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ORIGINAL ARTICLE

Statistical optimization of cellulases production by *Aspergillus niger* HQ-1 in solid-state fermentation and partial enzymatic characterization of cellulases on hydrolyzing chitosan

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Abstract Cultivation conditions of cellulases production by *Aspergillus niger* HQ-1 in solid-state fermentation (SSF) were optimized. Furthermore, partial enzymatic characterization of the crude cellulases on hydrolyzing chitosan was studied. The moisture content, cultivation temperature, and initial culture pH were identified by Plackett-Burman design (PBD) as the significant factors for cellulases activities. The method of steepest ascent was undertaken to determine the optimal regions of the three significant factors. Box-Behnken design (BBD) and response surface analysis were adopted to further investigate the interaction effects between the three variables on cellulases activities and to determine the optimal values of the variables. The optimal ranges of moisture content, cultivation temperature and initial culture pH were 70.3–70.6%, 33.5–33.7°C and 4.626–4.662, respectively. Under the optimized conditions, endoglucanase activity, filter paper activity (FPA) and β-glucosidase activity were 305.103, 42.432 and 158.527 U/g, respectively. The optimal pH and temperature of the crude enzymes for chitosan hydrolysis were determined to be 5.6 and 50°C, respectively. The chitosanolytic activity was enhanced by metallic ions in order of Mn^{2+}, Mg^{2+}, Ca^{2+} and K^{+}, but inhibited by Zn^{2+}, Ba^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+} and Ag^{+}.

Keywords *Aspergillus niger* HQ-1 · Cellulases · Optimization · Response surface methodology · Solid-state fermentation · Chitosan

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Introduction

Cellulases are industrially important enzymes, which are mainly three types: cellobiohydrolases (EC 3.2.1.91), endoglucanases or CMCases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21) (Li et al. 2010). The enzymes are widely used in food, animal feed, textile, pulp and paper, cellulosic ethanol process, starch processing, pharmaceutical, malting and brewing industries (Mandels 1985; Oksanen et al. 2000; Mach and Zeilinger 2003). In addition, cellulases can be applied to the chitosan industry because most cellulases have a non-specific hydrolytic action on chitosan, and some are even superior to specific chitosanases in hydrolyzing chitosan (Xia et al. 2008). After hydrolysis of the polymer by cellulases, chitosan oligosaccharides are obtained which can perform a variety of biological activities such as inhibiting the growth of bacteria and fungi (Xu et al. 2007; Hu et al. 2007; Tsai et al. 2004), exerting antitumor activity (Wang et al. 2008), antiatherosclerotic effects (Dou et al. 2007) and acting as immunopotentiating effectors (Zaharoff et al. 2007).

An important impediment to the application of cellulases is the fact that the production of cellulases is expensive, contributing as much as 50% to the overall cost of hydrolysis (Xia et al. 2008). This is due to the low specific activities of cellulases, which necessitates a large quantity of the enzymes for extensive hydrolysis. Despite the production of cellulases having been widely studied in submerged culture processes, the high cost of cellulases production limits the industrial use of the enzymes (Ahamed and Vermette 2008).

Although there are some disadvantages concerning solid-state fermentation (SSF), such as long cultivation

time and being difficult to detect fermentation parameters, SSF is a highly attractive and alternative process because of the low capital cost by using agricultural and food wastes as substrates, the high concentration of products, the simple isolation techniques of products and the simple pre-processing of substrates (Mo et al. 2004; Xia and Shen 2004). Production of cellulases in SSF using various substrates, microorganisms and nutrient solutions has been reported (Latifian et al. 2007). The optimization of fermentation conditions is also a crucial problem in the development of economically feasible bioprocesses. Some parameters should be optimized due to their impact on the economy and the practicability of the process, such as cultivation conditions (moisture content, initial culture pH, cultivation temperature and cultivation time) and the medium composition (particularly carbon and nitrogen sources).

Cellulases are produced by various microorganisms including bacteria, actinomycetes and fungi. Fungi, especially, are known to secrete cellulases in large amounts. Most bacteria cannot utilize crystalline cellulose, which can be done by many filamentous fungi such as the *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium* genera. Among different fungi, *Aspergillus niger* can produce three types of cellulases and exhibits strong hydrolytic activity in contrast to *Trichoderma reesei* which has been widely studied (Sohail et al. 2009). Studies regarding the optimization of cellulases production by *A. niger* strains in SSF using response surface methodology are still few in the scientific literature.

In the current work, the mixture of corn stover and wheat bran was used as substrate, and statistical methods included Plackett-Burman design (PBD), while the method of steepest ascent and Box-Behnken design (BBD) were applied to optimize cellulases production in SSF by *A. niger* HQ-1, which was isolated from the degrading paper. Furthermore, partial enzymatic characterization of the crude cellulases on hydrolyzing chitosan was studied.

Materials and methods

Microorganism

Aspergillus niger HQ-1 stain was isolated from the degrading paper and identified according to the morphological characterization and analysis of internal transcribed spacer (ITS) gene sequence. It was maintained at 4°C on potato dextrose agar (PDA) slants in microbiology laboratory, School of life Sciences, Liaocheng University.

Isolation of the fungus strain HQ-1

To isolate the cellulolytic fungi strains, degrading paper was used as a screening source. The strain was isolated by using the selective medium which consisted of (g/l): carboxymethyl cellulose (CMC) 10.0, $(NH_4)_2SO_4$ 1.0, KH_2PO_4 0.8, $MgSO_4{\cdot}7H_2O$ 0.4, $CaCl_2$ 0.02, Bengal red 0.03, streptomycin 0.03, and chlortetracycline 0.002. At first, 5.0 g of the sample was transferred to the fresh 50 ml selective medium in a 250-ml Erlenmeyer flask for incubation at 30°C for 72 h. After being enriched more than three times, the inoculums (0.1 ml, successively diluted to 10^{-4}, 10^{-5} and 10^{-6} times) were spread on the selective medium agar plates and each plate was cultivated at 30°C for 72 h. Then, each sole colony was repeatedly streaked on selective medium agar plates. After incubation, the plates were stained by 1 mg/ml Congo red solution for 30 min and decolored by 1 M sodium chloride solution for 60 min. Then, the diameters of the clearance zone (D) and the colony (d) were measured. As a result, five fungi strains with major D/d ratios were selected and incubated in the medium [wheat bran 10.00 g, $(NH_4)_2SO_4$ 0.30 g, KH_2PO_4 0.06 g, $MgSO_4{\cdot}7H_2O$ 0.03 g, moisture content 70%, initial pH 7.0] at 30°C for 72 h to produce cellulases. The comparison of cellulases activities by the five fungi strains is shown in Table 1 and the strain HQ-1 with maximum cellulases activities was selected.

Table 1 Comparison of cellulases activities by five fungi strains; data are expressed as mean±SD of three replicates

Strains	Endoglucanase activity (U/g)	FPA (U/g)	β-Glucosidase activity (U/g)
MB-1	46.609 ±2.987	4.687±0.639	31.489±1.896
QM-1	35.723 ±1.885	3.748±0.588	10.085±1.745
QM-2	53.123 ±2.310	7.896±0.741	25.774±2.013
DM-3	51.881±2.558	9.638±0.896	29.482±2.081
HQ-1	63.338±3.025	12.557±1.025	40.489±2.566

Identification of cellulolytic fungus strain HQ-1

Morphology examination was performed after incubating the strain on a Czapek′s medium agar plate for 72 h. The conidial head and conidiophores of the strain were observed by a light microscope (Olympus BX51, Japan). Molecular identification was performed by analyzing ITS gene sequence. Genomic DNA was extracted by the Hexadecyltrimethy Ammonium Bromide (CTAB) method described as elsewhere (Guo et al. 2000; Saghai-Maroof et al. 1984). ITS primers (ITS1: 5′-TCC GTA GGT GAA CCT GCG G-3′, ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′) were used for amplification of the ITS sequence by using Polymerase Chain Reaction (PCR). Following the first denaturation at 95°C for 2 min, PCR was done in 35 cycles of 1 min at 95°C, 1 min at 55°C, 1.5 min at 72°C, followed by a final elongation at 72°C for 10 min. The

Table 2 Cellulosic compositions of wheat bran and wheat straw and corn stover on dry basis; data are expressed as mean±SD of three replicates

Substrates	Cellulose (%)	Hemicellulose (%)	Protein (%)	Lignin (%)	Ash (%)
Wheat bran	7.40±0.18	32.38±0.41	17.89±0.15	4.01±0.08	6.40±0.08
Wheat straw	43.52±0.38	22.41±0.30	2.40±0.09	9.80±0.18	6.60±0.05
Corn stover	34.02±0.45	32.10±0.40	9.30±0.16	4.50±0.05	7.03±0.07

sequencing was accomplished by Sangon Biotech (Shanghai, China). The complete ITS sequence of strain HQ-1 was compiled by using MEGA version 4.1, after the performance of multiple alignments of the data by CLUSTAL_X (Thompson et al. 1997). A distance matrix method, including clustering using neighbor-joining, and a discrete character-based neighbor-joining method were used. In each case, bootstrap percentages based on 1,000 replications were calculated. The ITS gene sequences of related taxa were obtained from the GenBank Nucleotide Sequence database (NCBI).

Seed inoculum

Suspension of the spores was made from 7-day-old cultures that had been cultivated on PDA slants at 30°C. Sterile Tween 80-water (0.02%, v/v) was aseptically added to each slope, and suspension of the spores was made by lightly brushing the mycelium with a sterile wire loop. Then, the suspension was diluted with sterile Tween80-water (0.02%, v/v) to reach a final spore count of 10^7–10^8 spores/ml.

For inoculum preparation, the glucose pre-cultured medium [%, (w/v), glucose, 1.5; yeast extract, 0.4; K_2HPO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; pH 7.0] was used (Soni et al. 2010). The cultivation was done in a 250-ml Erlenmeyer flask, containing 50 ml of the medium. After inoculated with 2.5 ml spore suspension, the culture medium was cultivated at 30°C, 180 rpm for 24 h in a rotary shaking incubator.

Solid-state fermentation for cellulases production

Solid-state fermentation was carried out in a 250-ml Erlenmeyer flask containing the medium [substrates 10.00 g, $(NH_4)_2SO_4$ 0.30 g, KH_2PO_4 0.06 g, $MgSO_4{\cdot}7H_2O$ 0.03 g]. Prior to sterilization, the initial culture pH and moisture content of the medium were adjusted to 7.0 and 60%, respectively. After sterilization at 121°C for 30 min, the culture medium was inoculated with 10% inoculum (v/w) and cultivated at 30°C for 72 h.

Enzyme extraction

Fermented residues, 5.0 g, were suspended in 100 ml distilled water and shaken at 120 rpm for 2 h and filtered. The filtrate was centrifuged (13,980 *g*) using a high speed centrifuge (TGL-20 M, China) for 15 min and the clarified supernatant was used for enzyme assay.

Enzyme assay

Endoglucanase (carboxymethyl cellulase, CMCase) activity was assayed in a reaction mixture (2.0 ml) containing 0.5 ml of the enzyme and 1.5 ml of 1% (w/v) carboxymethyl cellulose (CM-cellulose) solution prepared in sodium acetate buffer (50 mM, pH 5.0). The reaction mixture was incubated at 50°C for 30 min. The reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method (Miller 1959). The total cellulase activity (filter paper activity, FPA) was determined as reported earlier (Ghose 1987).

β-Glucosidase activity was assayed by using *p*-nitrophenyl-β-D-glucoside (*p*NPG) as substrate. The reaction mixture (4.0 ml) containing 0.2 ml of the enzyme, 1.8 ml of 2 mM *p*NPG solution and 2.0 ml of sodium acetate buffer (50 mM, pH 5.0) was incubated at 50°C for 30 min. The reaction was stopped by adding 1.0 ml 1.0 M sodium carbonate solution and the colour that developed as a result of *p*-nitrophenol (*p*NP) liberation was measured at 405 nm.

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or *p*-nitrophenol (*p*NP) per minute under the assay conditions. The enzyme activity was expressed as U/g (units per gram of dry substrate).

Table 3 Levels of variables tested in Plackett-Burman design

Variables	Low level (−1)	High level (+1)
(A) Corn stover (g)	5.0	8.0
(B) Wheat bran (g)	5.0	8.0
(C) $(NH_4)_2SO_4$ (g)	0.2	0.4
(D) KH_2PO_4 (g)	0.04	0.08
(E) $MgSO_4{\cdot}7H_2O$ (g)	0.02	0.04
(F) Moisture content (%)	50	75
(G) Initial culture pH	5.0	7.0
(H) Cultivation temperature (°C)	25	35

Table 4 Plackett-Burman design for eight variables with coded values and results for cellulases activities in SSF; see text for explanation of codes A–H

Trial	A	B	C	D	E	F	G	H	Endoglucanase activity (U/g)	FPA (U/g)	β-Glucosidase activity (U/g)
1	1	−1	1	−1	−1	−1	1	1	103.099	15.953	59.431
2	1	1	−1	1	−1	−1	−1	1	188.734	29.626	103.641
3	−1	1	1	−1	1	−1	−1	−1	111.056	17.040	60.344
4	1	−1	1	1	−1	1	−1	−1	143.888	22.795	82.062
5	1	1	−1	1	1	−1	1	−1	79.643	12.288	40.240
6	1	1	1	−1	1	1	−1	1	228.264	35.510	127.360
7	−1	1	1	1	−1	1	1	−1	110.750	16.992	63.171
8	−1	−1	1	1	1	−1	1	1	104.133	15.802	54.890
9	−1	−1	−1	1	1	1	−1	1	218.693	34.327	128.578
10	1	−1	−1	−1	1	1	1	−1	137.330	21.614	78.810
11	−1	1	−1	−1	−1	1	1	1	154.635	23.693	81.295
12	−1	−1	−1	−1	−1	−1	−1	−1	115.021	17.347	63.448

The chitosanolytic activity of the crude cellulases used in this study was assessed by measuring enzyme-released reducing sugar in the hydrolyzing reaction through the dinitrosalicylic acid (DNS) method (Ilyina et al. 2000). An enzymatic hydrolyzing reaction was performed by mixing 1.0 ml of the crude enzymes with 2.5 ml of 1.0% (w/v) chitosan solution (deacetylation degree (DD) 90%, molecular mass 200 kDa) in acetate buffer (200 mM, pH 4.8) at 50°C for 15 min. The reducing sugars formed in the supernatant were estimated spectrophotometrically by using the modified dinitrosalicyclic acid (DNS) method (Miller 1959), with D-glucosamine as the calibration standard. The chitosanolytic activity was expressed as the amount of enzyme that could liberate 1 μmol of reducing sugar as D-glucosamine per min under the conditions described above.

Table 5 Statistical analysis of Plackett-Burman design for endoglucanase activity; outline criterion: 0.05

Term	Effect	Coeff	SE Coeff	*T*	*P*
Constant		141.27	4.181	33.79	0.000
(A) Corn stover	11.11	5.56	4.181	1.33	0.276[a]
(B) Wheat bran	8.49	4.24	4.181	1.01	0.385[a]
(C) $(NH_4)_2SO_4$	−15.48	−7.74	4.181	−1.85	0.161[a]
(D) KH_2PO_4	−0.59	−0.30	4.181	−0.07	0.948[a]
(E) $MgSO_4{\cdot}7H_2O$	10.50	5.25	4.181	1.26	0.298[a]
(F) Moisture content	48.65	24.32	4.181	5.82	0.010[b]
(G) Initial culture pH	−52.68	−26.34	4.181	−6.30	0.008[c]
(H) Cultivation temperature	49.98	24.99	4.181	5.98	0.009[b]

[a] Non-significant at $P<0.05$
[b] Significant positive effect
[c] Significant negative effect

Table 6 Statistical analysis of Plackett-Burman design for FPA; outline criterion: 0.05

Term	Effect	Coeff	SE Coeff	*T*	*P*
Constant		21.916	0.6559	33.41	0.000
(A) Corn stover	2.098	1.049	0.6559	1.60	0.208[a]
(B) Wheat bran	1.219	0.609	0.6559	0.93	0.421[a]
(C) $(NH_4)_2SO_4$	−2.467	−1.234	0.6559	−1.88	0.157[a]
(D) KH_2PO_4	0.112	0.056	0.6559	0.09	0.937[a]
(E) $MgSO_4{\cdot}7H_2O$	1.696	0.848	0.6559	1.29	0.287[a]
(F) Moisture content	7.813	3.906	0.6559	5.96	0.009[b]
(G) Initial culture pH	−8.384	−4.192	0.6559	−6.39	0.008[c]
(H) Cultivation temperature	7.806	3.903	0.6559	5.95	0.009[b]

[a] Non-significant at $P<0.05$
[b] Significant positive effect
[c] Significant negative effect

Table 7 Statistical analysis of Plackett-Burman design for β-glucosidase activity; outline criterion: 0.05

Term	Effect	Coeff	SE Coeff	*T*	*P*
Constant		78.61	3.041	25.85	0.000
(A) Corn stover	6.64	3.32	3.041	1.09	0.355[a]
(B) Wheat bran	1.47	0.74	3.041	0.24	0.824[a]
(C) $(NH_4)_2SO_4$	−8.13	−4.06	3.041	−1.34	0.274[a]
(D) KH_2PO_4	0.32	0.16	3.041	0.05	0.962[a]
(E) $MgSO_4{\cdot}7H_2O$	6.20	3.10	3.041	1.02	0.383[a]
(F) Moisture content	29.88	14.94	3.041	4.91	0.016[b]
(G) Initial culture pH	−31.27	−15.63	3.041	−5.14	0.014[c]
(H) Cultivation temperature	27.85	13.93	3.041	4.58	0.020[b]

[a] Non-significant at $P<0.05$

[b] Significant positive effect

[c] Significant negative effect

Evaluation of carbon and nitrogen sources for cellulases production

Different carbon sources including wheat straw (WS), corn stover (CS), wheat bran (WB), the mixture of wheat straw and wheat bran (WS/WB, 1/1) and the mixture of corn stover and wheat bran (CS/WB, 1/1) and different nitrogen sources including ammonium sulphate (AS), ammonium chloride (AC), potassium nitrate (PN), urea (U), yeast extract (YE) and peptone (P) were individually evaluated for their performances in cellulases production. Wheat straw and corn stover were chopped using a high speed disintegrator (QE-100, China) to a particle size smaller than 5 mm. The cellulosic compositions of wheat bran (WB), wheat straw (WS) and corn stover (CS) are shown in Table 2.

Plackett-Burman design (PBD)

A Plackett-Burman design experiment for eight variables was formulated by using the Minitab (14.12) statistical software package. The eight variables were as follows: corn stover (A), wheat bran (B), $(NH_4)_2SO_4$ (C), KH_2PO_4 (D), $MgSO_4{\cdot}7H_2O$ (E), moisture content (F), initial culture pH (G) and cultivation temperature (H). Each factor was tested at two levels, high (+1) and low (−1) (Table 3). A total of 12 experiments were generated and cellulases activities were measured and are shown in Table 4.

Steepest ascent method

Frequently, the initial estimate of the optimal operating conditions for the system will be far from the actual optimum. In such circumstances, variables that significantly influenced cellulases production were optimized with respect to the responses by applying a single steepest ascent experiment.

Response surface methodology (RSM)

Based on the results of Placket-Burman design and steepest ascent experiment (Tables 5, 6, 7 and 8), moisture content (x_1), initial culture pH (x_2) and cultivation temperature (x_3) were chosen as the variables. Box-Behnken design (BBD) was performed to optimize the optimal levels of the variables to obtain the maximum cellulases activities. The variables were studied at low, middle and high levels with coded values including −1, 0 and +1, respectively (Table 9). The behavior of the system was explained by the following quadratic model equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

where Y was the predicted response, x_1, x_2 and x_3 were coded independent variables, β_0 was the intercept, β_1, β_2 and β_3 were linear coefficients, β_{11}, β_{22} and β_{33} were

Table 8 Experimental design of steepest ascent and corresponding responses

Step	Moisture content (%)	Cultivation temperature (°C)	Initial culture pH	Endoglucanase activity (U/g)	FPA (U/g)	β-Glucosidase activity (U/g)
1	65.0	30	5.0	267.320	39.550	143.115
2	70.0	33	4.5	303.918	42.072	157.424
3	75.0	36	4.0	258.669	37.454	135.025
4	80.0	39	3.5	230.533	32.574	110.741

Table 9 Experimental design and results of Box-Behnken design (BBD)

Run	(x_1) Moisture content (%)	(x_2) Cultivation temperature (°C)	(x_3) Initial culture pH	Endoglucanase activity (U/g)	FPA (U/g)	β-Glucosidase activity (U/g)
1	−1 (65)	−1 (28)	0 (4.5)	215.330	30.907	116.594
2	−1	1 (38)	0	244.231	34.421	127.535
3	1 (75)	−1	0	254.218	35.808	129.489
4	1	1	0	250.254	35.952	134.210
5	0 (70)	−1	−1 (4.0)	230.058	32.208	120.108
6	0	−1	1 (5.0)	262.951	36.521	136.589
7	0	1	−1	259.560	35.800	133.892
8	0	1	1	273.908	38.904	143.335
9	−1	0 (33)	−1	218.477	29.844	114.360
10	1	0	−1	264.010	37.668	141.880
11	−1	0	1	270.180	38.525	139.084
12	1	0	1	265.176	35.830	136.541
13	0	0	0	305.453	42.374	158.580
14	0	0	0	303.379	42.036	157.520
15	0	0	0	302.897	42.019	157.203

quadratic coefficients and β_{12}, β_{13} and β_{23} were interactive coefficients. The software Minitab (14.12) and Statistical Analysis System (SAS, 8.0) were used for the experimental design and analysis of the experimental data.

Effect of temperature on chitosanolytic activity and thermal stability of the crude cellulases

The optimal temperature of the enzymes for hydrolyzing chitosan was evaluated by incubating the reaction mixtures for 15 min at different temperatures ranging from 30 to 65° C. Effect of temperature on chitosanolytic activity was expressed as relative activity, which was the percentage ratio of the activity under each reaction temperature to that under the optimal reaction temperature. Thermal stability was performed by incubating the crude enzymes at different temperatures from 30 to 65°C for 12 h. The residual chitosanolytic activity of each sample was then measured at 50°C for 15 min with the DNS method. The effect of temperature on enzyme thermal stability was also expressed as relative activity, which was the percentage ratio of the chitosanolytic activity of the crude cellulases incubated at each temperature for 12 h to that of the enzymes without being incubated.

Effect of pH on chitosanolytic activity and pH stability of the crude cellulases

The optimal pH of the enzymes for hydrolyzing chitosan was evaluated by incubating the reaction mixtures in the present of appropriate buffers using 200 mM sodium acetate (pH 3.6–5.8) and 200 mM sodium phosphate (pH 5.8–6.8), at 50°C and for 15 min. The pH stability was determined after diluted properly by the buffers described above and incubated for 24 h. The residual chitosanolytic activity of each sample was quantified. Expression of the effect of pH on enzyme activity and stability was similar to that of the effect of temperature.

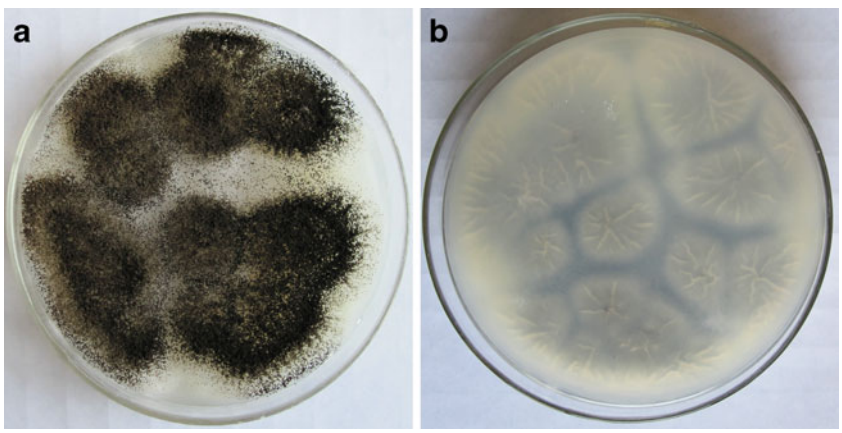

Fig. 1 Photos of culture of the strain HQ-1 on a Czapek′s medium agar plate (30°C, 72 h). **a** Front surface of the culture; **b** back surface of the culture

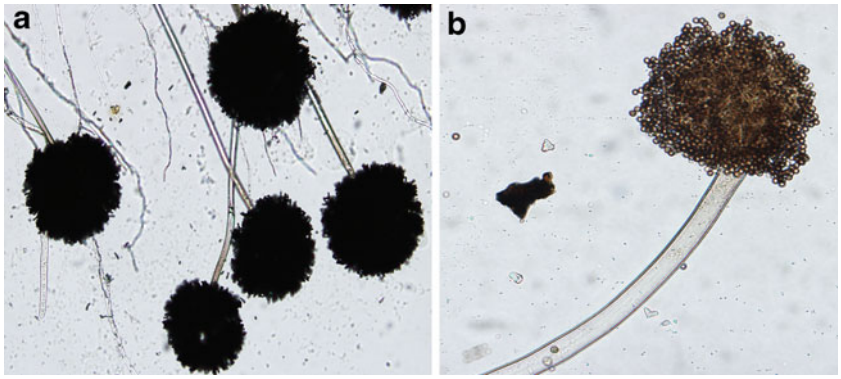

Fig. 2 Microphotographs of conidial head (**a**, ×200) and conidiospores (**b**, ×400) of the strain HQ-1

Effect of metallic ions on chitosanolytic activity of the crude cellulases

Effect of metallic ions on chitosanolytic activity of the crude cellulases was examined by adding each additive solution to the reaction system until the concentration of the metal ion was 5 mM. The additives used in this study were $CaCl_2$, $MgSO_4 \cdot 7H_2O$, $FeCl_3$, K_2SO_4, $ZnSO_4$, $CoCl_2$, $BaCl_2$, $AgNO_3$, $CuSO_4$, and $MnCl_2$. The reaction mixture with each additive was incubated at 50°C and for 15 min. The activity assayed in the absence of additives was defined as control. Effect of metallic ions on chitosanolytic activity was also expressed as relative activity, which was the percentage ratio of the activity with each metal ion to that of control.

Results and discussion

Isolation of the cellulolytic fungi strains

Five fungi strains with major D/d ratios were obtained and comparison of cellulases activities was shown in Table 1.

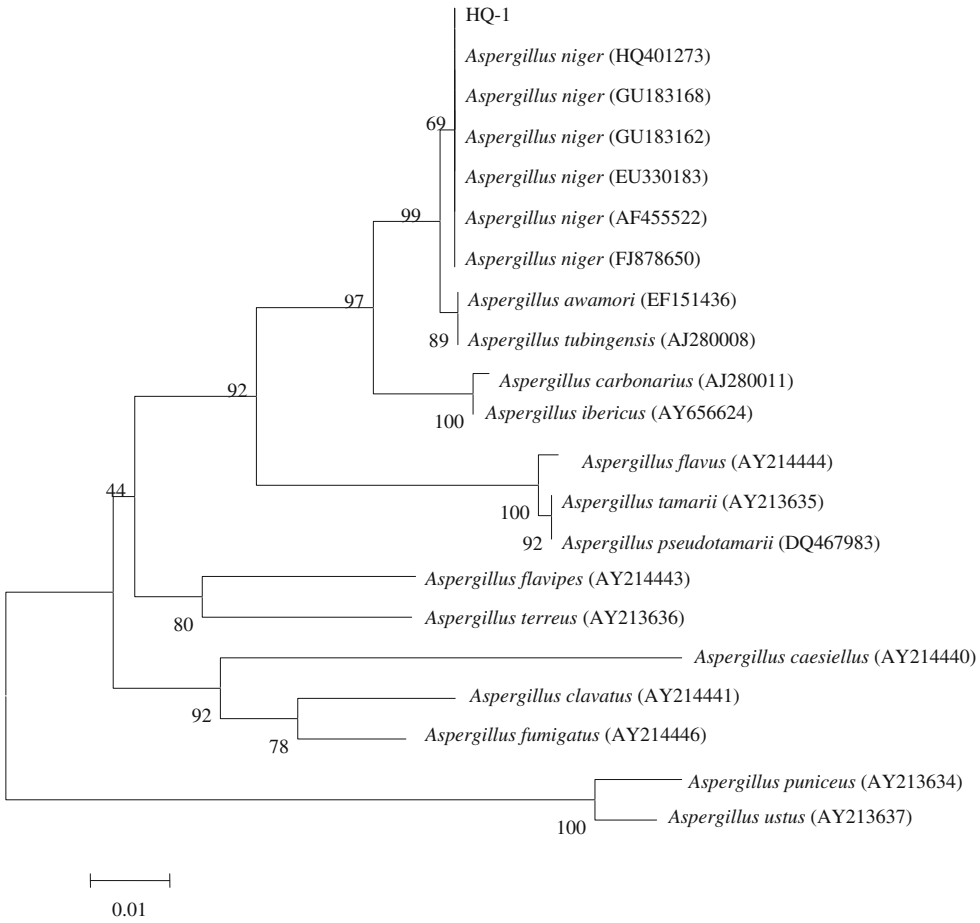

Fig. 3 Neighbor-joining tree based on phylogenetic analysis of the ITS gene sequence comparisons of different Aspergilli

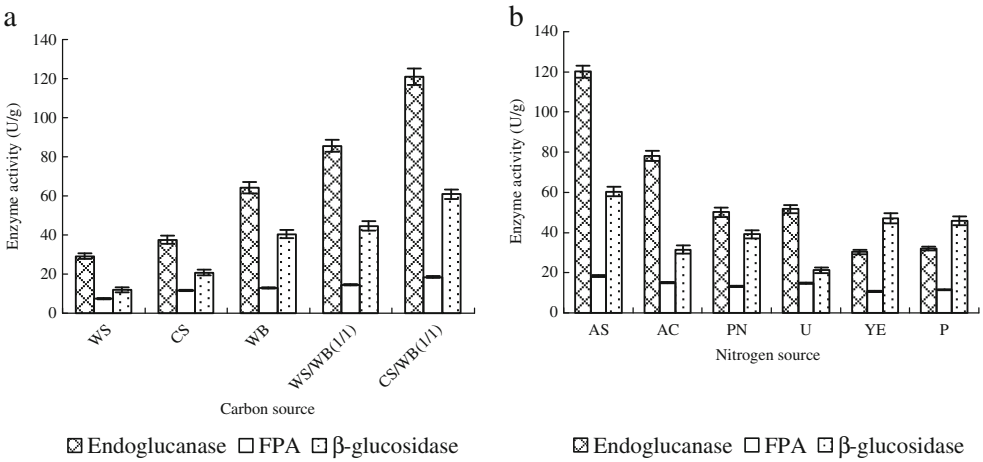

Fig. 4 Effect of different carbon sources (**a**) and nitrogen sources (**b**) on cellulases activities by *A. niger* HQ-1 in SSF. *WS* wheat straw, *CS* corn stover, *WB* wheat bran, *AS* ammonium sulphate, *AC* ammonium chloride, *PN* potassium nitrate, *U* urea, *YE* yeast extract, *P* peptone

The cellulases activities of the strain HQ-1 were relatively higher than those of the other strains. Therefore, the strain HQ-1 was selected to be identified and further studied.

Identification of the cellulolytic fungus strain HQ-1

Colors of front and back surface for the strain culture cultivated on a Czapek′s medium agar plate at 30°C for 72 h were black and white, respectively (Fig. 1). As shown in Fig. 2, the shape of spore was circular and ventricosus conidiophore apex looked like nearly spherical shape. The shape of conidial head looked like a chrysanthemum. There was no divarication of hyaline conidiophore. Therefore, the fungus strain HQ-1 was identified as primarily *Aspergillus* genus (Dai 1987).

The amplification of ITS sequence for the strain HQ-1 (Accession number of ITS sequence: HQ891869) resulted in a PCR product of 558 bp in size. Sequence-similarity calculations indicated that the strain HQ-1 was most closely related to *A. niger* HQ401273 (100% similarity), *A. niger* GU183168 (100% similarity), *A. niger* GU183162 (100% similarity), *A. niger* EU330183 (100% similarity), *A. niger* AF455522 (100% similarity) and *A. niger* FJ878650 (100% similarity), respectively (Fig. 3). Therefore, the cellulolytic fungus strain HQ-1 was identified as *A. niger*.

Table 10 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for endoglucanase activity

Source	*df*	SS	MS	*F*	*P*
Model	9	11,062.8	1,229.20	153.02	0.000
Linear	3	2,699.8	899.94	112.03	0.000
Square	3	7,368.5	2,456.16	305.76	0.000
Interaction	3	994.50	331.50	41.27	0.001
Residual error	5	40.2	8.03		
Lack of fit	3	36.5	12.16	6.59	0.135
Pure error	2	3.7	1.84		
Total	14	11103.0			

SS sum of squares, *df* degree of freedom, *MS* mean square

Effect of different carbon and nitrogen sources

Cellulases can be produced by cellulolytic microorganisms when medium contains cellulosic substrates which act as both carbon sources and inducers. Furthermore, hemicellulose as a whole is also a good inducer of cellulolytic enzyme system and the C/N ratio is crucial for the growth of microorganisms and for producing specific products under SSF (Brijwani et al. 2010).

While the mixture of corn stover and wheat bran acted as carbon source, maximum endoglucanase activity, FPA and β-glucosidase activity were 120.981, 18.516 and 60.894 U/g, respectively (Fig. 4a). This observation suggested that possible co-operation between various inducers present in corn stover and wheat bran resulted in high production of cellulases. The type and composition of the carbohydrates present in corn stover were suitable for induction of cellulases by *A. niger* HQ-1. Compared with wheat straw, corn stover contained lower amounts of lignin (Table 2) and that was beneficial for production of cellulases, as the presence of lignin would envelope the cellulose fraction making it unavailable for the fungus to act upon. Wheat bran was a nutrient-richer byproduct of the wheat-processing industry which could supply microorganisms with ample nutrition such as protein and hemicellulose (Kalogeris et al. 2003a). Furthermore, it remained loose even under moist conditions during the SSF mode of culturing thereby providing a large surface area and efficient aeration (Jatinder et al. 2006a). In addition, C/N ratio of the mixture of corn stover and wheat bran was more suitable for the strain HQ-1 to produce cellulases compared with that of the mixture of wheat straw and wheat bran.

As shown in Fig. 4b, cellulases activities were generally lower while complex organic sources including peptone and yeast extract were employed. Ammonium sulphate was the most effective nitrogen source for cellulases production by *A.*

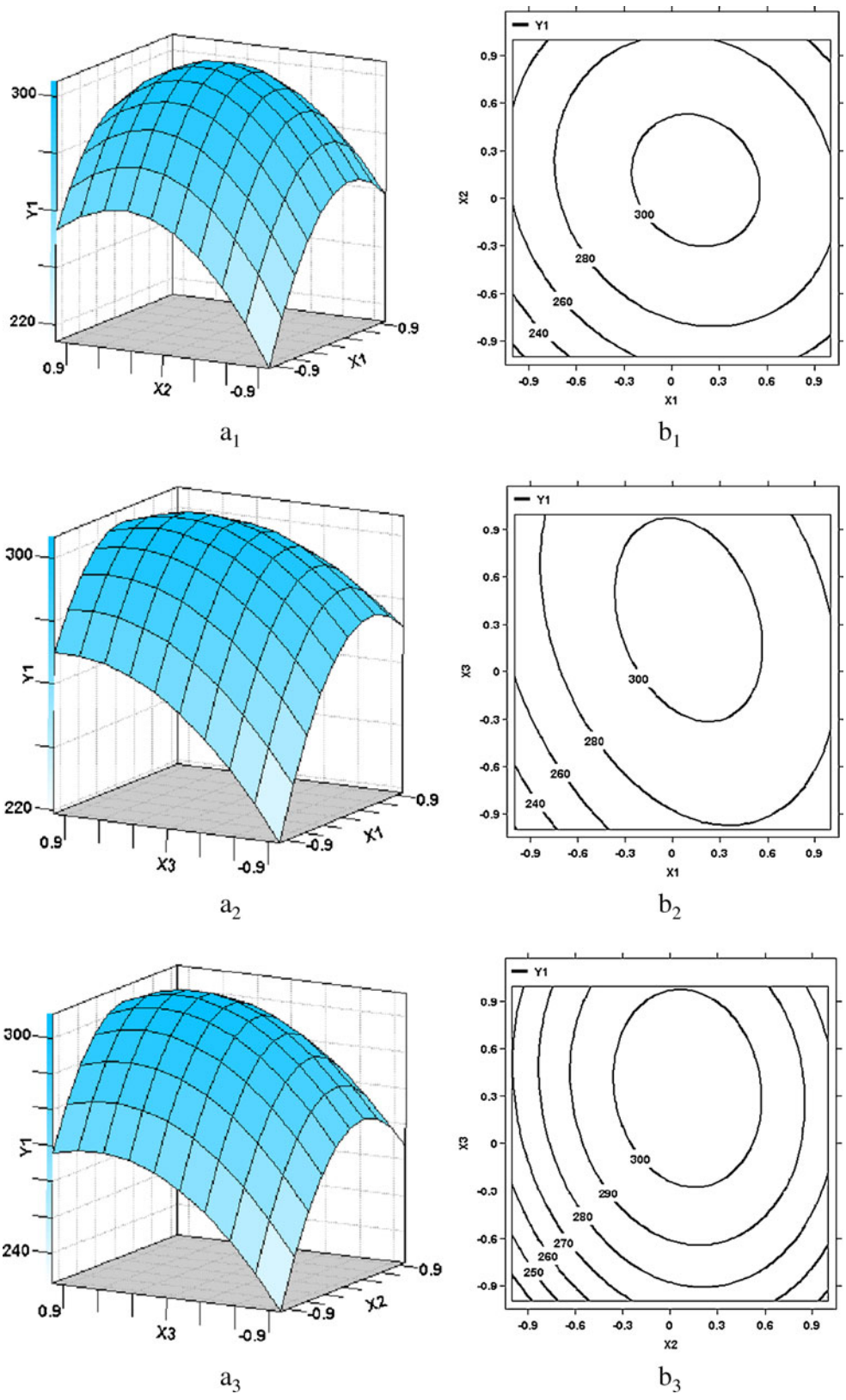

Fig. 5 Response surface plots (**a**) and contour plots (**b**) of interaction effect of each independent variable's pair on endoglucanase activity (Y_1). (**a_1**, **b_1**) x_1 (moisture content) and x_2 (cultivation temperature); (**a_2**, **b_2**) x_1 (moisture content) and x_3 (initial culture pH); (**a_3**, **b_3**) x_2 (cultivation temperature) and x_3 (initial culture pH)

niger HQ-1. Earlier reports had reported that ammonium salts in the form of sulfate facilitated cellulases production by *Thermoascus aurantiacus*, *Penicillium funiculosum*, *Chaetomium cellulolyticum*, *Trichoderma reesei*, *A. niger* and *A. terreus* (Kalogeris et al. 2003b; Jatinder et al. 2006a, b). Though yeast extract and peptone could supply the strain HQ-1 with abundant nutrient contents, there was no cellulose in them. When yeast extract, peptone and cellulosic substrates coexisted in the medium, yeast extract and peptone were more easily utilized by the strain compared with cellulosic substrates. Despite the two kinds of nutrient-richer substrates resulting in high biomass yield, they resulted in reduced cellulases activities as the enzymes belonged to inducible enzymes, whereas it was reported that the organic nitrogen sources favored *Aspergillus terreus* M11 to produce cellulolytic enzymes (Gao et al. 2008). Therefore, the ability to utilize particular nitrogen sources varied with different species.

Plackett-Burman design

The data listed in Table 4 indicated that there was a wide variation in cellulases activities in the 12 trials. The variation reflected the importance of optimization of fermentation parameters to attain higher production. As shown in Tables 5, 6 and 7, factors including moisture content and cultivation temperature had significant positive effects on three types of cellulases activities while initial culture pH had significant negative effect. The negative standardized effect meant that the shift of the variable from the low level to the high level produced a decrease of the responses while the positive one meant this change increased such responses. The factors including corn stover, wheat bran, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$, with *P* values more than 0.05, had no significant effect on cellulases activities. Earlier reports had shown that moisture content and temperature had a significant effect on FPA of the mixed-culture of *Trichoderma reesei* ATCC 26921 and *Aspergillus oryzae* ATCC 12892 (Brijwani et al. 2010). In addition, it was reported that only moisture content had a significant effect on cellulases production by *Trichoderma reesei* QM9414 and factors including cultivation temperature and moisture content were significant parameters for cellulases production by *T. reesei* MCG77 (Latifian et al. 2007). Therefore, all other insignificant variables were neglected and the optimal levels of the three significant variables were further determined by method of steepest ascent and RSM design.

The method of steepest ascent

Based on the results of Plackett-Burman design, the method of steepest ascent was employed to find the proper regions of the variables by increasing the moisture content and cultivation temperature and decreasing the initial culture pH. Table 8 listed the directions of changes for three variables. It was found that three types of cellulases activities reached the plateau on the second step. Therefore, this condition was chosen for further optimization.

Optimization of significant variables using response surface methodology

A total of 15 experiments with different combinations of moisture content (x_1), cultivation temperature (x_2) and initial culture pH (x_3) were performed in duplicates (Table 9). The results were submitted to ANOVA on software Minitab (14.12) and Statistical Analysis System (SAS, 8.0) and the regression models were given below:

$$Y_1 = 303.910 + 10.680x_1 + 8.174x_2 + 12.514x_3 - 32.530{x_1}^2 - 30.371{x_2}^2 - 16.919{x_3}^2 - 8.216x_1x_2 - 12.634x_1x_3 - 4.636x_2x_3 \quad (1)$$

$$Y_2 = 42.143 + 1.445x_1 + 1.204x_2 + 1.783x_3 - 4.131{x_1}^2 - 3.740{x_2}^2 - 2.545{x_3}^2 - 0.843x_1x_2 - 2.630x_1x_3 - 0.302x_2x_3 \quad (2)$$

$$Y_3 = 157.768 + 5.568x_1 + 4.524x_2 + 5.664x_3 - 15.663{x_1}^2 - 15.148{x_2}^2 - 9.139{x_3}^2 - 1.555x_1x_2 - 7.516x_1x_3 - 1.759x_2x_3 \quad (3)$$

where Y_1, Y_2 and Y_3 were the predicted endoglucanase activity, FPA and β-glucosidase activity, respectively. The

Table 11 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for FPA

Source	*df*	SS	MS	*F*	*P*
Model	9	205.780	22.865	113.79	0.000
Linear	3	53.725	17.908	89.13	0.000
Square	3	121.188	40.396	201.04	0.000
Interaction	3	30.867	10.289	51.21	0.000
Residual error	5	1.005	0.201		
Lack of fit	3	0.924	0.308	7.69	0.117
Pure error	2	0.080	0.040		
Total	14	206.785			

SS sum of squares, *df* degree of freedom, *MS* mean square

codes including x_1, x_2 and x_3 were moisture content, cultivation temperature and initial culture pH, respectively.

For endoglucanase activity, nine model terms (x_1, x_2, x_3, x_1^2, x_2^2, x_3^2, x_1x_2, x_1x_3 and x_2x_3) were significant terms.

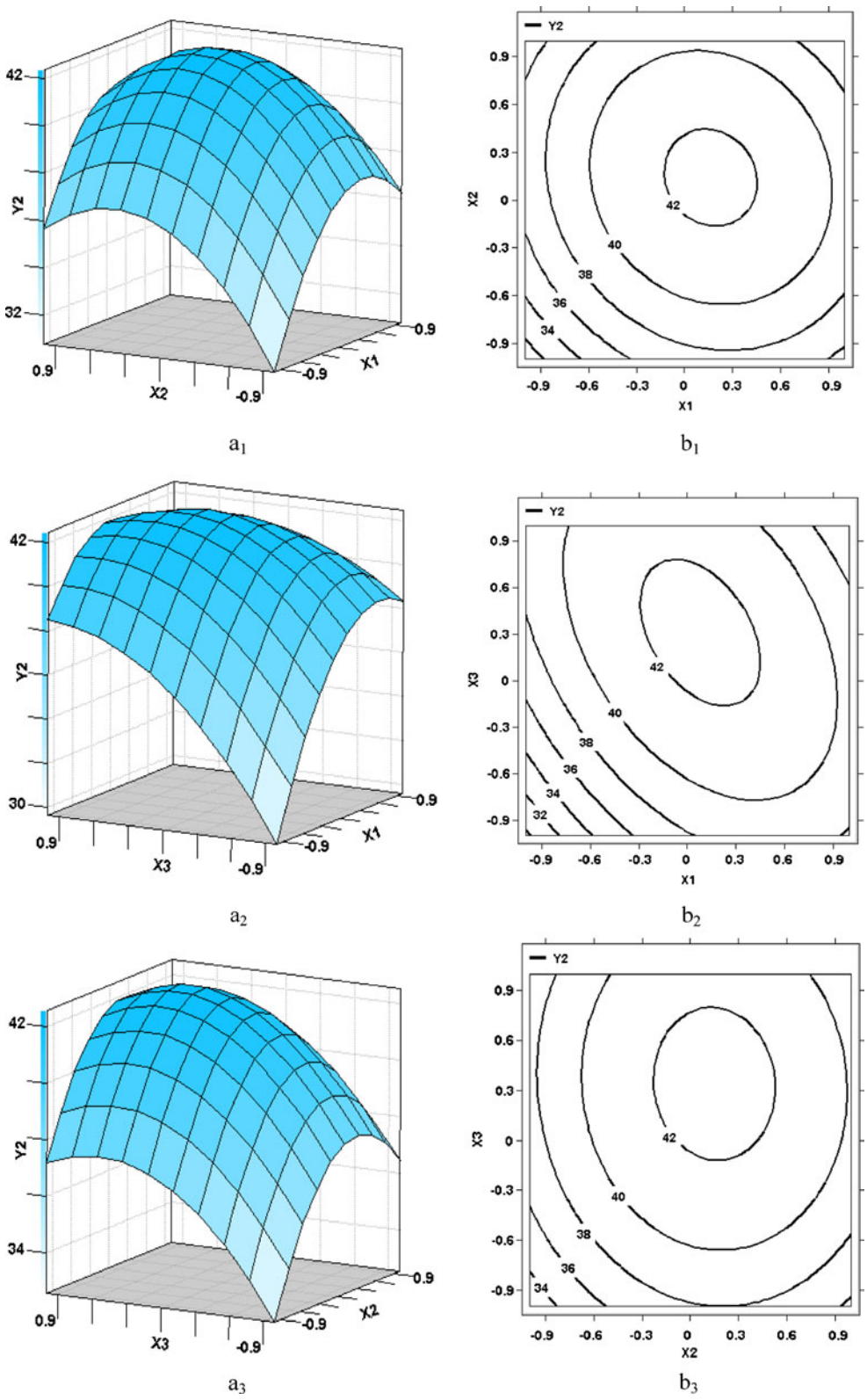

Fig. 6 Response surface plots (**a**) and contour plots (**b**) of interaction effect of each independent variable's pair on FPA (Y_2). (**a_1**, **b_1**) x_1 (moisture content) and x_2 (cultivation temperature); (**a_2**, **b_2**) x_1 (moisture content) and x_3 (initial culture pH); (**a_3**, **b_3**) x_2 (cultivation temperature) and x_3 (initial culture pH)

The value of the coefficient of determination (R^2=0.996) was in reasonable agreement with the adjusted determination coefficient (Adj R^2=0.990). As shown in Table 10, the *P* values for the model and lack of fit were 0.000 and 0.135, respectively. It indicated that the lack of fit was not significant and the equation was adequate for predicting endoglucanase activity by *A. niger* HQ-1. The response surface plots and their corresponding counter plots of the interaction effects between the three significant variables on endoglucanase activity are given in Fig. 5. Elliptical contour plots indicated that the interaction effect of each variable′ pair on endoglucanase activity was significant. According to the canonical analysis, the results predicted by the model showed that the maximum endoglucanase activity could be achieved when moisture content, cultivation temperature and initial culture pH were 70.5% (x_1=0.089), 33.5°C (x_2=0.098) and 4.662 (x_3=0.323), respectively. The maximal predicted endoglucanase activity was 306.807 U/g.

For FPA, eight model terms (x_1, x_2, x_3, x_1^2, x_2^2, x_3^2, x_1x_2 and x_1x_3) were significant terms. The values of R^2 and Adj R^2 were 0.995 and 0.986, respectively. The *P* values for the model and lack of fit were 0.000 and 0.117, respectively (Table 11). The response surface plots and their corresponding counter plots of the interaction effect of each independent variable's pair on FPA are given in Fig. 6. According to the canonical analysis, the results predicted by the model showed that the maximum FPA could be achieved when moisture content, cultivation temperature and initial culture pH were 70.3% (x_1=0.062), 33.7°C (x_2=0.142) and 4.655 (x_3=0.310), respectively. The maximum predicted FPA was 42.549 U/g.

For β-glucosidase activity, seven model terms (x_1, x_2, x_3, x_1^2, x_2^2, x_3^2 and x_1x_3) were significant terms. The values of R^2 and Adj R^2 were 0.995 and 0.987, respectively. The *P* values for the model and lack of fit were 0.000 and 0.118, respectively (Table 12). The response surface plots and their corresponding counter plots of the interaction effect of each independent variable's pair on β-glucosidase activity are given in Fig. 7. According to the canonical analysis, the results predicted by the model showed that maximum β-glucosidase activity could be achieved when moisture content, cultivation temperature and initial culture pH were 70.6% (x_1=0.111), 33.6°C (x_2=0.129) and 4.626 (x_3=0.252), respectively. The maximum predicted β-glucosidase activity was 159.082 U/g. Based on the above results, the optimal ranges of moisture content, cultivation temperature and initial culture pH for cellulases activities were 70.3–70.6%, 33.5–33.7°C and 4.626–4.662, respectively.

Validation of the experimental model

The time course of cellulases production by *A. niger* HQ-1 was examined under both the optimized and unoptimized conditions in SSF (Fig. 8). Endoglucanase activity, FPA and β-glucosidase activity under the optimized conditions from three replications were 305.103, 42.432 and 158.527 U/g, respectively. Increasing the harvesting time might result in the accumulation of hydrolysis products such as glucose and cellobiose, which were inhibitors for cellulases-system activity and could adversely affect the rate of cellulases production. Maximum endoglucanase activity, FPA and β-glucosidase activity under unoptimized conditions (moisture content 60%, cultivation temperature 30°C and initial culture pH 7.0) from three replications were 120.533, 18.511 and 60.238 U/g, respectively (Fig. 8).

Comparison of enzyme activity

Cellulases production by different fungi strains under SSF has been reported by various workers (Table 13). Optimal cultivation conditions including moisture content, temperature and initial culture pH of different cellulolytic fungi strains varied with different species. Different celluloic substrates were used by the strains and resulted in cellulases production. It was noteworthy that the optimal substrate for the strain HQ-1 was the mixture of corn stover and wheat bran, which was not adopted by the other researchers. The ability to utilize particular cellulosic substrates varied with different species. It was obvious that the pH range of cellulases production by cellulolytic fungi was from pH 4.5 to 6.0, except for pH 7.0 of *Aspergillus fumigatus* Fresenius (Soni et al. 2010). The cultivation temperatures of *Aspergillus fumigatus* Fresenius, *Scytalidium thermophilum* and *Melanocarpus* sp. MTCC 3922, higher than those of other strains, were 45°C without exception, as the three fungi strains were thermophilic or thermotolerant fungal strains (Soni et al. 2010; Jatinder et al. 2006a, b).

Table 12 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for β-glucosidase activity

Source	*df*	SS	MS	*F*	*P*
Model	9	2,724.00	302.667	116.40	0.000
Linear	3	668.40	222.800	85.68	0.000
Square	3	1,807.60	602.534	231.72	0.000
Interaction	3	248.00	82.667	31.79	0.001
Residual error	5	13.00	2.600		
Lack of fit	3	11.96	3.987	7.67	0.118
Pure error	2	1.04	0.520		
Total	14	2737.00			

SS sum of squares, *df* degree of freedom, *MS* mean square

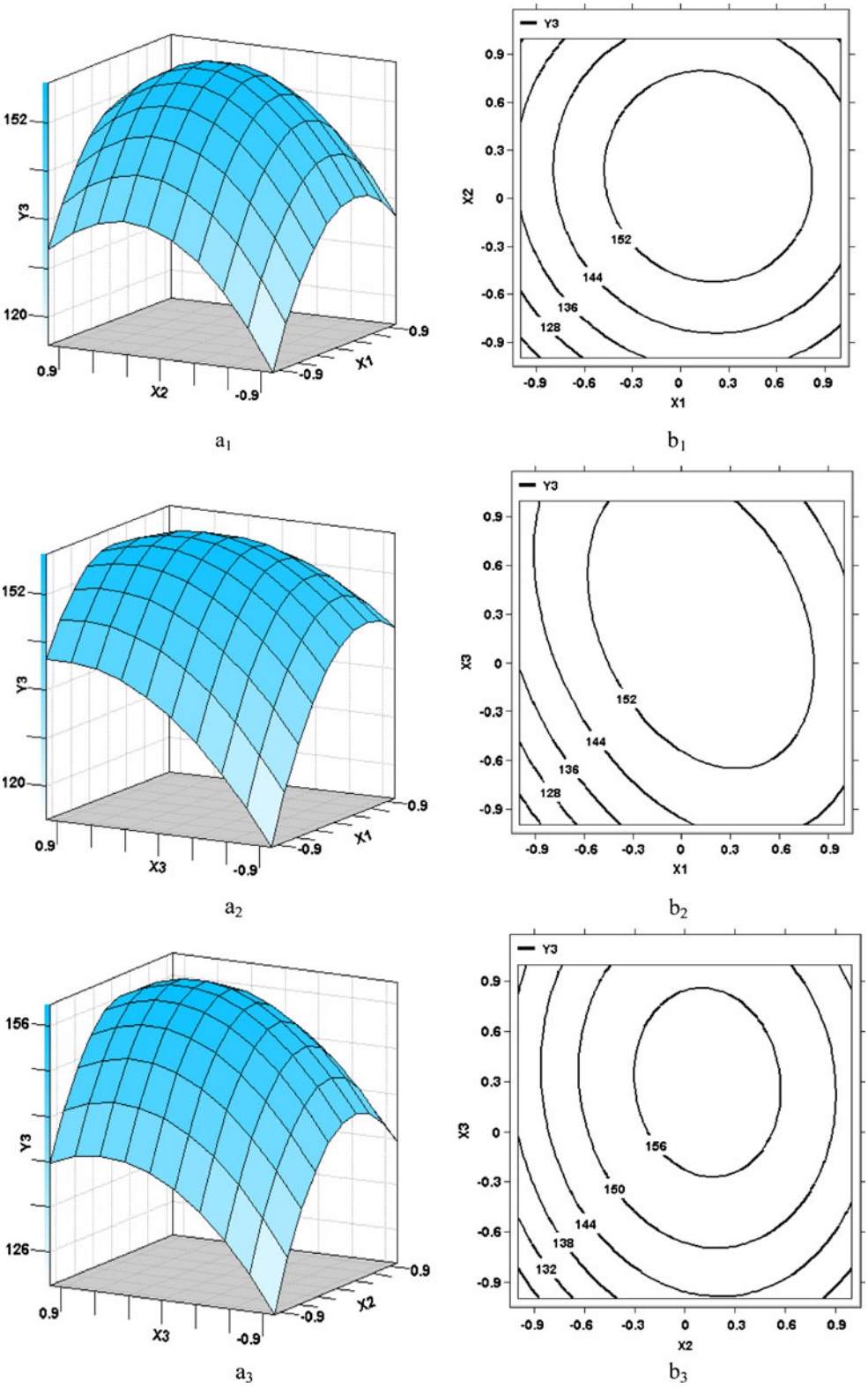

Fig. 7 Response surface plots (a) and contour plots (**b**) of interaction effect of each independent variable's pair on β-glucosidase activity (Y_3). (**a_1**, **b_1**) x_1 (moisture content) and x_2 (cultivation temperature); (**a_2**, **b_2**) x_1 (moisture content) and x_3 (initial culture pH); (**a_3**, **b_3**) x_2 (cultivation temperature) and x_3 (initial culture pH)

Enzyme assay conditions (including substrate, reaction temperature, pH and time) differed from each other. For example, the reaction substrate used in endoglucanase activity assay of *Trichoderma reesei* QM9414 (Rocky-Salimi and Hamidi-Esfahani 2010) was CMC solution (2%, w/v) prepared in sodium acetate buffer (50 mM, pH4.8), whereas that of *Aspergillus fumigatus* Fresenius (Soni et al. 2010) was CMC solution (1%, w/v) prepared in sodium

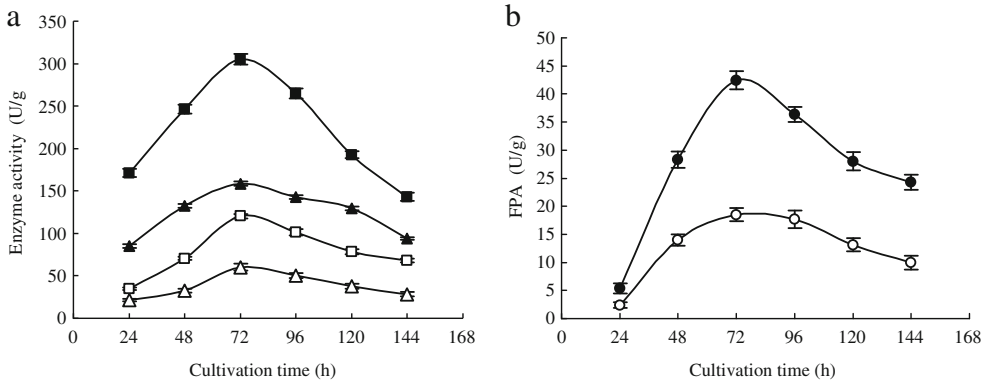

Fig. 8 The time course of cellulases production by *A. niger* HQ-1 under both optimized and unoptimized conditions. Endoglucanase activity (**a**): unoptitimized condition (□), optimized condition (■); FPA (**b**): unoptimized condition (○), optimized condition (●); β-glucosidase (**a**): unoptitimized condition (△), optimized condition (▲)

citrate buffer (50 mM, pH 6.0). Therefore, comparison of cellulase activities was not always possible as no standard enzyme assay conditions (including substrate) had yet been adopted. Under the optimal conditions found in this study, relatively high levels of endoglucanase activity, FPA and β-glucosidase activity were obtained, except that β-glucosidase activity was lower than that of *Aspergillus fumigatus* Fresenius (Soni et al. 2010). Cellulases activities by *A. niger* HQ-1 compared favorably with those reported in the literature for other cellulolytic fungi strains.

Table 13 Comparison of cellulases activities by cellulolytic fungi strains in SSF

Strain	Substrates	Moisture content (%)	Temperature (°C)	Initial culture pH	Cellulases activities (U/g)			Refs
					Endoglucanase	FPA	β-Glucosidase	
Aspergillus niger HQ-1	Corn stover and wheat bran	70.3–70.6	33.5–33.7	4.626–4.662	305.103	42.432	158.527	This study
Penicillium decumbens no.1	Steam-exploded wheat straw and wheat bran	69	30	-	-	17.7	52.8	(Mo et al. 2004)
Penicillium decumbens JUA10	Steam-exploded straw and wheat bran	71.43	27	4.5	-	20.4	-	(Xu et al. 2002)
Trichoderma reesei QM9414	Rice bran	70	25–28	5.0	94.210	11.648	-	(Rocky-Salimi and Hamidi-Esfahani, 2010)
Aspergillus fumigatus Fresenius	Rice straw	75	45	7.0	240.2	9.73	470	(Soni et al. 2010)
Co-culture of *Trichoderma reesei* and *Aspergillus oryzae*	Soybean hulls and wheat bran	70	30	5.0	108	10.7	10.7	(Brijwani et al. 2010)
Aspergillus niger KK_2	Rice straw	65	28	7.0	129	19.5	100	(Kang et al. 2004)
Penicillium decumbens L-06	Bagasse and wheat bran	70.44	30	5.28	-	3.97	-	(Long et al. 2009)
Trichoderma citrinoviride C-9	Sugarcane straw	70	30	-	35	16.22	26	(Guerra et al. 2006)
Scytalidium thermophilum	Rice straw and wheat bran	75	45	5.75	62.5	3.0	151	(Jatinder et al. 2006a)
Melanocarpus sp. MTCC 3922	Rice straw	80	45	6.0	142.4	39.9	109.0	(Jatinder et al. 2006b)
Trichoderma reesei Rut C-30	Kinnow pulp and wheat bran	-	30	6.0	25.2	13.4	18	(Oberoi et al. 2010)

Effect of temperature on the chitosanolytic activity and thermal stability

As shown in Fig. 9a, the crude enzymes had the optimal temperature at 50°C for chitosan hydrolysis. The result was in accordance with that of commercial cellulase in some previous reports (Lin and Ma 2003; Zhou et al. 2003). More than 50% of the optimal chitosanolytic activity was maintained after incubation at broad temperatures ranging from 30 to 60°C for 12 h.

Effect of pH on chitosanolytic activity and pH stability

As shown in Fig. 9b, the optimal pH for the chitosanolytic activity of the enzymes was pH 5.6 which was in accordance to that of the commercial cellulase in the previous report (Lin and Ma 2003). More than 80% of the original chitosanolytic activity of the enzymes was maintained at broad pH ranging from 4.4 to 6.0 after incubation at 30°C for 24 h.

Effect of metallic ions on chitosanolytic activity of crude cellulases

The relative activities for the crude cellulases on hydrolyzing chitosan in the presence of 5 mM $CaCl_2$, $MgSO_4$, $FeCl_3$, K_2SO_4, $ZnSO_4$, $CoCl_2$, $BaCl_2$, $AgNO_3$, $CuSO_4$ and $MnCl_2$ were 115.4±1.3, 116.1±1.1, 54.6±1.2, 106.6±1.1, 90.6±0.9, 76.3±1.1, 81.4±0.9, 21.5±0.8, 45.9±0.6 and 118.6±1.7, respectively. The chitosanolytic activity was enhanced in the presence of 5 mM Ca^{2+} and the result was in accordance with that reported in the literature (Liu and Xia 2006). As described previously, endoglucanase activity of *Mucor circinelloides* was enhanced by 5 mM Mg^{2+} and that of *Bacillus subtilis* subsp. *subtilis* A-53 was enhanced by 5 mM Mn^{2+} (Saha 2004; Kim et al. 2009). It was reasonable that the chitosanolytic activity of cellulases by *A. niger* HQ-1 could be enhanced by 5 mM Mg^{2+} and 5 mM Mn^{2+}, respectively, as the crude enzymes used for hydrolyzing chitosan were also cellulases.

Conclusion

The cellulolytic fungus *A. niger* HQ-1 was isolated from degrading paper and identified. Cultivation conditions for cellulases production by the strain were first optimized by employing response surface methodology (RSM). RSM appeared to be a valuable tool for cellulases production by *A. niger* HQ-1. Endoglucanase activity, FPA and β-glucosidase activity were increased from 120.533, 18.511 and 60.238 U/g to 305.103, 42.432 and 158.527 U/g, respectively. Cellulases activities by *A. niger* HQ-1 compared favorably with those reported in the literature for other cellulolytic fungi strains (Table 13). The substrate used in this study was the mixture of corn stover and wheat bran which might help in dropping the cost of enzyme production and the mixture was seldom used in the other reports. The strain has good potential in the production of cellulases in SSF and is worthy of further investigation in larger-scale operations for cellulases production.

Cellulases could hydrolyze chitosan (Xia et al. 2008). The study demonstrated the partial enzymatic characterization of crude cellulases produced by *A. niger* HQ-1 on hydrolyzing chitosan. To the best of our knowledge, this is the first report concerning the characterization of cellulases by *A. niger* strains on hydrolyzing chitosan. The optimal pH and temperature of the crude cellulases for chitosan hydrolysis were determined to be 5.6 and 50°C, respectively. It was

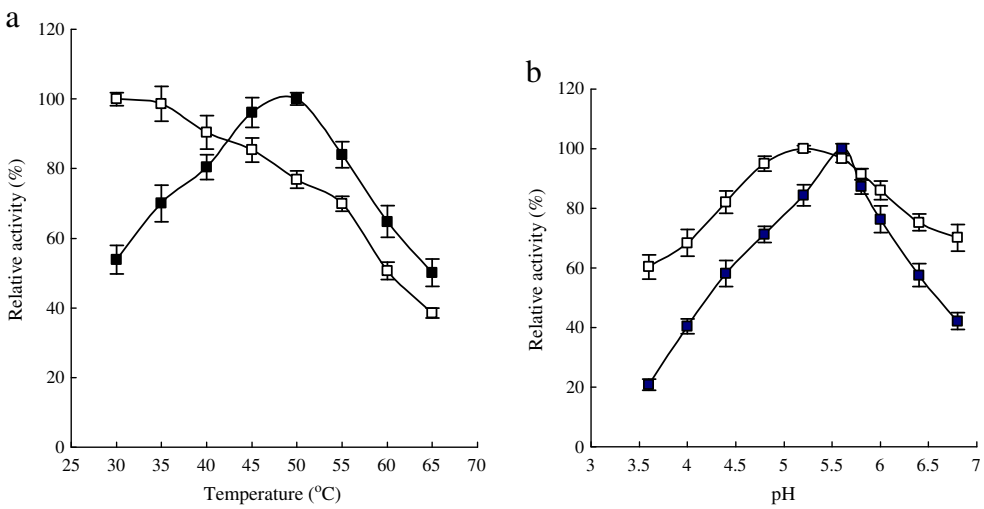

Fig. 9 Effect of temperature (**a**) and pH (**b**) on chitosanolytic activity (■) and stability (□) of the crude cellulases

noteworthy that the chitosanolytic activity of the crude cellulases could be enhanced in the presence of 5 mM Mn^{2+} and Mg^{2+}, whereas that of the cellulases produced by *Trichoderma viride* was enhanced in the presence of 3 mM Mn^{2+} and 2.5 mM Mg^{2+} (Liu and Xia 2006). Salts tolerance of cellulases on hydrolyzing chitosan varied with their origins. Perhaps, salts tolerance of cellulases produced by *A. niger* HQ-1 was relatively high compared with that of cellulases produced by other microorganisms.

This study provides correlative references for research and development of cellulase-producing microorganisms. The cellulases produced by the strain also provide a relatively new resource for bifunctional enzymes containing both chitosanase and cellulase activity. However, further investigation is required for purification of chitosanolytic cellulases produced by *A. niger* HQ-1 and the enzymes activation mechanism for hydrolyzing chitosan and analysis of the chitosan hydrolysates.

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References

Ahamed A, Vermette P (2008) Culture-based strategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. Biochem Eng J 40 (3):399–407

Brijwani K, Oberoi HS, Vadlani PV (2010) Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Process Biochem 45(1):120–128

Dai FL (1987) Fungal Forms and Class. Science Press, Beijing, pp 89–94

Dou JL, Tan CY, Du YG, Bai XF, Wang KY, Ma XJ (2007) Effects of chitooligosaccharides on rabbit neutrophils in vitro. Carbohydr Polym 69(2):209–213

Gao JM, Weng HB, Zhu DH, Yuan MX, Guan FX, Xi Y (2008) Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 99(16):7623–7629

Ghose TK (1987) Measurement of cellulose activities. Pure Appl Chem 59(2):257–268

Guerra G, Casado MRLG, Arguelles J, Sanchez MI, Manzano AM, Guzman T (2006) Cellulase production with Sugarcane straw by *Trichoderma citrinoviride* on solid bed. Sugar Tech 8(1):30–35

Guo LD, Hyde KD, Liew ECY (2000) Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytol 147(3):617–630

Hu Y, Du YM, Yang JH, Tang YF, Li J, Wang XY (2007) Self-aggregation and antibacterial activity of N-acylated chitosan. Polymer 48(11):3098–3106

Ilyina AV, Tikhonov VE, Albulov AI, Varlamov VP (2000) Enzymic preparation of acid-free-water-soluble chitosan. Process Biochem 35(6):563–568

Jatinder K, Chadha BS, Saini HS (2006a) Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using Response Surface Methodology. World J Microb Biotechol 22(2):169–176

Jatinder K, Chadha BS, Saini HS (2006b) Optimization of medium components for production of cellulases by *Melanocarpus sp.* MTCC 3922 under solid-state fermentation. World J Microb Biotechol 22(1):15–22

Kalogeris E, Iniotaki F, Topakas E, Christakopoulos P, Kekos D, Macris BJ (2003a) Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. Bioresour Technol 86(3):207–213

Kalogeris E, Christakopoulos P, Katapodis P, Alexiou A, Vlachou S, Kekos D, Macris BJ (2003b) Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes. Process Biochem 38(7):1099–1104

Kang SW, Park YS, Lee JS, Hong SI, Kim SW (2004) Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour Technol 91(2):153–156

Kim BK, Lee BH, Lee YJ, Jin IH, Chung CH, Lee JW (2009) Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53. Enzyme Microb Technol 44(6–7):411–416

Latifian M, Esfahani ZH, Barzegar M (2007) Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioresour Technol 98(18):3634–3637

Li XH, Yang HJ, Roy B, Park EY, Jiang LJ, Wang D, Miao YG (2010) Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. Microbiol Res 165(3):190–198

Lin Q, Ma KL (2003) Study of catalytic hydrolysis of chitosan by cellulase. China Surfactant Detergent Cosmetics 33(1):22–25

Liu J, Xia W (2006) Purification and characterization of a bifunctional enzyme with chitosanase and cellulase activity from commercial cellulase. Biochem Eng J 30(1):82–87

Long CN, Ou YQ, Guo P, Liu YT, Cui JJ, Long MN, Hu Z (2009) Cellulase production by solid state fermentation using bagasse with *Penicillium decumbens* L-06. Ann Microbiol 59(3):517–523

Mach RL, Zeilinger S (2003) Regulation of gene expression in industrial fungi: *Trichoderma*. Appl Microbiol Biotechnol 60 (5):515–522

Mandels M (1985) Applications of cellulases. Biochem Soc Trans 13:414–416

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428

Mo HT, Zhang XY, Li ZH (2004) Control of gas phase for enhanced cellulase production by *Penicillium decumbens* in solid-state culture. Process Biochem 39(10):1293–1297

Oberoi HS, Chavan Y, Bansal S, Dhillon GS (2010) Production of cellulases through solid state fermentation using kinnow pulp as a major substrate. Food Bioprocess Technol 3(4):528–536

Oksanen T, Pere J, Paavilainen L, Buchert J, Viikari L (2000) Treatment of recycled Kraft pulps with *Trichoderma reesei* hemicellulases and cellulases. J Biotechnol 78(1):39–48

Rocky-Salimi K, Hamidi-Esfahani Z (2010) Evaluation of the effect of particle size, aeration rate and harvest time on the production of cellulase by *Trichoderma reesei* QM9414 using response surface methodology. Food Bioprod Process 88(1):61–66

Saghai-Maroof MA, Solman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphi- sms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Nati Acad Sci USA 81(24):8014–8018

Saha BC (2004) Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem 39:1871–1876

Sohail M, Siddiqi R, Ahmad A, Khan SA (2009) Cellulase production from *Aspergillus niger* MS82: effect of temperature and pH. New Biotechnol 25(6):437–441

Soni R, Nazir A, Chadha BS (2010) Optimization of cellulase production by a versatile *Aspergillus fumigatus* fresenius strain (AMA) capable of efficient deinking and enzymatic hydrolysis of Solka floc and bagasse. Ind Crop Prod 31(2):277–283

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25(24):4876–4882

Tsai GJ, Zhang SL, Shieh PL (2004) Antimicrobial activity of a low molecular-weight chitosan obtained from cellulase digestion of chitosan. J Food Prot 67(2):396–398

Wang SL, Lin HT, Liang TW, Chen YJ, Yen YH, Guo SP (2008) Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. Bioresour Technol 99(10):4386–4393

Xia LM, Shen XL (2004) High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. Bioresour Technol 91(3):259–262

Xia WS, Liu P, Liu J (2008) Advance in chitosan hydrolysis by non-specific cellulases. Bioresour Technol 99(15):6751–6762

Xu FJ, Chen HZ, Li ZH (2002) Effect of periodically dynamic changes of air on cellulase production in solid-state fermentation. Enzyme Microb Technol 30(1):45–48

Xu JG, Zhao XM, Han XW, Du YG (2007) Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi in vitro. Pesticide Biochem Phys 87(3):220–228

Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW (2007) Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine 25 (11):2085–2094

Zhou G, He ZP, Deng GH, Huang ZY, Tan XC (2003) Enzyme kinetics of amylase and cellulase on hydrolyzing chitosan. China Mae Sci 27(11):59–63