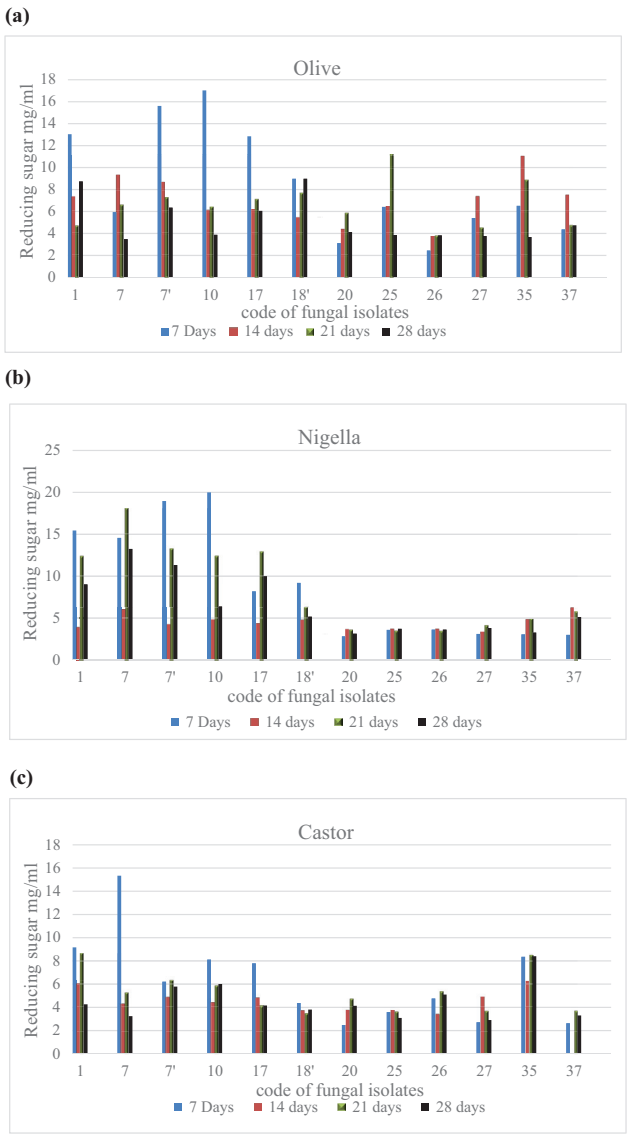
Table 1 Variables showing (+1) high and (−1) low levels in Plackett–Burman design

Variables	Symbol	Level	
		−1	+1
Temperature	X1	28	32
pH	X2	3	7
Incubation period (day)	X3	4	6
Inoculum density (ml)	X4	0.5	2.5
Aeration rate (rpm)	X5	100	200
Surfactant conc. (% Tween-80)	X6	0.1	0.5
Ammonium sulfate (g/l)	X7	0.5	2.5

Table 2 Matrix of the Plackett–Burman design experiments on seven variables

	Pattern	Temperature	pH	Incubation period (day)	Inoculum density	Aeration rate (rpm)	Surfactant conc. (Tween-80)	Ammonium sulfate (g/l)	Enzyme activity (U/ml)
1	+−−++−−	32	3	4	2.5	200	0.1	0.5	0.527
2	−−−−+++	28	3	4	0.5	200	0.5	2.5	0.248
3	−+−+−+−	28	7	4	2.5	100	0.5	0.5	0.697
4	+++++++	32	7	6	2.5	200	0.5	2.5	0.258
5	−−++−−+	28	3	6	2.5	100	0.1	2.5	0.212
6	++−−−−+	32	7	4	0.5	100	0.1	2.5	0.3454
7	+−+−−+−	32	3	6	0.5	100	0.5	0.5	0.336
8	−++−+−−	28	7	6	0.5	200	0.1	0.5	1.405

Figure 1



Assessment of reducing sugars (mg/ml) released by12 fungal isolates from (a) olive, (b) nigella, and (c) castor bean solid wastes after 7, 14, 21, and 28 days of incubation.

Box–Behnken design

BB is a class of rotatable second-order design based on three-level incomplete factorial design. The design of BB [23] was used to estimate the optimum level of the selected variables based on the results of the PBD. In this design, an experimental of 13 runs and one central point was constructed with the three significant independent variables [pH, aeration rate (rpm), and incubation time (day)]. Three different levels low (-), high (+), and basal (0) were done for each run. The average values of the enzyme activities were presented as a dependent response.

The second-order polynomial equation was used for predicting the optimal point. The interpretation of the correlation between the variables and the cellulase production response was as follows:

$$Y = B_o + \Sigma B_i X_i + \Sigma B_{ii} X_i 2 + \Sigma B_{ij} X_i X_j \quad (3)$$

where Y is the predicted cellulase production, ß0 is the model intercept, ßi is linear coefficient, ßii is quadratic coefficient, and ßij is cross-product coefficient, where Xi and Xj are the coded levels of the independent variables. The experiments were performed in triplicates and the mean values are given.

Results and discussion

Isolation of fungi and screening for production of cellulase enzyme

Thirty-five fungal isolates were isolated from different agriculture soil samples collected from different locations (Giza, Sharqia, Gharbia, and Elmenia) and from animal wastes collected from (Giza). Clear zones could be noticed around colonies of the active cellulase-producing isolates [27]. This area was measured for enzymatic index (EI) calculation from the following form [36]:

$$EI = \text{Diameter of clear zone}/\text{Diameter of colony}$$

In this regard, Ahmad *et al.* [37] previously evaluated cellulase activity by the measuring of a halo zone that is a light-yellow color encircling colonies. The primary screening indicated that 16 fungal isolates on Congo red plates might form cellulases that could be measured by clear-zone diameter (cm), among them, six fungal

isolates formed clear zone that ranged from 5.5 to 7.5 cm and 10 produced clear zone that ranged from 2.7 to 5 in diameter. Considering the largest clear-area diameter, the high level of cellulase activity was recorded by the fungal isolate no. 10 with enzymatic index of 3.25. In a previous report, Alnusaire and Farag [38] measured clear-zone diameters that ranged from 8 to 22 mm and selected the most potent celluase-producing fungal isolate with the largest (22 mm) clear zone finally identified as *Aspergillus ochraceus*. The colony-to-clear-zone ratio and enzyme activity are thought to have a poor association, and the plate screening approach is only qualitative rather than quantitative [39].

Cellulolytic enzyme assay

Data from Czapek–Dox broth medium showed that only 12 fungal isolates out of 35 fungal isolates have high cellulase activity. Fungal isolates with a code number (1, 7, 10, 17, 18, 18Ë, 30, 35, and 37) represent 25.7% of all fungal isolates that achieved high cellulase activities that ranged between 2.275 and 0.2454 IU/ml. Highly active Fpase, fungal isolates with a coded number (18Ë, 10, 19, 20, 23, 26, 27, 35, and 37) represent 25.71% of all fungal isolates that ranged between 0.924 and 0.209 IU/ml, while for CMCase activity, fungal isolates with code numbers (18Ë, 10, 20, 23, 26, 27, 30, 35, and 37), which represent 25.71% of all fungal isolates that ranged between 1.843 and 0.462 IU/m (the results are not shown(.

The fungal isolates 1, 7, 10, 17, 18, 18', and 19 were isolated from agriculture soil, while those coded 20, 23, 26, 27, 30, and 35 were isolated from animal wastes and one fungal isolate 37 was isolated from rotted grape.

AbdElrsoul and Bakhiet [40] isolated eight fungi from soil identified as *Aspergillus*, *Trichoderma*, *Fusarium*, and *Penicillium*. They selected three isolates, that is, *Aspergillus niger*, *Trichoderma viride*, and *Fusarium solani* were selected as active cellulase producers. In this context, El Baz *et al.* [41] recommended *T. viride* as a great source of cellulase.

Solid-state fermentation for fungal cellulase production from plant cakes

Twelve fungal isolates out of 35 fungal isolates were obtained from primary screening owing to their higher cellulase activity. The enzymes produced in SSF were determined after 7, 14, 21, and 28 days. Data presented in Fig. 1a show the amount of reducing sugars released from olive cake SSF by all 12 fungal isolates. After 7 days of incubation, 4 isolates 1, 7', 10, and 17 produced higher amounts of reducing sugars corresponding to 13.04, 15.61, 17.03, and 12.85 mg/ml, respectively, compared with other isolates. While enzyme activity of fungal isolates No. 35 and 25 reached upto 11.07 and 11.22 mg/ml after 14 and 21 days, respectively. In harmony with these findings, five fungal isolates were compared by Zaier *et al.* [42] utilizing olive wastes and they noted that *T. viride* and *A. niger* had high ability to produce cellulase activity of upto 14.55 and 9.92 U/gds, respectively.

Data illustrated by Fig. 1b show that isolate numbers 1, 7, 7', and 10 released high reducing sugars from black seeds (15.45, 14.57, 18.96, and 20.00 mg/ml, respectively) after 7 days and one isolate No. 7 produced high reducing sugars of 18.08 mg/ml after 21 days of incubation.

Figure 1c shows that castor bean waste material was not the appropriate substrate for almost all fungal isolates as only one fungal isolate No. 7 released 15.35 mg/ml reducing sugars after 7 days.

Higher cellulase activities of fungal isolate No. 10 *A. terreus* (OQ085169) might be due to the high growth of its mycelia on black seed waste. *A. niger* has been previously utilized to produce cellulase [43]. Pothiraj *et al.* [44] mentioned the fact that compared with *Rhizopus stolonifer* and *A. niger*, *A. terreus* shows low cellulolytic enzyme activity. This disagrees with Mirzaakhmedov *et al.* [45] who showed that the most active producer of cellulolytic enzymes was *A. terreus* compared with *Penicillium tigrinus* and *Penicillium ostreatus*. Ali *et al.* [46] reported high level of cellulase from *A. terreus* on water hyacinth after 6 days at 40°C. On the seventh day of this experiment, *A. terreus* displayed its highest cellulase activity.

There have been a lot of reports on *A. terreus* cellulase greater relative activity in cellulosic substrates [47]. Alnusaire and Farag [38] reported that cellulase activity by *A. ochraceus* varied significantly on different lignocellulose substrates under SSF with wheat bran yielding the highest CMCase and Fpase enzyme activity (52.55 and 50.37 U/mg, respectively). However, due to nutrient availability and the chemical composition of each substrate, direct comparison of these results would be difficult with orange peels that produced the lowest enzyme activities for CMCase and Fpase under SSF. Ismail and Hassan [48] showed that the cellulase activity of *A. terreus* RS2 was high (9.5 U/ml) using rice straw wastes. In the current study, the cellulase enzyme activity of *A. terreus* (OQ085169) was

improved by the activities than other fungal isolates that belong to other species.

Molecular identification of the most potent cellulose-decomposing isolate. Identification was performed on the basis of 18 S rRNA nucleotide sequencing, and the phylogenetic analysis was performed using MEGAX and neighbor-joining method [49]. The phylogenetic tree is shown in Fig. 2, and the data of the partial sequence were submitted under the name *A. terreus* with similarity of 98.67% to NCBI and accession number OQ085169.

Optimization of cellulase production by Plackett–Burman method

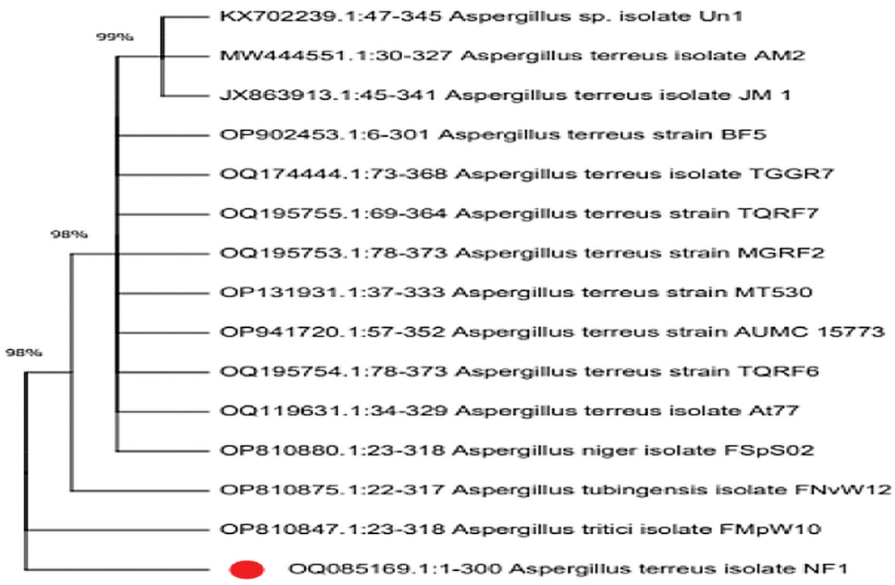
PBD [35] was used for screening and evaluating parameters that can influence enzyme activity. However, this model is unable to explain how different variables interact. Then after, the study was improved utilizing a BBD. The most significant variables that enhanced the cellulase production by *A. terreus* (OQ085169), seven variables were chosen in the PBB [incubation temperature, pH, incubation period (day), inoculum concentration (ml), aeration rate (rpm), surfactant concentration (%), and ammonium sulfate (g)].

As shown in Table 2, there was a wide range of changes in the results of cellulase activity from 0.212 to 1.405 U/ml. The highest activity was observed at run number 8 with 1.405 U/ml by applying cultural conditions of temperature 28°C, pH 7, incubation period (6 days), inoculum concentration (0.5 ml), aeration rate (200 rpm), surfactant concentration (0.1%), and ammonium sulfate (0.5 g). Whereas, the lowest effect (0.212 U/ml) was observed at run 5.

Several statistical techniques have been successfully used for the optimization of the production of many enzymes [50,51]. Dutt and Kumar [52] studied incubation time for production of cellulase by *A. niger* and *A. flavus* and they found that the best time was 5 days, and cellulase activity after that was decreased, whereas biomass was increased after 6 days. Hsu *et al.* [53] reported that the optimal conditions of pH 6.5, 37°C, and 30 h of incubation time produced the greatest enzyme activity. The synthesis of enzymes and the rate of fungal growth were both significantly impacted by the pH of the medium. Sivaramanan [54] noted that *A. niger* performs best in acidic media with pH 6, where Priyanka *et al.* [55] recorded that a pH of 7.0 was ideal for promoting increased cellulase enzyme activity. Also, Das *et al.* [56] recorded that when the pH value was lower than 7, the growth of the fungus was reduced.

The main effects of the tested variables on cellulase activity were calculated and presented in Fig. 3. At *P* value less than 0.05, the analysis indicated that out of seven variables, three variables had positive effects, pH, aeration rate, and incubation time, whereas temperature, inoculum concentration, and emulsifying agent (Tween-80) had negative effects. The coefficient of determination R^2 value of the model was 0.9232, which indicated that this model could explain 92.32% of the response variability.

Figure 2



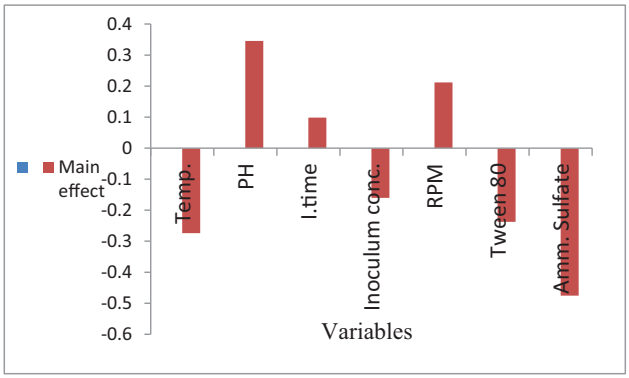
Phylogenetic tree of *Aspergillus terreus (OQ085169).*

Joglekar and May [57] demonstrated that the R^2 should be at least 80% to indicate the suitability of the used model. In this study, PBD analysis indicated that three variables out of seven have the highest significant influence on production of cellulase (pH, aeration rate, and incubation time).

Box–Behnken design

The matrix of BBD was used to investigate the influence of important variables on cellulase production and find the most combination of these variables resulting in maximum production [23]. Three variables (pH, aeration rate, and incubation time) identified by PBD having positive effect were used in the BBD with three levels [low (−), medium (0), and high (+)] that were chosen. Table 3, actual and coded units for the variables with observed and predicted values of the results are given in the matrix of BBD (Table 4).

Figure 3



Assessment of the main effect of independent variables on cellulase activity.

The second-order polynomial equation was used to predict the optimal point, within the experimental constraints:

$$Y=0.951+0.53735x_1-0.73325x_2+0.291375x_3 -0.0625x_1x_2-0.03075x_1x_3-0.1505x_2x_3+0.541875X_1^2 +0.331625X_2^2+0.613875X_3^2.$$

where Y is the cellulase activity, while X1, X2, and X3 are pH, aeration rate, and incubation time, maximal cellulase activity of 3.071 U/ml was predicted to be from pH 5, incubation times 7 days, and aeration ratio 150 rpm, compared with the actual value of 3.136 U/ml.

The scatterplot (Fig. 4) showed that the majority of the dots were around the regressed diagonal line because R^2 was 0.98. Additionally, the residuals are homoscedastic, which demonstrated the accuracy of the variance of the error through the three levels of the independent variables.

In the process of producing cellulase, agitation speed is essential, a significant change in cellulase enzyme activity was recorded when aeration rate increased from 100 to 200 rpm and then declined when aeration rate increased from 200 to 300 rpm. Ma *et al* [58] mentioned that the cellulase activity inhibition occurred with faster agitation speed. Many fungi produced cellulases at different shaking rates between 100 and 200 rpm [59]. A 72-h

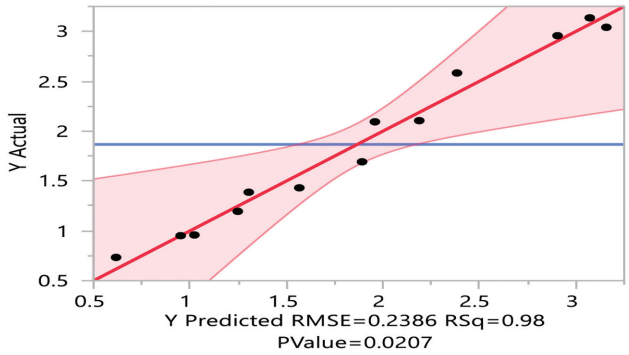
Table 3 Assessment of the three levels of the independent variables by Box–Behnken design

Symbols	Independent variables	Coded levels		
		−	0	+
X1	pH	4	5	6
X2	Aeration rate	150	200	250
X3	Incubation time	5	6	7

Table 4 Experimental values of cellulase activity by three variables through Box–Benhken design

Run numbers	Pattern	X1	X2	X3	Y	Predicted Y	Residual Y
1	−−0	4	150	6	2.093	1.957875	0.135125
2	0−+	5	150	7	3.136	3.071625	0.064375
3	+0+	6	200	7	2.957	2.90475	0.05225
4	0++	5	250	7	1.387	1.304125	0.082875
5	0+−	5	250	5	0.958	1.022375	−0.06438
6	−0−	4	200	5	1.195	1.24725	−0.05225
7	−0+	4	200	7	1.692	1.8915	−0.1995
8	0−−	5	150	5	2.105	2.187875	−0.08288
9	000	5	200	6	0.951	0.951	1.11E-16
10	−+0	4	250	6	0.733	0.616375	0.116625
11	++0	6	250	6	1.431	1.566125	−0.13513
12	+0−	6	200	5	2.583	2.3835	0.1995
13	+−0	6	150	6	3.041	3.157625	−0.11663

Figure 4



Predicted and actual cellulose activity plot for the BBD.

assessment of the incubation period's impact on cellulase production revealed a considerable impact. Enzyme activity increased with longer incubation time, reaching a high peak value of enzyme activity after 48 h, then it began to fall on the third day (72 h). After 24 h, the lowest enzyme activity was recorded. These results are in agreement with several researches that hypothesized that a decrease in enzymatic activity with increased time of incubation may be caused by utilizing nutrients in the medium, which might lead to fungal stress and inhibit the secretion of enzyme [60].

El Baz *et al.* [41] detected that cellulase activity increased by 64% (from 1.066 to 2.99 IU/ml) after the significant variables were discovered by PBD and their extended levels were optimized by BBD. These results demonstrated the efficacy of statistical methods to increasing cellulase activity in the economical enzyme production from *T. viride*.

Using BBD technique to determine the optimum level of the highest significant three variables, the R^2 was 0.98. Ghorbannezhad *et al.* [61] demonstrated that the suitable value of R^2 indicates that the model represents accurate results, and it can be successfully used in the optimization experiments [62]. Moreover, the residual analysis referred that the model is accurate for all of the observed findings on average.

Conclusion

Production of cellulase enzyme by local fungal isolate *A. terreus* (OQ085169) using untreated medicinal plant solid wastes was investigated in this study. The results indicated the black seed plant waste was the best for cellulase production on SSF process. By using PBD followed by BBD for optimizing the parameters of culture, the production of cellulase reached 3.136 U/ml. This study was highlighting focus on using of medicinal plant wastes for economical production of one of the important industrial enzymes (cellulase enzyme).

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Conflicts of interest

There are no conflicts of interest.

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