



# Assessment and evaluation of cellulase production using ragi (*Eleusine coracana*) husk as a substrate from thermo-acidophilic *Aspergillus fumigatus* JCM 10253

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## Abstract

The cellulase production by filamentous fungi *Aspergillus fumigatus* JCM 10253 was carried out using agro-industrial waste ragi husk as a substrate in the microbial fermentation. The effect of the process parameters such as temperature, substrate concentration, pH, and incubation process time and their interdependence was studied using response surface methodology. The optimum cellulase activities were obtained at 50 °C under the conditions with 1–2% of substrate concentration at pH 2–4 for the incubation period of 7–8 days. The maximum carboxymethyl cellulase (CMCase) and  $\beta$ -glucosidase activities with optimized process variables were 95.2 IU/mL and 0.174 IU/mL, respectively. The morphological characterization of fungus by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) revealed the presence of secondary protein structures. Furthermore, this study demonstrated that the application of ragi husk could be a promising feedstock for value-added industrial products. The thermo-acidophilic nature of isolated strain *Aspergillus fumigatus* JCM 10253 possessed a significant potential for higher titer of cellulase production that could be further employed for lignocellulosic bioethanol production.

**Keywords** Filamentous fungi · Ragi husk · Fermentation · Response surface methodology

## Introduction

Lignocellulosic materials have gained more importance as a raw material for biofuel production due to worldwide availability of low-cost renewable feedstock in abundance; fossil fuel usage causes global and greenhouse gas emission; uncertainty supply of petroleum due to high consumption and present no food production conflict. Lignocellulosic polysaccharide polymers are cross-linked with strong bonds that lead to a robust structure. Due to the highly complex structure of these natural polymers, economically feasible production of lignocellulosic bioethanol is challenging. Cellulases are a combination of three major enzyme endoglucanases (EC 3.2.1.4), exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and  $\beta$ -glucosidases

(EC 3.2.1.21). Cellulases are the enzymes that catalyze the hydrolysis of cellulose into glucose by attacking the glycosidic linkages [1]. Due to the complex structure of lignocellulosic biomass, the enzyme quantity required for hydrolysis is onefold higher than for starch [2]. The production cost and low yield of these enzymes are the foremost issues for industrial applications. The purpose of agro-waste as a solid substrate in the fermentation system for the production of the enzymes has many advantages due to its lower operating expenses and lower capital assets relative to other techniques [3]. Another advantage of fermentation processes allows the utilization of forestry, agriculture, and agro-industrial residues as substrates, which maintains the microbial growth and metabolism by allowing the microorganisms to grow on moist solid substrates [4, 5].

Since commercial processes require high temperature, there is a rising demand for a supply of thermotolerant enzymes mainly those which are resistant to inhibition of product and are extremely thermostable [6, 7]. Presently, thermophilic fungal enzymes have received more attention due to its industrial biotechnology relevant applications as a potential biocatalyst such as stability of their protein at

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elevated temperatures and oxidative ligninolytic enzymes for lignocellulosic biomass degradation at high temperatures. Thus, these enzymes have substantial industrial potential, even though under extreme operational conditions provide better yields [8].

In preliminary studies, *Aspergillus fumigatus* JCM 10253 was isolated from a soil sample and screened for cellulase activity by the plate assay method using carboxymethyl cellulose (CMC) agar medium with Congo red staining and  $\beta$ -glucosidase activity was screened by gel diffusion plate assay method. It was observed that strain JCM 10253 has shown the highest cellulolytic enzyme producer among other isolates [9]. In the present study, ragi (*Eleusine coracana*) husk is used as a substrate for enzyme production. Ragi husk was received after the dehusking process from the agriculture field of Chikmagalur District, State of Karnataka, which is a major ragi (or finger millet) producing state in India [10]. To optimize process parameters, different approaches were implemented such as one-factor-at-a-time (OFAT) and statistical design to maximize the individual responses of CMCase and  $\beta$ -glucosidase with process variables such as temperature, substrate concentration, pH, and process time duration [11–15].

To our knowledge, there are no reports of using ragi husk together with fungal cultures for cellulase production. Therefore, the objective of the present study was to investigate CMCase and  $\beta$ -glucosidase production by directly utilizing agro-industrial waste ragi husk as a substrate for the microbial fermentation process. Besides, a statistical design was used to optimize the process variables that have a significant role in enzyme production to reduce energy requirements and cost.

## Materials and methods

### Microbial strain

The fungal strain was isolated from a soil sample collected from Warangal District, Telangana, India. The strain was identified as *Aspergillus fumigatus* JCM 10253 (Accession no. LC228711) based on 18S rRNA sequencing performed by the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India. The isolated fungal strain was maintained on Emerson YSS agar and stored at 4 °C for further studies [9].

### Scanning electron microscopy analysis

Czapek–Dox broth medium with addition of 1% (w/v) carboxymethyl cellulose (CMC) was prepared in 250 mL of Erlenmeyer flasks. The prepared medium was autoclaved and then inoculated a loopful of isolated fungal spores into

a prepared medium was incubated for 20 days at 30 °C, 150 rpm. After 20 days of incubation, fungal biomass was filtered by Whatman filter paper and treated with 3% glutaraldehyde and 2% formaldehyde for 5 h. The samples were washed with distilled water three-to-four times and treated with ethanol for dehydration. After that, the samples were kept in the oven to remove the moisture content. The prepared samples were carried out for high magnification images under scanning electron microscopy (SEM) at different voltage and magnifications [16].

### Processing of substrate

Ragi husk is an outer covering or protective layer of ragi grains. The ragi husk was obtained after the dehusking process from the agriculture field. The dried husk with the particle size of 1.33 mm was sieved and stored at room temperature.

### Enzyme production in shake flasks

Cellulase production was performed in the Mandels and Weber medium [17]. The Mandels and Weber production medium was carried out in 250 mL of Erlenmeyer flasks which composed of:  $\text{KH}_2\text{PO}_4$ , 2 gm/L;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3 gm/L; Urea, 0.3 gm/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 gm/L;  $(\text{NH}_4)_2\text{SO}_4$ , 1.4 gm/L; Peptone, 0.25 gm/L; Yeast extract, 0.1 gm/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005 gm/L;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.0016 gm/L;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0014 gm/L;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.002 gm/L; Tween-80, 1 mL; pH 2.0, and supplemented with 1.5% (w/v) ragi husk as a substrate and 1% (w/v) CMC as a carbon source. The prepared medium was sterilized by autoclaving at 121 °C under a pressure of 15 psi for 15 min. A loopful of isolated fungal spores from the agar plate was inoculated into the above-sterilized medium and

**Table 1** Experimental setup and independent variables used in the central composite design for (A) CMCase and (B)  $\beta$ -glucosidase

Independent variables	Code	Levels		
		−1	0	+1
(A) CMCase				
Temperature (°C)	A	40	50	60
Substrate (% w/v)	B	1	1.5	2
pH	C	1	2	3
Incubation time (days)	D	6	7.5	9
(B) $\beta$ -glucosidase				
Temperature (°C)	A	40	50	60
Substrate (% w/v)	B	0.5	1	1.5
pH	C	3	4	5
Incubation time (days)	D	6	7.5	9

incubated at 50 °C, 160 rpm for 12 days. During the fermentation process, enzyme activity was measured by collecting the sample for every 24 h of interval and centrifugation at 10,000 rpm for 20 min at 4 °C to separate the pellet from a liquid medium. Therefore, the supernatant was collected for further analysis.

### Protein quantification and enzyme assays

Bradford assay was used to determine the extracellular total protein content [18]. Carboxymethyl cellulase (CMCase) and filter paper activity (FPase) were measured at 540 nm wavelength using UV–visible spectrophotometer (Model: Jasco-V-630) as the method described by Ghose [19].  $\beta$ -glucosidase activity was analyzed at a wavelength of 400 nm using the substrate as *p*-nitrophenyl- $\beta$ -D-glucopyranoside (pNPG) [20]. Enzyme activity was evaluated in international units (IU) per mL. The quantity of enzyme required to liberate 1  $\mu$ mol of glucose or *p*-nitrophenol from their respective substrates per min under the standard assay conditions was defined as one unit (IU) of enzyme activity [21].

### Optimization of cellulase production

Optimization of the process variables influencing the enzyme production was performed using the one-factor-at-a-time (OFAT) method. The process variables selected for investigation were temperature (30–60 °C), substrate concentration [0.25–3% (w/v)], pH (1–6), and incubation time (1–12 days). All experimental tests were performed in triplicate at an agitation rate of 160 rpm.

### Experimental design

Based on the result obtained from the one-factor-at-a-time approach, response surface methodology (RSM) was employed. The experimental central composite design (CCD) was used to determine the effect of variables and optimize the variables that had a significant effect on enzyme production. Statistical software Design-Expert7<sup>®</sup> was used to analyze and fit a second-order quadratic model to four independent variables [i.e., temperature (A), substrate concentration (B), pH (C), and incubation time (D)], with the experimental ranges denoted in Table 1A and B. Selections of actual and coded factors were based on the premise that has a significant influence on enzyme production. Table 2A and B shows the 30 experimental setup of CCD with respect to the variables. The response surface plots were obtained to determine their optimum levels and

**Table 2** Effect of different variables on (A) CMCase and (B)  $\beta$ -glucosidase production as determined using the central composite design

A: temperature (°C)	B: substrate % (w/v)	C: pH	D: incubation time (days)	Cellulase activity (IU/mL)
<b>(A) CMCase</b>				
50	1.5	2	7.5	97.43
40	2	1	9	71.88
60	1	3	9	64.8
60	2	1	9	59.31
40	1	1	6	45.21
60	2	3	6	54.2
50	1	2	7.5	89.34
60	1	3	6	61.7
60	1	1	6	47.13
50	1.5	2	7.5	93.56
50	1.5	2	9	98.61
50	1.5	2	7.5	104.54
40	2	1	6	64.21
50	1.5	2	7.5	95.22
40	1	3	9	76.8
50	1.5	2	7.5	101.34
60	2	3	9	56.42
40	1	3	6	70.23
40	1.5	2	7.5	79.4
50	1.5	2	6	82.9
50	1.5	2	7.5	99.2
40	2	3	6	67.33
60	1	1	9	52.22
50	1.5	3	7.5	76.5
60	2	1	6	61.43
60	1.5	2	7.5	68
50	1.5	1	7.5	84.32
50	2	2	7.5	91.8
40	2	3	9	67.6
40	1	1	9	54.76
<b>(B) <math>\beta</math>-glucosidase</b>				
50	1	3	7.5	0.14
60	1.5	3	9	0.064
50	1	4	9	0.138
40	0.5	3	9	0.0262
40	1.5	5	9	0.09
40	1.5	5	6	0.072
60	1	4	7.5	0.072
50	1	4	7.5	0.183
50	1	4	7.5	0.218
60	0.5	5	6	0.022
60	0.5	3	9	0.024
60	1.5	3	6	0.057
50	1.5	4	7.5	0.142
50	1	4	7.5	0.22
50	1	4	7.5	0.175

**Table 2** (continued)

A: temperature (°C)	B: substrate % (w/v)	C: pH	D: incubation time (days)	Cellulase activity (IU/mL)
40	1.5	3	6	0.092
40	0.5	5	6	0.021
40	1	4	7.5	0.12
50	1	4	7.5	0.232
60	0.5	3	6	0.016
50	0.5	4	7.5	0.116
60	1.5	5	9	0.034
50	1	4	6	0.164
40	0.5	3	6	0.025
50	1	5	7.5	0.13
60	0.5	5	9	0.023
50	1	4	7.5	0.226
60	1.5	5	6	0.03
40	0.5	5	9	0.023
40	1.5	3	9	0.098

also to understand the effect of variables individually and in combination [22].

### Fourier transform infrared spectroscopy

The characterization of the crude enzyme was performed by Fourier transform infrared spectroscopy (FTIR) (Model: 3000 Hyperion Microscope with Vertex 80 FTIR system, Bruker, Germany). The pellet was obtained by adding the sample to KBr salt (5% sample, 95% KBr), and the spectrum scan range was from 4000 to 400  $\text{cm}^{-1}$  [23]. Disks were prepared in triplicates to obtain a constant spectrum.

## Results and discussion

### SEM analysis

The morphological characterization of fungal biomass from *Aspergillus fumigatus* JCM 10253 was grown on the CMC-based medium for 20 days which found to be the most significant for micro- or nano-scale visualizing of surface morphology and structures such as conidial heads, conidia, and ascospores. The SEM analysis determined that it can reproduce sexually by developing cleistothecium with ascospores and asexually by developing conidia (Fig. 1). Such observations play a crucial role in the effective cultivation of fungi and also in optimization at different parameters for fermentation processes [16, 24]. The previous results described different stages of *Aspergillus* sp. biofilm that was analyzed through SEM [16, 25]. The reports revealed that

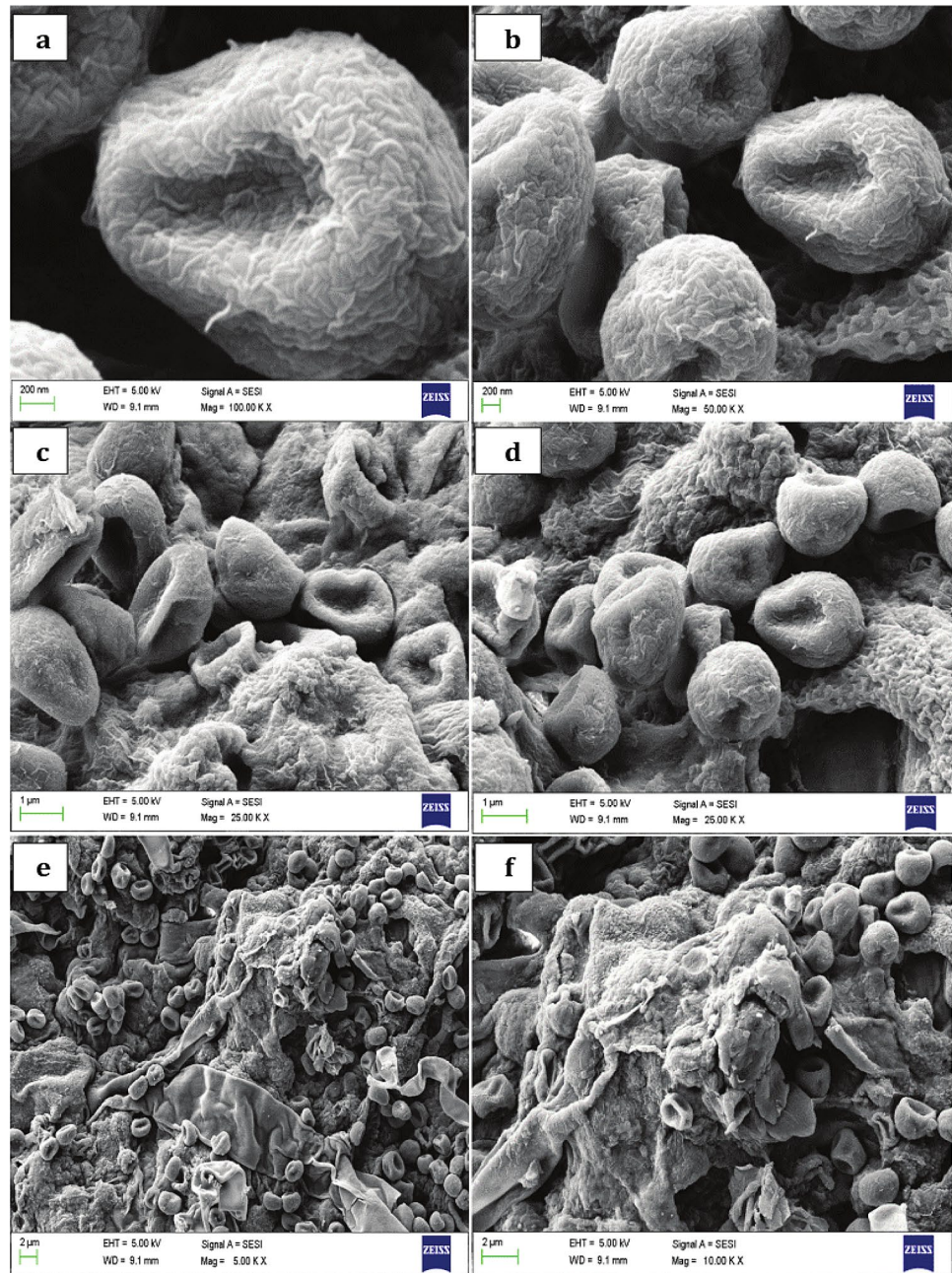
the morphological and physiological studies of *Aspergillus* biofilms are beneficial for industrial aspects [26, 27].

### Effect of physiological parameters on cellulase production

Temperature is the most significant physical variable affecting enzyme production, because enzymes are sensitive to temperature [28, 29]. The optimal production of CMCCase (17.1 IU/mL), FPase (4.40 IU/mL), and  $\beta$ -glucosidase (0.14 IU/mL) were obtained at 50 °C which was in the temperature range favorable to thermophilic fungi [30]. Enzyme production gradually increased in the temperature range from 30 to 50 °C, and maximum enzyme production occurred at high temperature, which is suitable for the hydrolysis of cellulosic materials. However, a further increase in temperature beyond 50 °C results in a drastic decline in enzyme activities (Fig. 2a). The thermostable enzymes have been reported previously as a great potential towards lignocellulosic biomass conversion into simple sugars for the bioenergy sector due to persisting non-denatured under a longer duration of hydrolysis condition [31]. Gautam et al. reported optimum cellulase production using municipal solid waste residue by *Trichoderma* sp. at 45 °C [32]. Liu et al. exhibited optimum CMCCase and FPase activities from *Aspergillus fumigatus* Z5 were obtained at 50 °C [33]. On the other hand, Soni et al. [34] described optimum cellulase level achieved at 45 °C and Mehboob et al. demonstrated the maximal cellulase activity from *Aspergillus fumigatus* at 55 °C [35]. Mallek-Fakhfakh et al. also reported that maximum  $\beta$ -glucosidase activity was achieved at 50 °C [36]. The obtained results suggest that cellulases from *Aspergillus fumigatus* JCM 10253 perform well under thermophilic conditions. Hence, these types of enzymes are favored to employ for commercial purposes or other biotechnological applications.

The level of the solid substrate acts as a crucial role in enzyme production during fermentation. Substrate concentration ranging from 0.25 to 3% (w/v) was used to determine the substrate effect on enzyme production in the medium (Fig. 2b). The optimal substrate concentrations for cellulase production were determined at 1.5% (w/v) for CMCCase (34.4 IU/mL), 1% (w/v) for FPase (21.7 IU/mL), and  $\beta$ -glucosidase (0.151 IU/mL). The different substrate concentrations were used to enhance the production of enzymes. The utilization of agro-waste materials in fermentation provides aeration and growth to the microbes which survive in between substrate particles. Moreover, the availability of the nutrients in medium absorbed by a solid substrate through which microbes maintain their growth in the medium for enzyme production [37, 38]. There are various factors which influence enzyme production like accessibility,

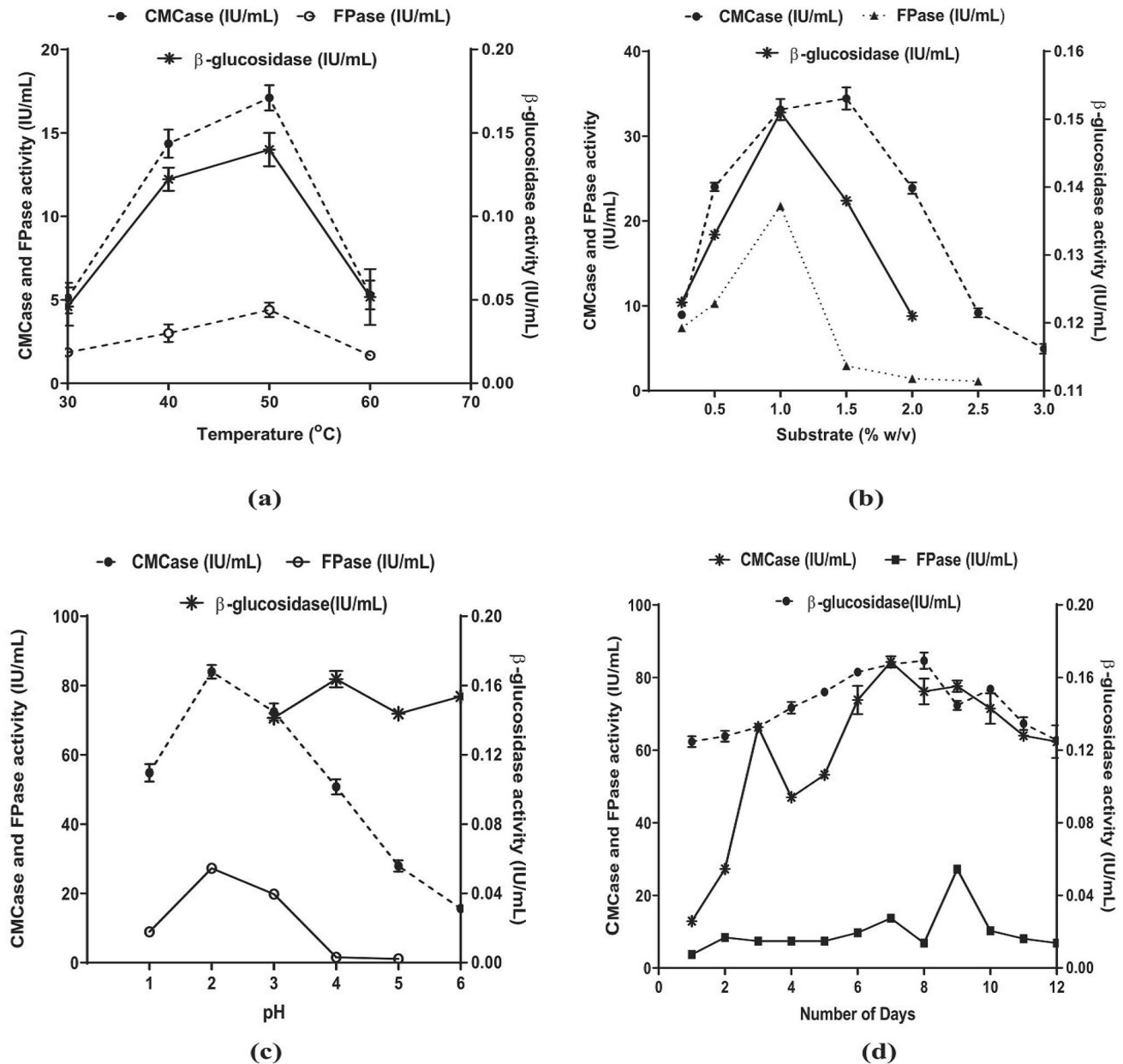
**Fig. 1** Morphological characterization of *Aspergillus fumigatus* JCM 10253 grown on carboxymethyl cellulose (CMC) medium for 20 days demonstrates nanostructural and microstructural images under scanning electron microscopy. Scale bars: 200 nm (a, b), 1  $\mu\text{m}$  (c, d), and 2  $\mu\text{m}$  (e, f)



biodegradability, chemical structure, and the physiochemical linkage between the components of the substrate [37, 39–41]. Kumar et al. described cellulase producing fungi *Schizophyllum commune* NAIMCC-F-03379 which showed optimum CMCase and FPase activities with 1% (w/v) wheat bran [42]. The previous reports also showed that the supplement of 1% (w/v) sawdust supported optimum CMCase, FPase, and  $\beta$ -glucosidase activities by *Aspergillus niger* [43, 44]. The CMCase and FPase activities enhanced with 1% (w/v) soybean hulls by *Penicillium* sp. [45].

The pH is a critical environmental parameter that influences fungal growth and plays a major role in enzyme stability

and production. The cellulase production by *Aspergillus fumigatus* JCM 10253 was optimized by varying pH from 1 to 6. The maximum CMCase (84.2 IU/mL) and FPase activities (27.2 IU/mL) occurred when the initial pH was 2, and  $\beta$ -glucosidase (0.163 IU/mL) showed the maximum activity at pH 4 (Fig. 2c). The results demonstrated that the cellulase activities decreased as the pH increased towards alkalinity. The optimum cellulase production determined at low pH indicates that fungal strain is acidophilic in nature. This property suggests that the enzymes could be useful to degrade cellulosic materials in acidic conditions. In the previous studies, optimum pH values ranging from 1 to 6 were determined for



**Fig. 2** Profiles of cellulase production by *Aspergillus fumigatus* JCM 10253 using ragi husk as a substrate. **a** Effect of temperature, **b** effect of substrate concentration, **c** effect of pH, and **d** effect of time course on CMCase, FPase, and  $\beta$ -glucosidase activity. Cellulase production

measured by its activity as described in the Materials and methods section. Results are the mean of triplicate experiments with standard deviation represented by error bars

cellulase production [46–48]. El-Nahrawy et al. reported optimum cellulase production at pH 4.0 by *Aspergillus tubingen-sis* KY615746 [49]. Liu et al. described maximum CMCase and FPase production by *Aspergillus fumigatus* Z5 at pH 4.0 [33]. Simultaneously, Xu et al. described  $\beta$ -glucosidase production by *Inonotus obliquus* exhibited maximum activity at pH 3.5 [50]. The report revealed by Delabona et al. that the maximum FPase and  $\beta$ -glucosidase activities were obtained at pH 4.0 and 3.0, respectively [51]. Similarly, the

thermo-acidophilic fungus *Aspergillus terreus* M11 showed high-level cellulase activity at pH 3 [39].

The effect of the incubation period on cellulase production was investigated and the results are shown in Fig. 2d. CMCase activity (84.6 IU/mL) showed optimum on the 7th day of the incubation period; whereas the maximum FPase and  $\beta$ -glucosidase activities were 27.6 IU/mL and 0.169 IU/mL occurred on the 9th and 8th day of incubation period, respectively. Extending the incubation time

**Table 3** Analysis of variance of response surface quadratic model for (A) CMCase and (B)  $\beta$ -glucosidase production

ANOVA for response surface quadratic model						
Source	Sum of squares	df	Mean square	F value	p value prob > F	Statistical significance
<b>(A) CMCase<sup>a</sup></b>						
Model	8606.93	14	614.78	22.54	<0.0001	Significant
A—temperature	289.68	1	289.68	10.62	0.0053	
B—substrate	56.85	1	56.85	2.08	0.1693	
C—pH	168.73	1	168.73	6.19	0.0251	
D—incubation time	128.32	1	128.32	4.71	0.0465	
AB	21.41	1	21.41	0.79	0.3895	
AC	52.09	1	52.09	1.91	0.1872	
AD	15.54	1	15.54	0.57	0.462	
BC	456.78	1	456.78	16.75	0.001	
BD	16.54	1	16.54	0.61	0.4481	
CD	4.03	1	4.03	0.15	0.7061	
A <sup>2</sup>	926.3	1	926.3	33.97	<0.0001	
B <sup>2</sup>	10.76	1	10.76	0.39	0.5393	
C <sup>2</sup>	385.51	1	385.51	14.14	0.0019	
D <sup>2</sup>	8.9	1	8.9	0.33	0.5763	
Residual	409.04	15	27.27			
Lack of fit	327.71	10	32.77	2.01	0.2275	Not significant
Pure error	81.33	5	16.27			
Cor total	9015.98	29				
<b>(B) <math>\beta</math>-glucosidase<sup>b</sup></b>						
Model	0.13	14	9.688E-003	12.98	<0.0001	Significant
A—temperature	2.818E-003	1	2.818E-003	3.78	0.0710	
B—substrate	8.141E-003	1	8.141E-003	10.91	0.0048	
C—pH	5.249E-004	1	5.249E-004	0.70	0.4148	
D—days	2.497E-005	1	2.497E-005	0.033	0.8573	
AB	1.537E-003	1	1.537E-003	2.06	0.1718	
AC	1.764E-005	1	1.764E-005	0.024	0.8799	
AD	3.240E-006	1	3.240E-006	4.342E-003	0.9483	
BC	4.285E-004	1	4.285E-004	0.57	0.4603	
BD	3.249E-005	1	3.249E-005	0.044	0.8375	
CD	4.900E-007	1	4.900E-007	6.566E-004	0.9799	
A <sup>2</sup>	0.012	1	0.012	15.99	0.0012	
B <sup>2</sup>	3.148E-003	1	3.148E-003	4.22	0.0579	
C <sup>2</sup>	2.157E-003	1	2.157E-003	2.89	0.1097	
D <sup>2</sup>	4.282E-004	1	4.282E-004	0.57	0.4605	
Residual	0.011	15	7.463E-004			
Lack of fit	8.342E-003	10	8.342E-004	1.46	0.3536	Not significant
Pure error	2.852E-003	5	5.704E-004			
Cor total	0.15	29				

<sup>a</sup> $R^2$ -0.9546, Adj  $R^2$ -0.9123, Pred  $R^2$ -0.8417

<sup>b</sup> $R^2$ -0.9238, Adj  $R^2$ -0.8526, Pred  $R^2$ -0.8015

beyond these values did not have any significant effect on enzyme production due to the various reasons behind it such as spontaneous depletion of nutrients or either toxic substances produced in the medium during the fermentation process which affects fungal growth as well as enzyme

production [37, 40]. Silva et al. demonstrated optimum cellulase activity by thermophilic fungus *Myceliophthora heterothallica* F.2.1.4 exhibited after 192 h of incubation period [52]. The previous report showed that optimum cellulase production by *Trichoderma reesei* was achieved

after the 8th day of incubation period [53]. However, in another study, CMCCase, FPase, and  $\beta$ -glucosidase from *Penicillium oxalicum* HC6 showed maximal activities on the 8th day of incubation time [54]. Simultaneously, *Penicillium* sp. AKB-24 exhibited maximum  $\beta$ -glucosidase activity obtained on the 9th day of the incubation period [16].

### Optimization of enzyme production using Design-Expert® statistical software

Table 3A and B shows the analysis of variance (ANOVA) for the response surface quadratic model to evaluate the significant effects of variables and to confirm the adequacy of the model. The regression models developed showed responses as functions of temperature (A), substrate concentration (B), pH (C), and incubation time (D). A polynomial equation for the model describes the relationship between the responses and the independent variables:

CMCase activity (IU/mL)

$$\begin{aligned} &= +95.58 - 4.01 \times A + 1.78 \times B + 3.06 \times C + 2.67 \\ &\times D - 1.16 \times A \times B - 1.80 \times A \times C - 0.99 \times A \\ &\times D - 5.34 \times B \times C - 1.02 \times B \times D - 0.50 \times C \times D \\ &- 18.91 \times A^2 - 2.04 \times B^2 - 12.20 \times C^2 - 1.85 \times D^2 \end{aligned} \quad (1)$$

$\beta$ -glucosidase activity (IU/mL)

$$\begin{aligned} &= + 0.19 - 0.013 \times A + 0.021 \times B - 5.400E - 003 \times C \\ &- 1.178E - 003 \times D - 9.800E - 003 \times A \times B - 1.050E \\ &- 003 \times A \times C - 4.500E - 004 \times A \times D - 5.175E - 003 \\ &\times B \times C + 1.425E - 003 \times B \times D + 1.750E - 004 \times C \times D \\ &- 0.068 \times A^2 - 0.035 \times B^2 - 0.029 \times C^2 - 0.013 \times D^2 \end{aligned} \quad (2)$$

Low probability value of  $p$  [ $(p_{\text{model}} > F) < 0.0005$ ] shows that the model terms are significant. The fitness of the models was indicated by the coefficients ( $R^2$ ) of 95.46% for CMCCase and 92.38% for  $\beta$ -glucosidase activity. Furthermore, only 4.5% and 7.6% of the variation in the enzyme activities are not described by the model, respectively. In the current study, the pred.  $R^2$  values for Eq. 1 (84.1%) and Eq. 2 (80.1%) show that the sample variations for CMCCase and  $\beta$ -glucosidase of 95.46% and 92.38%, respectively, were attributed to independent variables. The adjusted coefficient (adj.  $R^2 = 91.2\%$  and  $R^2 = 85.2\%$  for Eq. 1 and Eq. 2, respectively) was also satisfactory and corroborated the significance of the models.

### Interactive effects of independent process variables on cellulase activity

The interactive effect of process variables on cellulase activity is illustrated in Fig. 3a–l. The highest cellulase activity

**Fig. 3** Three-dimensional surface plots used to explore the interactions between the variables: CMCCase activity vs. substrate and temperature (a),  $\beta$ -glucosidase activity vs. substrate and temperature (b), CMCCase activity vs. pH and temperature (c),  $\beta$ -glucosidase activity vs. pH and temperature (d), CMCCase activity vs. incubation time and temperature (e),  $\beta$ -glucosidase activity vs. incubation time and temperature (f), CMCCase activity vs. pH and substrate (g),  $\beta$ -glucosidase activity vs. pH and substrate (h), CMCCase activity vs. incubation time and substrate (i),  $\beta$ -glucosidase activity vs. incubation time and substrate (j), CMCCase activity vs. incubation time and pH (k),  $\beta$ -glucosidase activity vs. incubation time and pH (l)

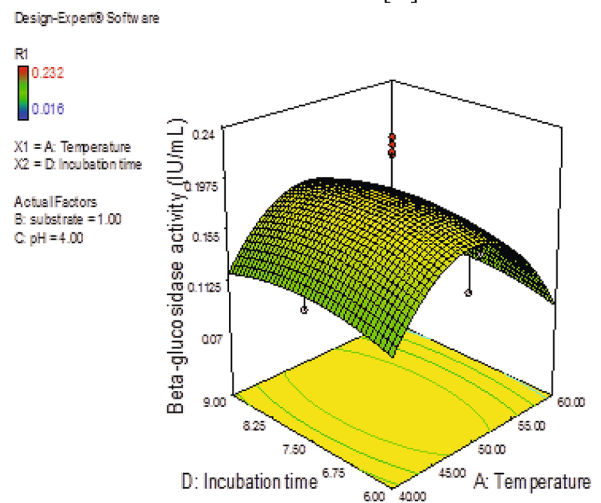
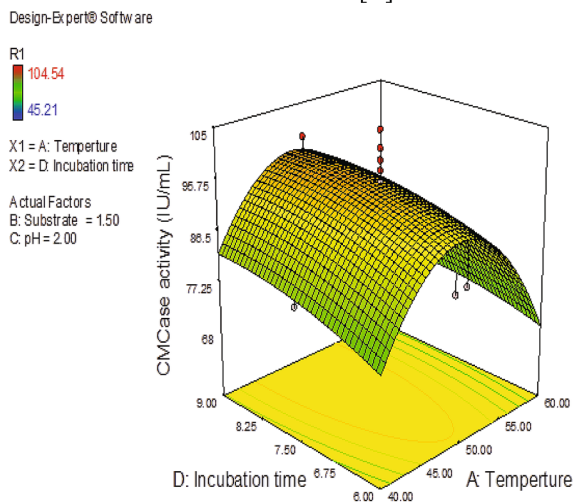
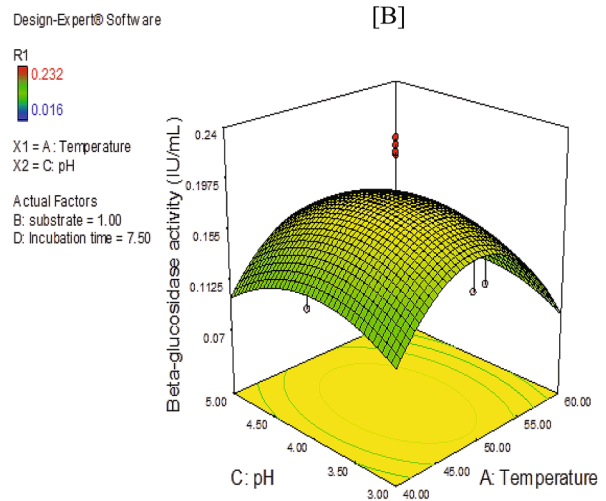
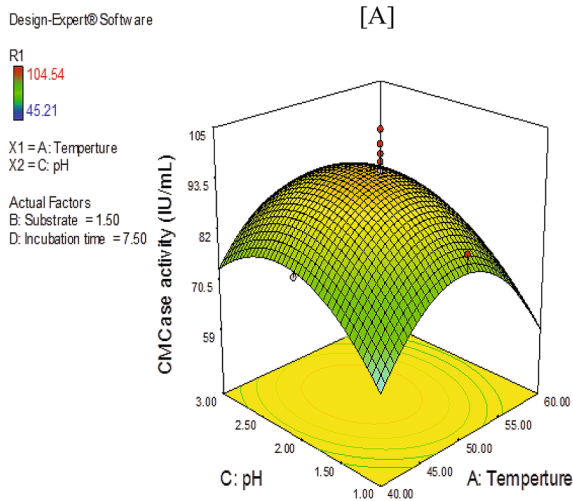
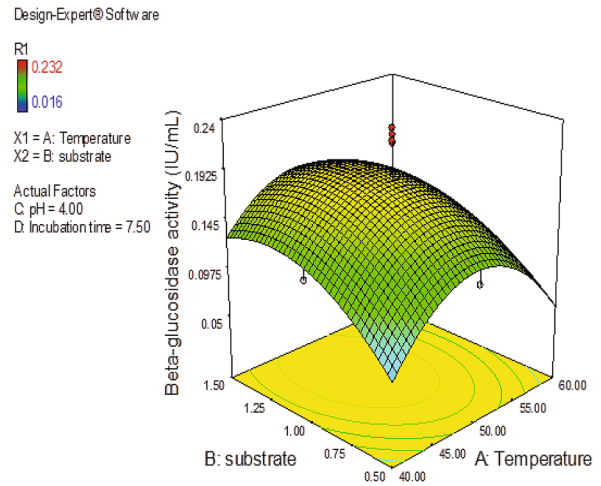
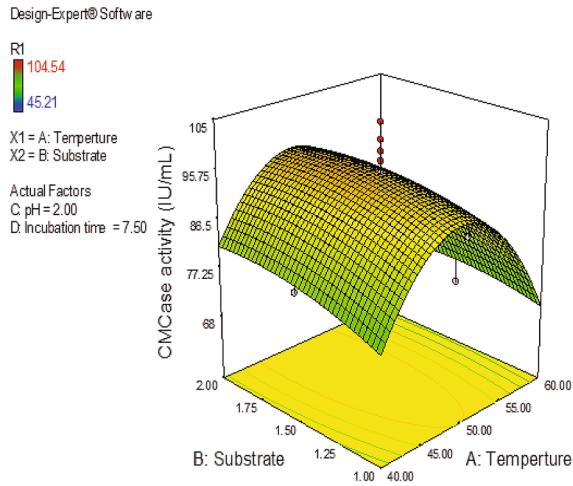
was achieved in the temperature range of 40–60 °C, substrate concentration from 0.5 to 2% (w/v), pH range of 1–5, and incubation time from 6 to 9 days. The three-dimensional surface plots were employed to investigate the mutual interactions among the independent variables to develop the correlation between the selected parameters. Each response surface for a particular enzyme represents different combinations of two test variables simultaneously while holding the other variables at zero.

### Model validation

The statistical response surface methodology proposed the desirable theoretical models for enzyme production. The most highly desirable theoretical model was selected to validate the models. The optimal values for CMCCase production were 48.6 °C at 1.6% (w/v) substrate concentration, pH 2.0, and 8.5 days of process time. Correspondingly, this combination resulted in a predicted maximum CMCCase activity of 97.06 IU/mL, which was quite close to the experimental observation of 95.2 IU/mL. Similarly, a temperature of 48.8 °C at 1.17% (w/v) substrate concentration, pH 3.8, and 7.6 days of process time resulted in a predicted maximum  $\beta$ -glucosidase activity of 0.19 IU/mL, which was relatively close to the experimental observation of 0.174 IU/mL, as described in Table 4. The predicted values from optimized parameters attain from the RSM models are closely agreed with the experimental observations, validating that the statistical software for experimental design can effectively optimize the process variables, and identify the significance of the individual, cumulative, and interaction effects of the independent variables in enzyme production [55].

The incorporation of the statistical method has proven to be a strong and beneficial tool for the enhancement of standard culture condition and to obtain minimal data with the less number of test trials for high titer production. However, the potential utilization of agricultural by-products ragi husk as a cheap commodity along with selecting an efficient fungal strain *Aspergillus fumigatus* JCM 10253 that effectively employed for microbial fermentation to scale up the production of cellulase that





[A]

[B]

[C]

[D]

[E]

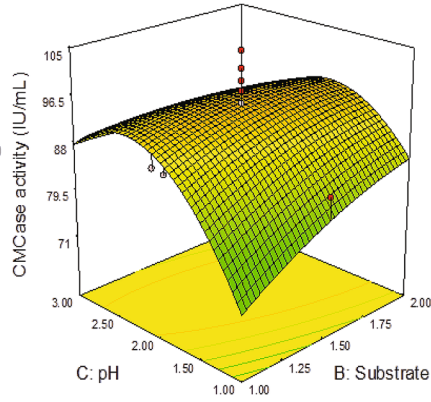
[F]

Design-Expert® Software



X1 = B: Substrate  
X2 = C: pH

Actual Factors  
A: Temperature = 50.00  
D: Incubation time = 7.50



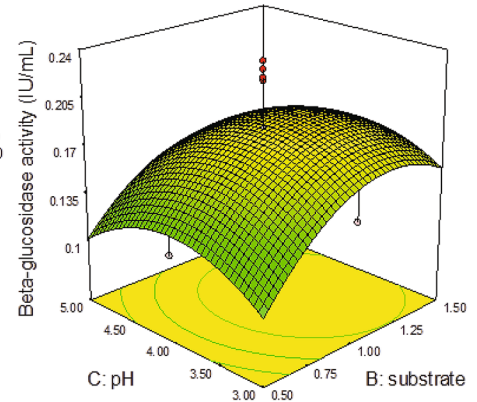
[G]

Design-Expert® Software



X1 = B: substrate  
X2 = C: pH

Actual Factors  
A: Temperature = 50.00  
D: Incubation time = 7.50



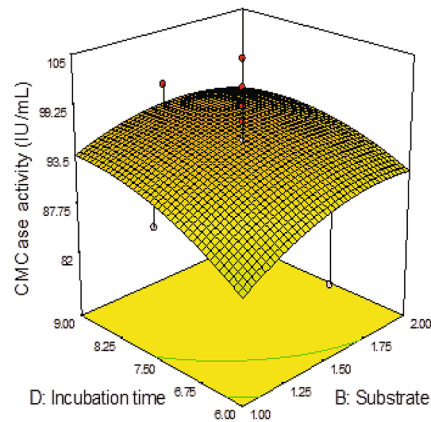
[H]

Design-Expert® Software



X1 = B: Substrate  
X2 = D: Incubation time

Actual Factors  
A: Temperature = 50.00  
C: pH = 2.00



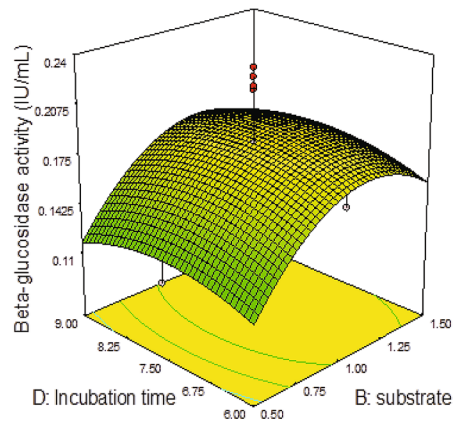
[I]

Design-Expert® Software



X1 = B: substrate  
X2 = D: Incubation time

Actual Factors  
A: Temperature = 50.00  
C: pH = 4.00



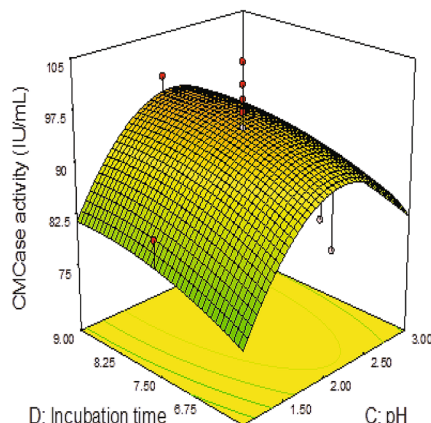
[J]

Design-Expert® Software



X1 = C: pH  
X2 = D: Incubation time

Actual Factors  
A: Temperature = 50.00  
B: Substrate = 1.50



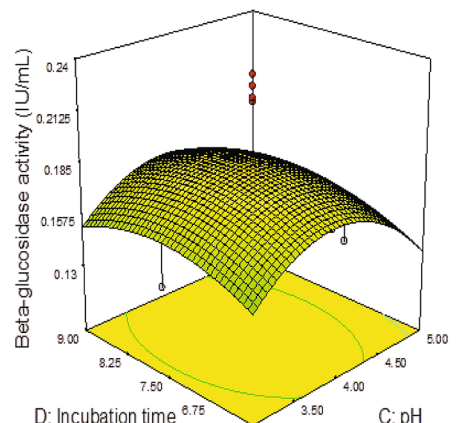
[K]

Design-Expert® Software



X1 = C: pH  
X2 = D: Incubation time

Actual Factors  
A: Temperature = 50.00  
B: substrate = 1.00



[L]

Fig. 3 (continued)

**Table 4** Optimum process variables for maximum cellulase production

A: temperature (°C)	B: substrate (%)	C: pH	D: incubation time (days)	Predicted CMCase and $\beta$ -Glucosidase (IU/mL)	Experimental
48.6	1.6	2.0	8.5	97.06	95.2
48.8	1.17	3.8	7.6	0.19	0.174

**Table 5** Comparison of cellulases production by *Aspergillus fumigatus* JCM 10253 with that by other strains of *Aspergillus fumigatus*

Fungal strain	Substrate	Enzymes		References
		CMCase	$\beta$ -glucosidase	
<i>Aspergillus fumigatus</i>	Mixed substrate (Rice straw and wheat bran)	14.7 IU/g	8.5 IU/g	[56]
<i>Aspergillus fumigatus</i> NITDGPKA3	Rice straw	6.53 IU/mL	80.1 IU/mL	[57]
<i>Aspergillus fumigatus fresenius</i>	Rice straw	240.2 IU/g	470 IU/g	[34]
<i>Aspergillus fumigatus</i> Z5	Corn stover	526.3 IU/g	–	[33]
<i>Aspergillus fumigatus</i>	Sawdust	5.54 IU/g	–	[58]
<i>Aspergillus fumigatus</i> JCF	Apple waste	2.28 IU/mL	–	[59]
<i>Aspergillus fumigatus</i>	Sweet sorghum	26 IU/gds	6 IU/gds	[60]
<i>Aspergillus fumigatus</i> SK1	Oil palm trunk	54.2 IU/g	4.5 IU/g	[61]
<i>Aspergillus fumigatus</i> (CWSF-7)	Dennanath grass	1.0 IU/mL	–	[62]
<i>Aspergillus fumigatus</i>	Wheat straw	16.9 U/g	11.8 U/g	[56]
<i>Aspergillus fumigatus</i> JCM 10253	Ragi husk	95.2 IU/mL	0.174 IU/mL	Present study

revealed as a potential feedstock for enzyme production. In the development of more robust processes, these thermostable enzymes can play a key role as a biocatalyst in sustainable bio-based processes for the production of fuels and chemicals. Therefore, the production values of CMCase and  $\beta$ -glucosidase were compared with those for previously reported enzymes produced by other strains of *Aspergillus fumigatus* using different substrates, which are described in Table 5.

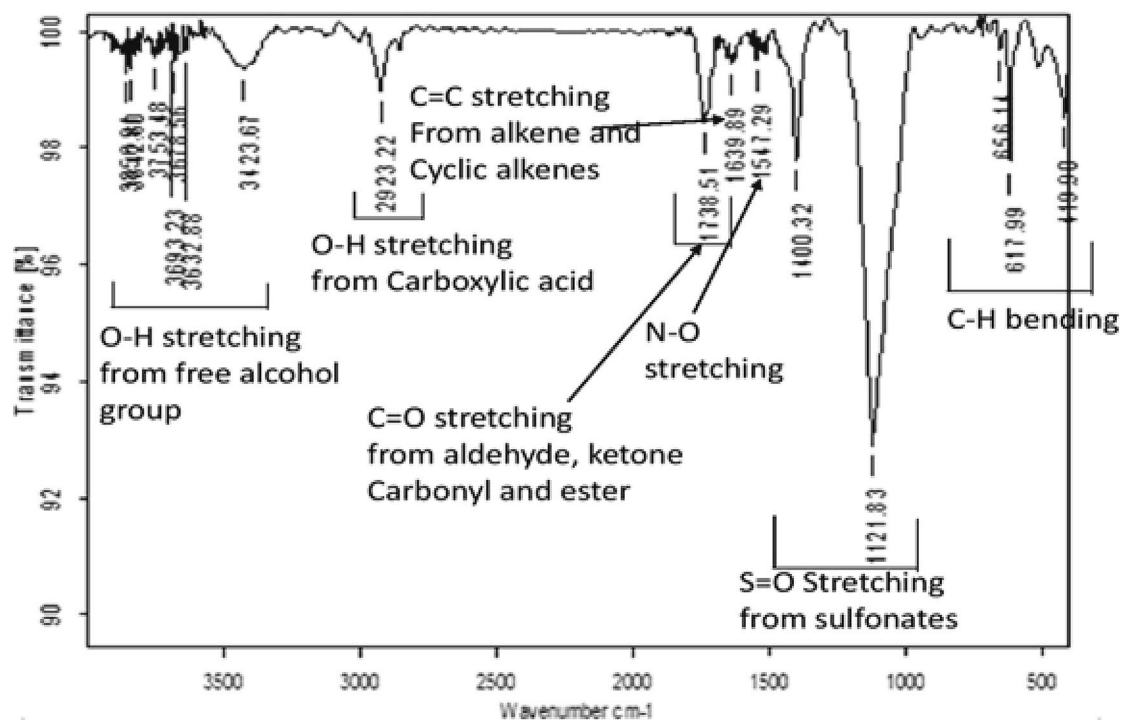
### FTIR characterization

The characterization of optimized crude CMCase and  $\beta$ -glucosidase enzymes was performed by FTIR spectroscopy. The FTIR spectrum of the crude enzymes showed the most intense absorption band for protein helical structures (Fig. 4a, b). For alpha structure, the frequency of 1639.89 and 1607.37  $\text{cm}^{-1}$  for CMCase and  $\beta$ -glucosidase showed amide I vibration, respectively, which are considered for protein secondary structure components [23]. It is primarily governed by the stretching vibration of C=O which is related to backbone conformation. Amide II is found in the 1500 and 1400  $\text{cm}^{-1}$  region which derives N–H-bending and C–N-stretching vibration [63, 64]. The degradation process of lignocellulosic materials to glucose required synergistic

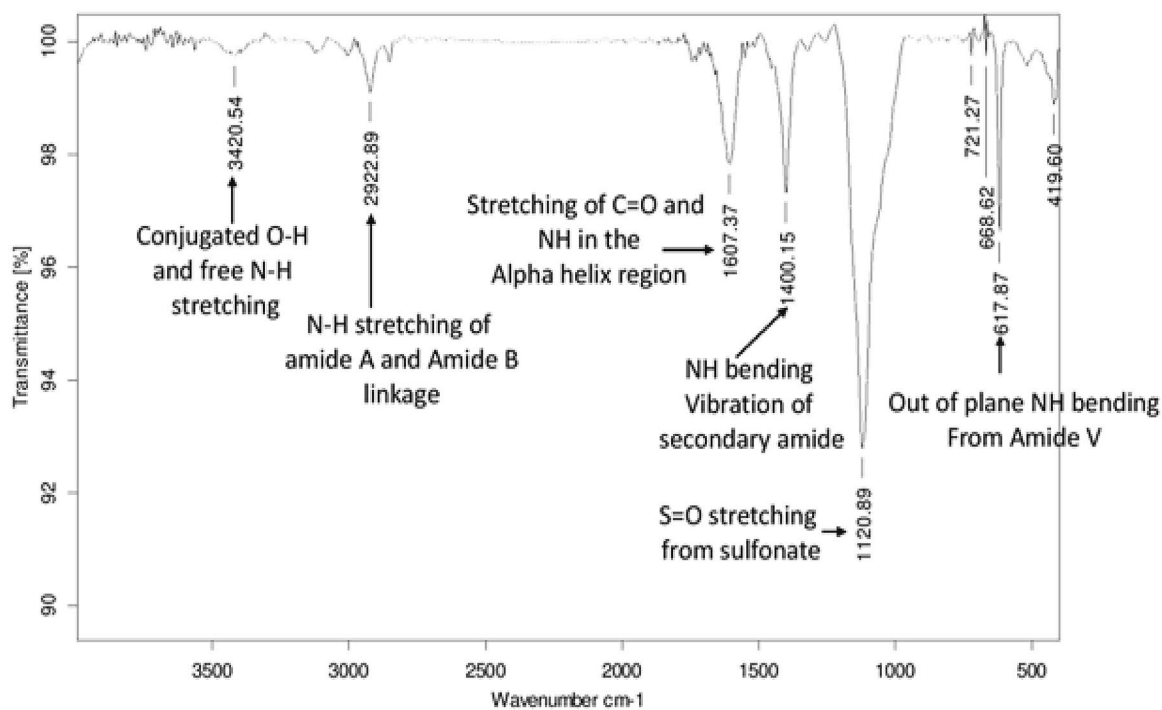
action between different enzymes. Therefore, the characterization of co-related enzymes is mandatory.

### Conclusion

This study evaluated the potential use of ragi husk as a raw material for cellulase production. The present study highlighted the ability of an isolated thermo-acidophilic fungus strain *Aspergillus fumigatus* JCM 10253 which could produce cellulase under production medium with untreated ragi husk as a substrate which is an abundant and cost-effective commodity. Statistical design response surface methodology was employed to maximize CMCase and  $\beta$ -glucosidase production. The parameters (temperature, substrate, pH, and process time) showed significant effects on CMCase and  $\beta$ -glucosidase production. The experimental yield of CMCase and  $\beta$ -glucosidase was 95.2 IU/mL and 0.174 IU/mL, respectively, using the optimized process parameters which were closely predicted by models developed through RSM. Consequently, these results demonstrated that the agro-industrial waste ragi husk has potential use as a substrate to scale up the production of the highly efficient thermophilic enzymes for converting lignocellulosic wastes into fermentable sugars.



(a)



(b)

**Fig. 4** FTIR spectrum of crude CMCase (a), and  $\beta$ -glucosidase (b) enzyme from *Aspergillus fumigatus* JCM 10253

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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