Optimizing the production of rice straw hydrolytic cellulase under solid-state fermentation using *Aspergillus terreus* RS2

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Received: 5 September 2019
Revised: 24 November 2019
Accepted: 4 February 2020
Published: 24 March 2020

Egyptian Pharmaceutical Journal 2020, 19:7–17

Background
The enzymatic hydrolysis of lignocelluloses into fermentable sugars is the key step in biorefining, and cellulases are the key enzymes. The low titer of cellulase production and their high cost remain the most significant barriers to their industrial applications. The aim of this study was the economic production of cellulase by an Egyptian fungal isolate under solid-state fermentation by using rice straw as a carbon source. Additionally, the produced enzyme can be applied in the hydrolysis of rice straw and production of free sugars that can be used in several biotechnological industries.

Materials and methods
The isolated fungus was identified according to its cultural and morphological features followed by 18S rDNA sequencing. Optimization of the enzyme productivity was performed by applying Plackett–Burman and Box–Behnken designs. Finally, the hydrolysis of rice straw for the production of fermentable sugars using the produced enzyme was carried out, confirmed by scanning electron microscope and thin-layer chromatography analysis.

Results and conclusion
The optimum cellulase activity produced by the isolated fungus *Aspergillus terreus* RS2 was 124.94 U/g dry substrate. It was achieved at the optimum conditions that were conducted to be as follows: 3.75 g (1.5%w/v) of rice straw moistened with 11.25 ml (1 : 3 biomass to moistening agent) of modified Mandel's medium [1% $(NH_4)_2SO_4$ was the only constituent nitrogen source] of pH 7, incubated at 30°C for 8 days. Finally, the activity of the produced enzyme in the degradation of rice straw indicated the release of reducing sugars of 343.98 mg/g dry substrate with a saccharification percentage of 34.4% recorded after 4-h hydrolysis period.

Keywords:
Aspergillus terreus RS2, carboxymethyl cellulase, rice straw, saccharification, solid-state fermentation

Egypt Pharmaceut J 19:7–17

1687-4315

Introduction

The overall energy shortage and the environmental problems enforce global concerns for the development and utilization of renewable energy sources [1]. Lignocellulosic biomass is one of the most abundant but underutilized biomass that is considered to be an attractive option for the development of today's biorefineries from the perspective of its low cost, renewability, and its minimal effect in food production [2]. It consists of ~35–50% cellulose, ~20–35% hemicelluloses, and ~5–30% lignin of the plant dry weight [3]. The conversion of the polysaccharide constituent of this biomass to its constituent fermentable sugars is a crucial step in the commercial success of biorefineries [4]. The use of enzymes in the conversion process is the most convenient, desirable, and ecofriendly method, with a satisfactory yield of free sugars [5].

Cellulolytic enzymes are the biorefineries key enzymes, as they are the enzymes that catalyze the hydrolysis of cellulose. They are produced naturally by wood-degrading fungi and bacteria as a part of the energy transfer and the carbon cycle. Commercially, filamentous fungi of *Trichoderma* spp. and *Aspergillus* spp. are the main source for their production [6,7], but their high cost is still the most significant barrier to their industrial applications [8], reflecting the importance of their low-cost production.

The production of cellulase using fungi via solid-state fermentation (SSF) is one of the most desirable and cost-effective techniques [7]. SSF is an old technology that has been emphasized to be efficient in the production of hydrolytic enzymes in which the cultivating conditions of the fungi simulate the natural environment [9]. The selection of the substrate for SSF processes that can

facilitate the anchorage of fungi, along with providing sufficient nutrients, remained a subject of intense research for many decades [10]. Rice straw is one of the most abundant lignocellulosic crop residue that is generated and collected directly from the fields [2]. According to the Food and Agriculture Organization of the United Nations points system, the annual global production of rice straw is ~600–900 million tons [6]. The low bulk density and high silica and mineral contents are characteristics that limited its application [11]. So the burning of rice straw in open fields is a common practice for its disposal all over the world, leading to air pollution that consequently affects public health [6]. The utilization of rice straw as a carbon source for the production of cellulase instead of using the refined cellulosic carbon source can decrease the total cost of the enzyme and increases its economic viability.

The conditions of the fermentation processes have a crucial effect on the growth of the microorganisms and their released metabolic products as well as the cost of the production process. For multivariable processes, statistical optimization is advantageous to save time and effort, decrease the number of the required experiments, and consequently, decrease the total cost of the process [12]. Response surface methodology was originally described by Box and Wilson [13], and in the past few years, it has been applied in the optimization processes in various microbiological and biotechnological fields [14,15]. It has been successfully applied in the optimization of the microbial production of cellulase [8,16,17].

The current study concerned with the production of cellulase by SSF of rice straw using locally isolated fungus in addition to the identification of the isolated fungus on the basis of its cultural and morphological features followed by 18S rDNA sequencing. Optimization of the enzyme productivity by applying Plackett–Burman and Box–Behnken designs was performed. Finally, the ability of the produced enzyme for rice straw hydrolysis and production of free sugars was examined.

Materials and methods

Materials

Carboxymethyl cellulose (CMC) and glucose standard were purchased from Sigma-Aldrich, (Saint Louis, MI, USA). Potato dextrose agar (PDA) and thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). Dinitrosalicylic acid (DNS) was obtained from Panreac (Barcelona, Spain). All other chemicals were of analytical or high-performance liquid chromatography grade.

Rice straw was collected after air drying from fields situated in Al Sharqiya, Egypt, which was then cut into pieces, grounded using a standard grinder, and used directly without any further processing.

Microorganism

The fungus used in this study was previously isolated during the intensive course of the screening program concerned with the isolation of microorganisms from the soil secreting nonstarch hydrolyzing enzymes (unpublished data). It was identified according to its cultural and morphological features. Molecular identification of the strain was carried out by 18S rDNA sequencing performed in Sigma Scientific Services Co.

Culture conditions and enzyme production

The fungus was cultivated on PDA slants and incubated at 30°C for 7 days unless other conditions were described. After cultivation, spore suspension was prepared by scratching of each slant with 10 ml distilled water containing 0.1% tween 80. SSF was initially performed, in which 2 ml of the spore suspension was used to cultivate 250 ml Erlenmeyer flask containing 5 g of rice straw (with 0% of initial moisture content) moistened with 10 ml tap water then incubated at 30°C for 7 days. At the end of the fermentation process, extraction was performed by adding 50 ml of distilled water to each flask, shaken at 150 rpm and 30°C for 1 h, and then centrifuged at 5000 rpm for 10 min. The resulted supernatant was subjected to further analysis.

Enzyme activity and protein content assays

The cellulolytic activity in terms of carboxymethyl cellulase (CMCase) activity was determined by DNS method [18] in a reaction mixture consisted of 500 µl of 1% CMC (dissolved in 0.05 mol/l acetate buffer, pH 5) and 500 µl of the culture supernatant incubated at 50°C for 30 min. At the end of the assay time, 2.5 ml of DNS was added to stop the reaction, and the color developed after boiling for 10 min was measured at 540 nm. One unit of the enzyme was defined as the amount of enzyme that released 1 µmol of glucose per minute under the assay conditions. The amount of the produced enzyme was expressed as units per gram dry substrate (U/gds).

The protein content was determined as described by Lowry *et al.* [19], using bovine serum albumin as a standard.

Effect of pretreatment of rice straw

The effects of microwave heating for 1 min and alkaline (soaking in 5% NH_4OH for 24 h at 4°C [20] and in

2.75% NaOH for 3 h at 55°C [21] in the ratio 1 : 20 w/v) pretreatments of rice straw were investigated.

Effect of moistening agent

Overall, 10 ml of Mandel's medium [composed of (g/l) $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2, urea 0.63, $CaCl_2$ 0.3, $MgSO_4.7H_2O$ 0.3, peptone 0.75, and 1 ml of trace element solution] [22] at pH 5 was used as a moistening agent instead of tap water. After that the constituent mixture of nitrogen sources was replaced by equivalent nitrogen content of each nitrogen source separately (peptone, urea, and $(NH_4)_2SO_4$), and then the most suitable concentration of the selected nitrogen source was examined in the range of 0.1–2%.

Statistical optimization of cellulase production

In this study, two-step optimization approaches were carried out to enhance cellulase production. Initially, the variables that influence cellulase production were selected by applying Plackett–Burman design. In the second step, Box–Behnken design was applied to optimize the selected variables.

Selecting the variables that influenced cellulase production

For multivariable processes, the screening of the variables that influenced any process is the initial step of its optimization. To identify the variables that have the highest influence on cellulase production, Plackett–Burman design [23], including seven variables, was applied, in which the experimental runs were calculated as n+1, where n is the number of the selected variables. The seven independent variables were substrate concentration (% w/v), moisture level (biomass to moistening agent ratio), pH of the moistening agent, time of autoclaving (min), age of the fungus (days), temperature (°C), and fermentation period (days); each variable has been represented in terms of high (+1) and low (−1) levels, as shown in Table 1.

Table 1 The levels of the independent variables screened in Plackett–Burman design

Variables	Level	
	−1	+1
Substrate concentration (% w/v)	2	3
Moisture level (biomass to moistening agent ratio)	1 : 2	1 : 3
pH of the moistening agent	5	7
Time of autoclaving (min)	15	30
Age of the fungus (days)	7	9
Temperature (deg.)	25	30
Fermentation period (days)	5	11

Each generated response was calculated according to the first-order linear equation:

$$Y = B_0 + \Sigma B_i X_i \qquad (1)$$

where Y is the response (cellulase production), B_0 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 determined by the following equation:

$$E_{(Xi)} = 2(\Sigma M_{i+} - M_{i-})/N \qquad (2)$$

where $E_{(}Xi_{)}$ is the effect of the tested variable. Mi_{+} and Mi_{-} represent cellulase production from the experimental runs where the independent variable (Xi) measured was present at high and low values correspondingly, and N is the number of runs.

Box–Behnken design

In the second step of optimization, Box–Behnken design [24] was applied to determine the optimum level of the selected variables. In this model (Table 3), an experimental design of 15 runs and three central points was constructed with three independent variables. Each variable was examined at three different levels: low (−), high (+), and control or basal (0). The second order polynomial equation used for the interpretation of the correlation between the variables and the response (cellulase production) was as follows:

$$Y = B_0 + \Sigma B_i X_i + \Sigma B_{ii} X_i 2 + \Sigma B_{ij} X_i X_j \qquad (3)$$

Table 2 Plackett–Burman design

Run number	Substrate concentration (% w/v)	Moisture level (biomass to moistening agent ratio)	pH of the moistening agent	Time of autoclaving (min)	Age of the fungus (days)	Temperature (deg.)	Fermentation period (days)	Cellulase (U/gds)
1	−	−	−	+	+	+	−	110.814
2	+	−	−	−	−	+	+	64.070
3	−	+	−	−	+	−	+	101.418
4	+	+	−	+	−	−	−	81.434
5	−	−	+	+	−	−	+	95.895
6	+	−	+	−	+	−	−	79.546
7	−	+	+	−	−	+	−	116.898
8	+	+	+	+	+	+	+	78.24

Table 3 Analysis of Plackett–Burman design

Variables	Cellulase analysis			
	Coefficient	*t*-statistics	*P* value	Confidence level (%)
Intercept	146.407			
Substrate concentration (% w/v)	–37.35	–32.446	4.99*E*–16	100
Moisture level (biomass to moistening agent ratio)	1.107	8.496	2.52*E*–07	100
pH of the moistening agent	1.605	3.944	0.00116	99.884
Time of autoclaving (min)	–0.018	–0.326	0.748715	25.129
Age of the fungus (days)	1.465	3.599	0.002402	99.76
Temperature (deg.)	0.587	3.603	0.002386	99.761
Fermentation period (days)	–2.045	–15.071	7.12*E*–11	100
Summary of the model				
Multiple *R*			0.995	
R^2			0.991	
Adjusted R^2			0.987	
SE			1.994	

where *Y* is the predicted cellulase production, ß$_0$ is the model intercept, ß*i* is linear coefficient, ß*ii* is quadratic coefficient, and ß*ij* is cross-product coefficient, where *Xi* and *Xj* are the coded levels of the independent variables.

Hydrolysis of rice straw

Saccharification activity of the produced enzyme

Initially, the filter paper hydrolyzing (FPase) activity was determined according to Ghose [25], in which 0.5 ml of the culture supernatant was incubated with a 1×6 cm (5 mg) rolled filter paper strip (Whatman No.1) in 0.5 ml of 0.05 mol/l acetate buffer, pH 5, at 50°C for 30 min. In addition xylanase, mannanase, and pectinase activities of the produced enzyme were estimated using xylan, locust bean gum, and citrus fruit pectin, respectively, as substrates. The amount of the released reducing sugars was determined by DNS method [18].

The saccharification of rice straw using the produced enzyme was examined in a 50 ml screw-capped bottle, in which 10 ml of the enzyme was added to 1 g of rice straw in 10 ml of 0.05 mol/l acetate buffer at pH 5 and incubated at 50°C for different incubation periods (1–6 h). The amount of the released reducing sugars was determined using DNS. The percentage of saccharification was calculated as follows [26]:

$$\text{Saccharification}\% = \frac{\text{Releasedreducingsugars(mg/ml)}}{\text{Amountofricestraw(mg/ml)}} \times 100 \quad (4).$$

Thin-layer chromatography analysis

The rice straw-resulted hydrolyzate with the maximum amount of released reducing sugars was analyzed by TLC using a mixture of propanol : water : ammonia (7 : 2 : 1 v/v) as a mobile phase [27]. The resulted sugars were visualized with diphenyl amine-aniline reagent [28].

Scanning electron microscope

The rice straw before and after enzymatic treatment was examined by field emission high-resolution scanning electron microscope (Quanta 250; FEI, Czechoslovakia, Czech Republic).

Statistical analysis

The experiments were carried out in triplicates, with three measurements per replicate, and the results were reported as average±SD.

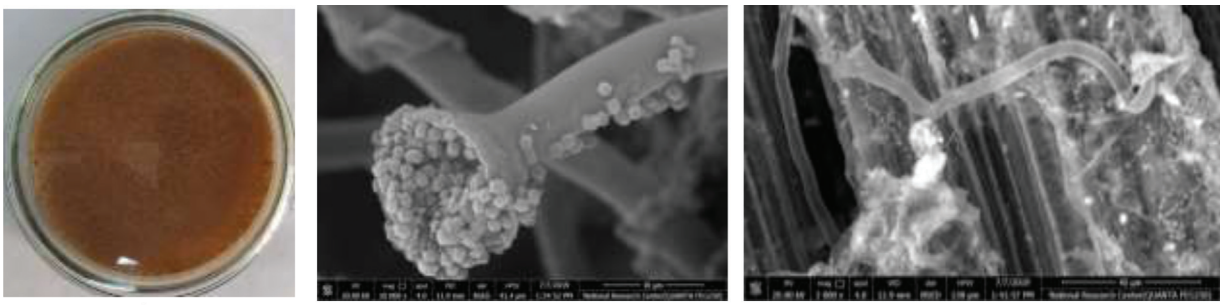
Results

Cultural and morphological features of the isolated fungus

The cultural feature of the used fungus on PDA is shown in Fig. 1a, indicating that the fungus colonies were cinnamon to brown in color. Morphological features of the isolate in terms of sporangia were examined under scanning electron microscope, as shown in Fig. 1b, indicating that the fungus is *Aspergillus* spp. Moreover, the culture of the isolated fungus in the optimized fermentation medium containing rice straw as a carbon source was examined under scanning electron microscope (Fig. 1c), manifesting the ability of the isolated fungus for the utilization of rice straw for growth.

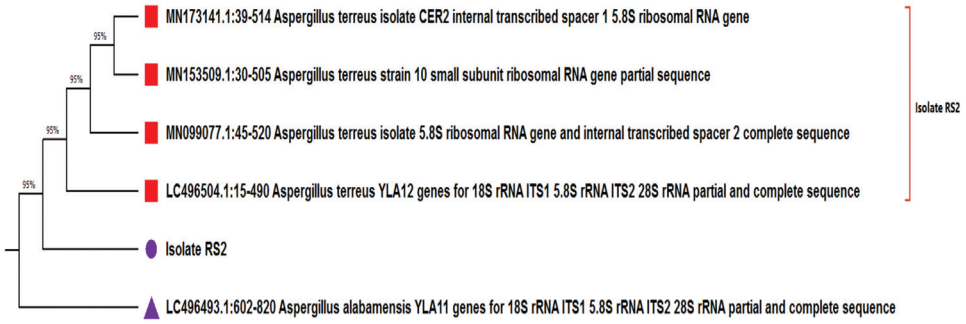
The confirmation for the isolate identification was performed on the basis of 18S rDNA nucleotide sequencing, and the phylogenetic analysis was performed using MEGAX and neighbor-joining method [29]. The phylogenetic tree is shown in Fig. 2, and the data of the partial sequence were submitted under the name *Aspergillus terreus* strain

Figure 1



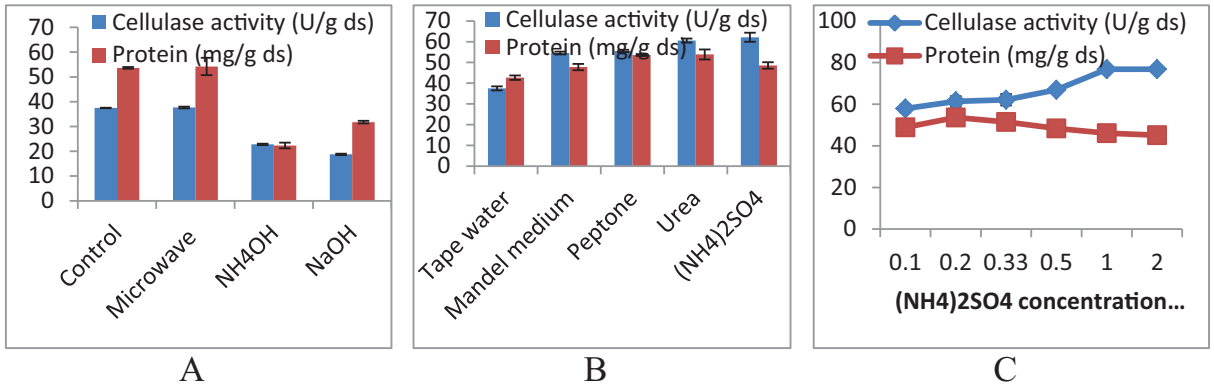
Cultural and morphological features of the used fungus. (a) The culture on potato dextrose agar; (b) scanning electron microscopy of the fungus cultured on potato dextrose agar; (c) scanning electron microscopy of the fungus cultured on rice straw (c).

Figure 2



Phylogenetic tree.

Figure 3



The effect of (a) rice straw pretreatment (b) different moistening agent and (c) different concentration of $(NH_4)_2SO_4$.

RS2 to NCBI and received accession number of MN368221.

Rice straw pretreatment effect

The effect of pretreatment of rice straw either by microwave heating or by soaking in alkaline solutions did not improve the productivity of the enzyme. The alkaline pretreatment led to almost 50% decrease in the total protein content and enzyme activity, as shown in Fig. 3a.

Effect of moistening agent

The substitution of tap water with Mandel's medium as a moistening agent improved the productivity from 37.481 to 54.536 U/gds and increase the total protein content from 42.676 to 47.776 mg/gds.

On replacement of the constituent mixture of nitrogen source with the equivalent nitrogen content of each individual nitrogen source, the highest productivity was observed by using $(NH_4)_2SO_4$ only, in which the

productivity increased to 62.149 U/gds, with a slight increase in total protein content (48.551 mg/gds) (Fig. 3b). By using different concentrations of $(NH_4)_2SO_4$ (0.1–2%), the productivity increased as the concentration increased to reach 76.828 U/gds by using $(NH_4)_2SO_4$ of concentration 1%. After that, no increase in productivity was observed by increasing the concentration. Although the total protein content increased as the concentration increase from 0.1 to 0.2%, the overincrease in concentration did not increase the total protein content (Fig. 3c).

Statistical optimization

Selecting of the variables that influenced cellulase production

By applying PBD, seven variables were screened for their influence on cellulase production, and the mean value of the observed enzyme activity is illustrated in Table 2. A wide variation in the results ranging from 64.07 to 116.898 U/gds was observed, reflecting the importance of the initial screening of the variables that influenced the enzyme productivity. The maximum activity was observed at run number 7, with 116.898 U/gds by applying fermentation conditions of substrate concentration, 2%; moisture level; 1 : 3 biomass to moistening agent ratio; pH: 7; time of autoclaving: 15 min; age of the fungus: 7 days; temperature: 30°C; and fermentation period: 5 days.

The main effects of the examined variables were calculated and are presented graphically in Fig. 4. The calculated values indicated that the highest main effects were investigated with the substrate concentration, moisture level, and fermentation period, in which both the substrate concentration and the fermentation period had negative values, whereas the moisture level had positive value.

The analysis of the data by multiple regression (Table 3) indicated that all of the tested variables except the time of autoclaving significantly affected the enzyme productivity. The highest confidence level was observed to be with the substrate concentration, moisture level, and fermentation period. The coefficient values were used to estimate the effect of the seven independent variables on the enzyme production. The variable that possessed positive effect was maintained at positive level whereas the one that possessed negative effect was maintained at negative level to achieve maximum production of the enzyme in the second phase of optimization. The first-order equation that described the correlation between the selected seven variables and the enzyme activity could be presented as follows:(5)

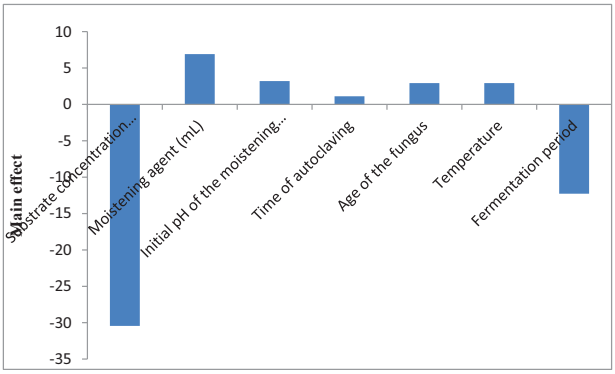
$$Y = 146.407 - 37.35X_1 + 1.107X_2 + 1.605X_3 0.018X_4 + 1.465X_5 + 0.586X_6 - 2.045X_7,$$

where Y is the cellulase activity, and X_1, X_2, X_3, X_4, X_5, X_6, X_7 are substrate concentration, moisture level, pH of the moistening agent, time of autoclaving, age of the fungus, temperature, and fermentation period, respectively.

The coefficient of determination (R^2) value of the selected model was 0.991, and the calculated analysis of variance indicated the overall high significant of the model since the model terms had probability greater than F value of 4.12 E-15 (<0.05).

From the aforementioned results of PBD, the substrate concentration, moisture level, and fermentation period were the variables with the highest main effect and the highest confidence level, so they were selected for the second phase of optimization.

Figure 4



The independent variables main effects on cellulase production.

Box–Behnken design

The observed and predicted mean values of Box–Behnken design results are represented in Table 4. The second-order polynomial equation, concluded from the multiple regression analysis (Table 5), was used in the prediction of the enzyme activity as follows:(6)

$$Y = 2.590215 + 9.353578X_1 + 6.647487X_2 + 24.02699X_3 - 12.7299X_1 2 - 0.27016X_2 2 - 1.73563X_3 2 + 1.481921X_1X_2 - 3.00523X_1X_3 + 0.150345X_2X_3,$$

where Y is the cellulase activity and X_1, X_2 and X_3 are the substrate concentration, moisture level, and fermentation period, respectively.

The R^2 value of the applied model was 0.86, indicating that 86% of the variation in the cellulase activity was

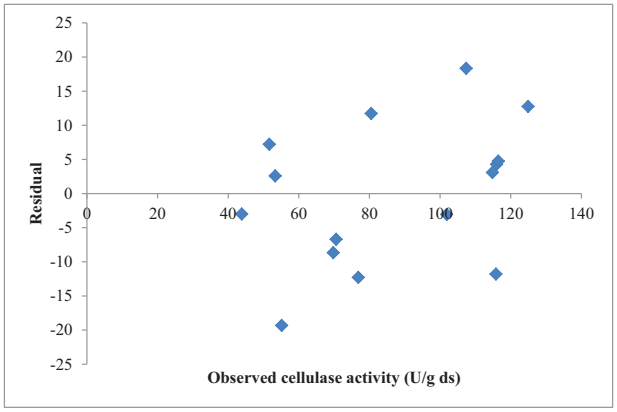
Table 4 Box–Behnken design

Run number	Independent variable			Observed cellulase (U/gds)	Predicted cellulase (U/gds)	Residual
	X_1 Substrate concentration (% w/v)	X_2 Moisture level (biomass to moistening agent ratio)	X_3 Fermentation period (days)			
1	1.5(–)	1 : 1(–)	5(0)	55.153	74.467	–19.314
2	2.5(+)	1 : 1(–)	5(0)	51.663	44.438	7.225
3	1.5(–)	1 : 5(+)	5(0)	115.833	127.619	–11.787
4	2.5(+)	1 : 5(+)	5(0)	80.505	68.763	11.741
5	1.5(–)	1 : 3(0)	2(–)	107.400	89.056	18.344
6	2.5(+)	1 : 3(0)	2(–)	70.574	77.263	–6.689
7	1.5(–)	1 : 3(0)	8(+)	124.940	112.182	12.758
8	2.5(+)	1 : 3(0)	8(+)	76.848	89.122	–12.274
9	2(0)	1 : 1(–)	2(–)	43.841	42.274	–3.004
10	2(0)	1 : 5(+)	2(–)	69.770	78.419	–8.649
11	2(0)	1 : 1(–)	8(+)	53.327	50.746	2.581
12	2(0)	1 : 5(+)	8(+)	101.869	104.932	–3.063
13	2(0)	1 : 3(0)	5(0)	116.021	111.730	4.292
14	2(0)	1 : 3(0)	5(0)	116.487	111.730	4.758
15	2(0)	1 : 3(0)	5(0)	114.819	111.730	3.09

Table 5 Box–Behnken design analysis

Term	Regression coefficient	*t*-Test	*P* value
Intercept	2.590215	0.042015	0.966725
X1	9.353578	0.157018	0.876133
X2	6.647487	4.104687	0.00023
X3	24.02699	3.938312	0.000373
X12	–12.7299	–0.82875	0.412862
X22	–0.27016	–8.3026	8.67*E*–10
X32	–1.73563	–4.37498	0.000104
X1 ×*2*	1.481921	1.72719	0.09295
X1 ×*3*	–3.00523	–1.23306	0.225772
X_2X_3	0.150345	1.317639	0.196185
Summary of the model			
Multiple *R*	0.92831		
R^2	0.861759		
Adjusted R^2	0.826211		
SE	11.85782		

Figure 5



Residual plot.

owing to the selected independent variables. The absolute average deviation (AAD) was calculated to be 10.938% by Eq. 7.(7)

$$\mathrm{AAD} = \left\{\left[\sum_{i=1}^{p}(|Y_{exp} - Y_{prd}|/Y_{exp})\right]/P\right\} \times 100,$$

where P, Y_{exp}, and Y_{prd} are the number of the experiment, observed, and predicted cellulase activity, respectively.

The analysis of variance of the regression indicated that the model terms used in this study are highly significant, as the model terms had F value of 24.242 and P value of 1.64 E–12 (<0.05). Moreover, the residual analysis (Fig. 5) obtained by plotting of the observed–predicted values (residuals) vs the response (observed cellulase activity) indicated that the residuals were symmetrically and constantly spread throughout the range.

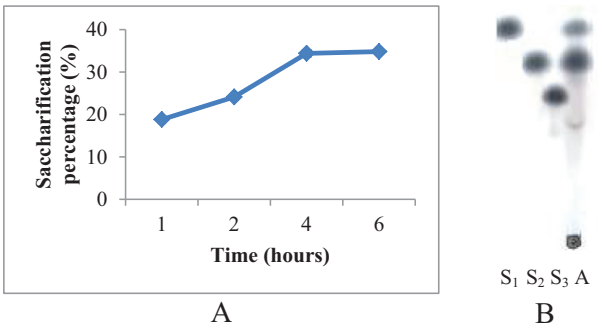
The validation of the applied model was confirmed by performing an experiment under the optimized conditions. The result indicated that the experimental cellulase activity (120.18 U/gds) was in accordance with the predicted value 112.183 U/gds.

Hydrolysis of rice straw

Saccharification activity

Initially, the FPase activity of the produced enzyme under the optimized culture conditions was determined as described previously in the material and method section. The result demonstrated that the produced enzyme possessed 1.1 U/gds Fbase activity at 50°C. Moreover, it possessed 2.3 U/gds mannanase activity,

Figure 6



Hydrolysis of rice. (a) Saccharification percentage; (b) thin-layer chromatography analysis of the resulted hydrolyzate after saccharification of untreated rice straw by using the produced enzyme for 4 h at 50°C in which S_1, S_2, S_3, and A are mannose, glucose, cellobiose standards, and the resulted hydrolyzate, respectively.

without the determination of any xylanase and pectinase activity.

The saccharification of untreated rice straw was carried out using the produced enzyme for different incubation periods, and the amount of the released reducing sugars was determined then the calculated saccharification percentage was represented in Fig. 6a. It was observed that the saccharification percentage increased up to 4 h, recording 34.4% with 343.98 mg reducing sugars/gds, and then by increasing the time, there was no improvement in the saccharification percentage.

Analysis

Thin-layer chromatography

The resulted reducing sugars were analyzed by TLC (Fig. 6b), and the results indicated that glucose is the main constituent followed by mannose without the detection of cellobiose, confirming the ability of the produced enzyme to hydrolyze rice straw to its constituent monosugars.

Scanning electron microscopy

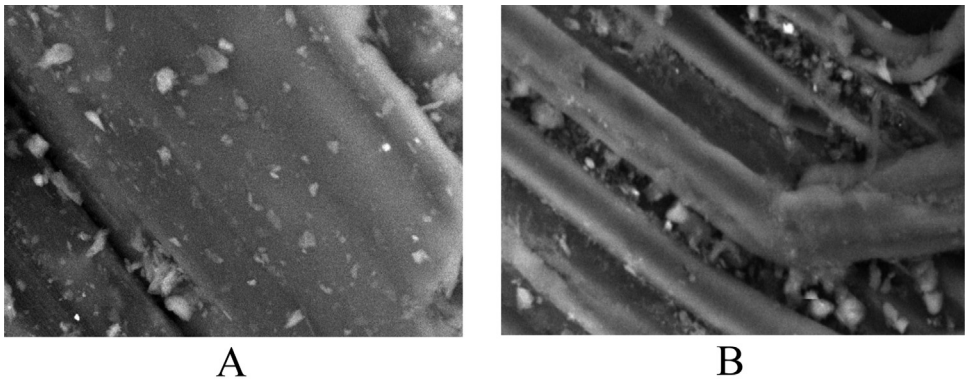
The examination of the surface of rice straw before and after enzymatic treatment by scanning electron microscope (SEM) is shown in Fig. 7, indicating that the morphology of rice straw was greatly altered after enzymatic hydrolysis.

Discussion

The utilization of lignocellulosic biomass for the production of value-added compounds in biorefineries depends mainly on their hydrolysis to its constituent soluble sugars [4]. Cellulases are the biorefineries key enzymes [6], and the production cost of the enzyme is the first crucial step in the determination of the economic feasibility of its industrial application. The cost of the enzyme production can be reduced by using crude substrates instead of using the purified substrates [30]. In the current study, cellulolytic potential in terms of CMCase activity of *A. terreus* RS2 isolated from soil was evaluated under SSF of 5 g rice straw moistened with 10 ml of tap water. The produced enzyme showed activity of 37.481 U/gds (3.7481 U/ml) after 7 days of fermentation period, which was further optimized to reach 124.94 U/gds. *A. terreus* has been previously reported as CMCase producers by SSF of different crude substrates, such as banana peels, with the production of maximum activity of 1.11 and 1.5 U/ml in mono and in co-culture with *Aspergillus niger* MS23, respectively [31]; sugarcane-bagasse with maximum activity of 13.83 U/gds [32]; and sweet sorghum bagasse with the production of 105.2 U/gds [33]. Rice straw has been also used as a carbon source for the production of 96.6 [34] and 141.29 U/gds [35] CMCase after optimization of the cultural conditions of SSF using *A. terreus*. The productivity of the enzyme using the locally isolated strain in the current study is comparatively high, indicating the efficiency of the isolated strain to utilize rice straw as a carbon source for the production of cellulase. The utilization of rice straw as the fermentation carbon source in the fungal production of cellulase attracted the research focus from the perspective of its low cost and reliable supply and to reduce its disposal environmental pollution hazards [20,21,36–38].

The conditions of the fermentation process have a crucial effect on the yield of the produced enzyme. Initially, the effects of microwave and alkaline pretreatment of rice straw were investigated. Although the use of microwave heating was reported by Hassan *et al.* [39] as one of the solutions to overcome the intrinsic recalcitrant nature of lignocellulosic biomass before the production of valuable compounds, the use of microwave pretreatment of rice straw in the current study did not achieve any improvement in the productivity of the enzyme. In addition, alkaline pretreatments did not improve the productivity, but they decreased the total protein content and consequently decreased the activity of the enzyme. This decrease may be attributed to the excess decomposition of the constitutional sugars and/or the release of compounds that inhibit the fermentation process [40]. In the present study, an overall increase of 3.3-fold was achieved in which the optimum conditions were conducted to be as follows: 3.75 g (1.5%w/v) of rice straw moistened with 11.25 ml (1 : 3 biomass to moistening agent) of modified Mandel's medium [1% $(NH_4)2SO_4$ was the only constituent nitrogen source] of pH 7, incubated at 30°C for 8 days. Ammonium salts as the nitrogen sources were

Figure 7



SEM of rice straw. (a) Untreated; (b) enzymatically treated using the produced enzyme.

favorable for the production of cellulase as reported by Aggarwal *et al.* [36], whereas Narra *et al.* [35] reported that the use of inorganic nitrogen sources led to poor production of the enzyme. Nguyen *et al.* [41], reported that both organic and inorganic nitrogen sources can be optimally utilized for the production of CMCase. In this study, the stimulation of cellulase production in the presence of ammonium salts may be attributed to their direct entry in the synthesis of protein [42].

The optimization of the fermentation process is necessary to increase the yield of the enzyme that consequently decreases the total cost of the enzyme. For the optimization of multivariable process, statistical techniques are advantageous as they save time and reduce the number of the required experiments, resulting in a decrease in the overall cost of the process. Several statistical techniques have been successfully employed in the fermentation technology for the optimization of the production of various enzymes [43–45]. In the current study, PBD was initially applied, in which seven variables were screened for their influence on the production of the enzyme. The R^2 value of the selected model was 0.991, indicating the high accuracy of the model, as it suggested that a variation of 99.1% occurs owing to the independent variables. Joglekar and May [46] reported that the R^2 value should be at least 80% to indicate the good fitness of the applied model. The analysis of PBD results indicated that the substrate concentration, moisture level, and fermentation period were the highest significant influencing variables. The substrate concentration and fermentation period exerted negative effects on the enzyme production (the enzyme productivity increased when the values of the variables changed from high to low), whereas moisture level exerted positive effect. The second step of optimization included the application of Box–Behnken design to determine the optimum level of the highest significant three variables, in which the R^2 and AAD values were 86.2 and 10.938%, respectively. Yolmeh and Jafari [15] and Ghorbannezhad *et al.* [47] reported that the suitable values of R^2 and AAD indicated that the applied model depicts the correct behavior, and it can be successfully used in the optimization process. Moreover, the residual analysis confirms that the model is correct on average for all the observed results.

Optimum concentration of the substrate in SSF is an essential requirement to ensure the appropriate growth of microorganisms [36]. In the present study, the production of the enzyme increased by decreasing the concentration of rice straw from 2 to 1.5%. This may be attributed to the fact that increasing the substrate concentration led to a decrease in the enzyme yield owing to the inhibitory effect of the increased amount of the released byproducts [48]. This result was in contrast with the result reported by Aggarwal *et al.* [36], in which increasing the concentration to 6% of alkali-assisted acid pretreated rice straw was the optimum concentration for maximum CMCase production by using *A. niger* BK01.The fungal production of cellulase by SSF generally starts by the fungal colonization of the substrate to ensure its depolymerization for the induction of the production of enzymes [10]. Accordingly, the optimal incubation period varied based on the type of the used substrate and the species of the fungus employed in the fermentation process. The result of the current study (8 days) was in accordance with that reported by Sohail *et al.* [32], in which 240 h was indicated as the optimum period for the production of the enzyme by the fermentation of sugarcane-bagasse using *A. terreus* MS105.

In SSF, moisture is the most significant variable. Behnam *et al.* [16] and Vu *et al.* [49] reported that

the increase in the moisture content results in a decrease in the porosity of the substrate and consequently reduces the oxygen transfer. On the contrary, the decrease in the moisture content leads to poor accessibility to nutrients and consequently poor microbial growth. Therefore, estimating the suitable moisture content for the optimum production of the enzyme is necessary. Fatma *et al*. [50] estimated a ratio of 1 : 3 (substrate: moistening agent) as an optimal ratio for maximum enzyme production by *Trichoderma reesei*. Similar result was achieved in the current study, whereas Aggarwal *et al*. [36] reported a ratio of 1 : 2 to be the best for the optimal production of the enzyme by *A. niger* BK01. Moisture contents of 50, 70, 75, and 80% were also reported for optimum fungal production of the enzyme [8,16,41,49,51,52].

The produced enzyme at the optimized culture conditions was used for the hydrolysis of untreated rice straw for different incubation period, and the results demonstrated a saccharification percentage of 34.4% with released reducing sugars of 16.9 mg/ml (343.98 mg reducing sugars/gds) recorded after 4 h of hydrolysis period. Aggarwal *et al*. [36] reported 35.96% as the highest saccharification value of alkali-assisted acid pretreated rice straw using crude *A. niger* BK01 cellulase for 2.5 h. Kobkam *et al*. [53] reported maximum yield of sugars of 0.54 g/g ds achieved by the enzymatic hydrolysis of hydrothermal-alkali pretreated rice straw using commercial cellulase after 120 h of hydrolysis period. Prajapati *et al*. [54] reported the production of 131 mg reducing sugar/g by the hydrolysis of hydrothermal-alkali pretreated rice straw using crude *Aspergillus tubingensis* cellulase. Chang *et al*. [55] reported 8.88 mg/ml as the highest amount of the released sugars resulting from the hydrolysis of $TiO_2/UV/H_2O_2$ pretreated rice straw by cellulase. The result of the current study suggests that the produced enzyme can be efficiently used in the hydrolysis of rice straw without the need of pretreatment.

Conclusion

This study demonstrated an economically and ecofriendly approach for rice straw discard valorization. Untreated rice straw was efficiently used as a carbon source for the production of cellulase by SSF using locally isolated *A. terreus* RS2. The production of the enzyme was statistically optimized to reach 124.94 U/gds. Moreover, the produced enzyme was efficiently used for rice straw hydrolysis without any pretreatment. Hence, the results obtained in this study hold promise in bio-processing industrial applications.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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