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A high-throughput screening method for breeding *Aspergillus niger* with $^{12}C^{6+}$ ion beam-improved cellulase

Bo-Ling Jiang[1,2] · Shu-Yang Wang[1,3] · Yu-Chen Wang[1,4] · Ji-Hong Chen[1] · Wen-Jian Li[1] · Jing Liu[1] · Wei Hu[1] · Guo-Qing Xiao[1] · Miao-Ying Dong[1] · Fu-Qiang Xu[1]

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Abstract In this study, a high-throughput screening method was established through the 24-square deep-well microliter plate (MTP) fermentation and micro-plate detection for large-scale screening of the mutants. It was suitable for screening a large number of mutants and improving the breeding efficiency after heavy-ion beam irradiation. Seventeen strains showed higher cellulase activity compared with the initial strain after the screening of plate and MTP fermentation. The filter paper activity and β-glucosidase activity of *Aspergillus niger* H11201 had increased 38.74 and 63.23 % separately compared with *A. niger* H11 by shaking flask fermentation, and it was genetically stable after being passaged to nine generations. The results indicate that the high-throughput screening method can be used for the quick breeding of *A. niger* with high cellulase activity.

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✉ Shu-Yang Wang
wangsy@impcas.ac.cn

[1] Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Rd, Lanzhou 730000, China

[2] University of Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing 100049, China

[3] Lanzhou University, 222 South Tianshui Road, Lanzhou 730000, Gansu Province, China

[4] Gansu Agricultural University, Lanzhou 730000, Gansu Province, China

Keywords High-throughput screening method · *Aspergillus niger* H11 · Cellulase · $^{12}C^{6+}$ ion beam

1 Introduction

Cellulases are important enzymes in industries, such as food, textiles, detergent, animal feed, biofuel, paper and pulp, waste management [1]. This group of enzymes mainly composes endoglucanases (EC 3.2.1.4, EG), cellobiohydrolases (EC 3.2.1.91, CBH) and β-glucosidase (EC 3.2.1.21, BGL), which act synergistically in the conversion of cellulose into glucose and then can be subsequently fermented to biochemicals for resource recycling [2, 3].

Among the cellulolytic fungi, *Aspergillus niger* (*A. niger*) has been mainly used for the production of extracellular cellulases including BGL, and EG. *A. niger* produces strong activity of BGL which causes deglycosylation of substrates to produce gentiobiose, a strong inducer of cellulases [4–6]. However, the low activity of cellulase hinders industrial use of the enzymes [7]. FPA can reflect the synergistic ability of the three components, and the activity of EG, CBH and BGL represents the capability of attacking amorphous cellulose, releasing cellobiose from crystalline cellulose and hydrolyzing cellobiose into glucose, respectively [8].

It is essential to find an effective method to improve the activity of cellulase for its commercial importance. Physical mutagens with high LET value, namely heavy-ion beams, can induce stronger biological effects than other physical methods, such as X- and γ-rays [9–11]. Therefore, heavy-ion beam irradiation can produce improved mutants capable of producing cellulases with high activity. The beam has a broad mutation spectrum and high mutation frequency

[12–14], and as such, it can produce a large number of mutants, which will cause the difficulties in screening.

This work was aimed at establishing a high-throughput screening method by MTP fermentation to primarily screen mutants and micro-plate reader to detect cellulase activity. The difficulties in screening after $^{12}C^{6+}$ ion beam irradiation were overcome to make the breeding process more efficiently. To our knowledge, this is the first report of the establishment of the high-throughput screening method by MTP fermentation and micro-plate detecting of *A. niger* using a $^{12}C^{6+}$ heavy-ion beam. And finally we screened out the mutants of *A. niger* with high cellulase activity by high-throughput screening and shaking flask fermentation.

2 Materials and methods

2.1 Microorganism

Initial *A. niger* H11 was provided by biophysics laboratory at the Institute of Modern Physics, Chinese Academy of Sciences (CAS). It was mutant strain induced by $^{12}C^{6+}$ ion beam irradiation of strain *A. niger* H, and maintained on potato dextrose agar (PDA) slants and stored at 4 °C in a refrigerator, and spores of *A. niger* H11 were inoculated in the bran medium for 6 d. *A. niger* GSTCC 60108 (H) was obtained from the industrial microbial culture collection center of Gansu Province, China.

2.2 Heavy-ion beam progressive irradiation mutagenesis

The conidial suspension of 6 d old slant culture of *A. niger* H11 in saline water was transferred to irradiation dish. The colony-forming units/mL (CFU/mL) were maintained at 1×10^6 cells/mL [15]. The spores were irradiated with $^{12}C^{6+}$ ions of 80 MeV/u at the Heavy Ion Research Facility of Lanzhou (HIRFL, Institute of Modern Physics). Nine groups of initial *A. niger* H11 strains were prepared and irradiated to 0, 40, 80, 100, 120, 140, 160, 200 and 250 Gy. The $^{12}C^{6+}$ ion beams of 80 MeV/u have an LET of 40 keV/μm. Three parallel groups for each sample were irradiated at each dose.

2.3 Screening of mutant strains for higher cellulase activity

2.3.1 Plate screening

Colony suspensions irradiated to different doses were diluted to 10^{-5} gradient. Then, 0.1 mL diluted colony suspensions was daubed on the carboxymethyl cellulose (CMC) agar plate (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone and 1.7% agar) and was stained by Gram's iodine after a 3-d incubation at 30 °C [16].

After irradiation, the survival and mutation rates were calculated using [8] Survival rate = $(T/U) \times 100\%$, where T and U are number of colonies after and before heavy-ion beam irradiation.

Positive mutation rate = $(M1/T) \times 100\%$, where $M1$ is the total number of colonies of the positive mutant strain. A transparent circle-to-colony (HC) ratio greater than 1.30 on CMC agar screening plates indicated a positive mutation.

Negative mutation rate = $(M2/T) \times 100\%$, where $M2$ is the total colony-forming units of the mutant strain. An HC value less than 1.10 on CMC-Na Agar screening plates indicates a negative mutation.

2.3.2 MTP fermentation screening

The strains with high HC value were selected for MTP fermentation and shaking flask fermentation [17]. A highly efficient method was established for a preliminary screening for hyper-cellulase-producing mutants. The screening medium was inoculated with the mutated spore suspensions and cultured in MTP at 30 °C and 200 r/min. The standard filter paper assay is not suitable for high-throughput determination of enzyme activity. By referring to the literature, we developed a micro-plate-based method for assaying large sample volumes to screen the mutants. The reaction volume was reduced by 25 times from the 0.5 mL used in the International Union of Pure and Applied Chemistry (IUPAC) method, and the absorbance was recorded with a 96 microplate reader using a test wavelength of 520 nm [18].

2.3.3 Enzyme production by shaking flask fermentation

Aspergillus niger H11 strains were pre-cultured for 12 h until adequate biomass was obtained. This biomass was used for subsequent enzyme production. The previous step was conducted in 250-mL Erlenmeyer flasks with 50 mL medium. The composition of the pre-culture medium (per flask) was 2 mL/L Tween-80, 7.5 g/L CMC-Na, 1.4 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.0016 g/L $MgSO_4 \cdot H_2O$, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.0016 g/L $MnSO_4 \cdot H_2O$, 0.0014 g/L $ZnSO_4 \cdot 7H_2O$, 0.002 g/L $CoCl_2$; the medium was autoclaved at 121 °C for 30 min and then aseptically inoculated with *A. niger* H11 maintained on substrates such as PDA. After inoculation, the pre-culture was incubated in a shaker at 30 °C and 200 r/min for 12 h. Pre-cultured *A. niger* H11 mycelium (2.5 mL) was inoculated aseptically into a flask, which was then incubated in a shaker (200 r/min) at 30 °C. Enzyme production was performed in 250-mL Erlenmeyer flasks with 50 mL of medium which was the same to the pre-culture medium. The

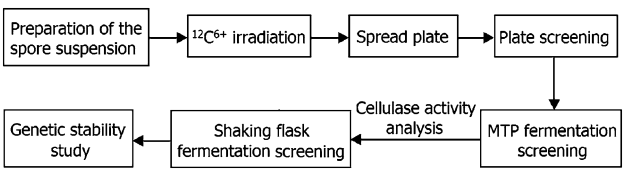
prepared medium was autoclaved at 121 °C for 30 min prior to use.

2.3.4 Cellulase activity assays

Cellulase activities were determined as previously reported with minor modification [19, 20]. FPA was determined using the dinitrosalicylic acid (DNS) method described by Ghose [21] and the reaction volume was reduced by 25 times. A 3 mm × 4 mm filter paper disk diluted in 40 μL 0.05 M citric buffer (pH 4.8) was digested by 20 μL of culture supernatant. The samples were incubated at 50 °C for 60 min. The enzymatic reaction was terminated by adding 140 μL of DNS, followed by incubation at 95 °C for 5 min. The samples were cooled in an ice bath. And then 40 μL of reaction fluid was transferred to 160 μL distilled water in a 96 microwell plate, and the absorbance was measured at 520 nm. One unit of FPA is defined as the amount of enzyme that produces 1 μg of glucose in 1 min. BGL activity was determined using the method described by Wu [22] and the reaction volume was reduced by 25 times. The reaction mixture containing 30 μL of D-salicin solution and 30 μL of enzyme solution was incubated at 50 °C for 30 min. The reaction was terminated by adding 140 μL of DNS, followed by incubating at 95 °C for 5 min. The samples were cooled in an ice bath. Then, 40 μL of reaction fluid was transferred to 160 μL distilled water in a 96 microwell plate, and the absorbance was measured at 520 nm. One unit of BGL activity is defined as the amount of enzyme that produces 1 μg of glucose in 1 min.

2.4 Flowchart of experiment

The experimental procedures of the study were as follows:



3 Results and discussion

3.1 Heavy-ion progressive irradiation mutagenesis

Progressive irradiation of 80 MeV/u $^{12}C^{6+}$ ion beams markedly affected the survival rate of *A. niger* H11 spores, as shown in Fig. 1a as a plot of survival rate against irradiation dose. At 40, 80, 120, 140,160, 200 and 250 Gy, the survival rates are 96.58, 54.79, 91.10, 77.40, 62.33, 51.37, 42.47 and 26.03%, respectively. Generally, the survival rate of spores decreased with increasing doses, dropping sharply to 50% at 80 Gy. The semi-lethal dose was 160 Gy, and the lethality rate of spores approached 73% at 250 Gy. The correlation between the ratio of mutants and the irradiation dose is shown in Fig. 1b.

The results of plate screening using CMC-Na as carbon source and Gram's iodine as a stain are shown in Table 1. Gram's iodine formed a bluish-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct zone around the cellulase-producing microbial colonies within 3–5 min. This method can be easily performed for screening large numbers of microbial cultures of both bacteria and fungi rapidly and efficiently. The HC value and the average FPA can be changed and improved after heavy-ion irradiation.

Figure 2 shows the HC ratio and FPA of cellulase correlated well and linearly ($R^2 = 0.9902$). The mutants with HC > 1.3 are defined as positive mutants, and the mutants with HC < 1.1 are defined as positive mutants, because FPA of the mutants is significantly higher than the initial strain if HC > 1.3, while it was significantly lower if HC < 1.1. The positive mutation rates are 16.28% at 80 Gy and 19.64% at 250 Gy, while the negative mutation rates are 9.30% at 80 Gy and 13.64% at 250 Gy. At 80 Gy and 250 Gy, the positive mutation rates are higher and the negative mutation rates are lower compared to those at other doses, so the two irradiation doses are good for screening *A. niger* H11 for higher cellulase production.

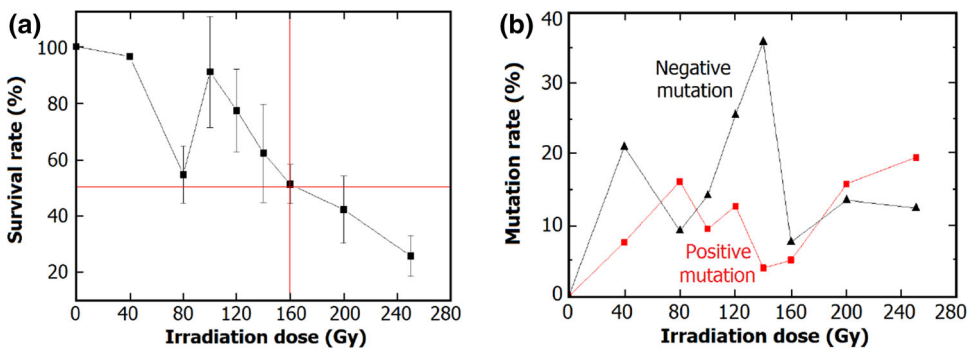


Fig. 1 Effect of 80 MeV/u $^{12}C^{6}$ ion progressive irradiation on mutation rate of *A. niger* H11. **a** The survival rate of *A. niger* H11 as a function of the irradiation doses; **b** the positive mutation rate and negative mutation rate of the irradiated *A. niger* H11

Table 1 Screening results for *A. niger* H11 after $^{12}C^{6+}$ irradiation

Irradiation dose (Gy)	Colony count	HC value range	Count of positive mutation	Count of negative mutation	Maximum HC value	Maximum FPA
40	52	1.06–1.38	4	11	1.38	190.62
80	43	1.06–1.33	7	4	1.33	277.86
100	42	1.02–1.40	4	6	1.40	152.29
120	39	1.00–1.40	5	10	1.40	140.29
140	50	1.08–1.46	2	18	1.46	125.67
160	39	1.06–1.31	2	3	1.31	218.55
200	44	1.14–1.40	7	6	1.40	259.10
250	56	1.14–1.36	11	7	1.36	254.56
0	38	1.05–1.26	The average FPA of *A. niger* H11: 116.71 ± 30.20			

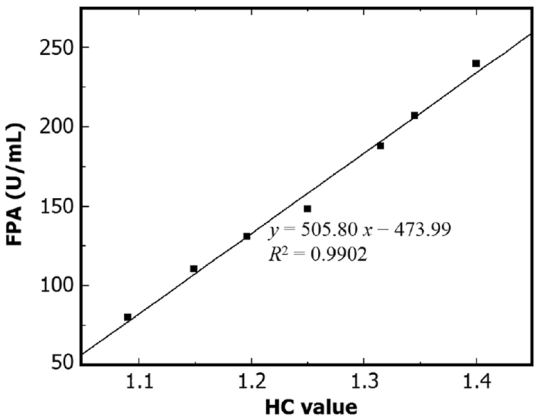


Fig. 2 Correlation between the HC value and FPA of cellulose

In Fig. 1a, while the survival rate decreases from 96.58% at 40 Gy to 26.03% at 250 Gy, the survival rate increases to 91.10% at 100 Gy, with the whole curve showing a saddle-type change. This may be due to the synthetic effects of energy deposition and momentum transfer, leading to the DNA damage and membrane damage at doses from 40 to 80 Gy, and further to the decrease in survival rate. This may activate the repair enzyme and induce new repair mechanism along with increasing doses, hence the increased survival rate at 100 Gy. With further increases in the irradiation dose, the damage caused by energy deposition and momentum transfer exceeds the repair capability, and the survival rate decreases again [23–26].

The positive mutation rate at 80 and 250 Gy was higher than others, which may be caused by the DNA damage and inactive repair enzymes at low doses, and the DNA damage caused by the high-dose irradiation cannot be repaired by the repair mechanism. So, we can obtain more mutants at 80 and 250 Gy [27].

3.2 The establishment of a high-throughput screening method

3.2.1 The micro-cultivation technique

The micro-cultivation technique is based on MTP. The relationship between the FPA of cellulase produced by MTP fermentation and shaking flask fermentation for *A. niger* H11 is shown in Fig. 3a, which proves that the MTP fermentation can replace the shaking flask fermentation for large-scale strain selection. The optimization of key factors of the MTP fermentation, i.e., the loading volume and the fermentation time, is shown in Fig. 3b. The FPA of cellulase is the highest at loading volume of 1.5 mL and the fermentation time of 60 h.

3.2.2 The high-throughput assay technique

The high-throughput assay technique is based on the micro-plate reader. The relative standard deviation (RSD) examined for the feasibility study of detecting the reducing sugar content using 96-well MTP is 4.19%. An RSD of <5% demonstrates that the 96-well MTP can be used for detecting the reducing sugar content, and further calculating FPA of cellulase (Table 2). The correlation determination of reducing sugar content between micro-plate reader and ultraviolet spectrophotometer (Fig. 4a) proves that the micro-plate reader can replace the ultraviolet spectrophotometer for detecting the reducing sugar content. The detection results under different wavelengths (Fig. 4b) and additive amounts of DNS (Fig. 4c) show that, judged from the correlation coefficients, the detective wavelength of 520 nm and DNS additive amount of 140 μL are the best for detecting the reducing sugar content. Figure 5 shows the glucose standard curve plotted under the two optimum conditions, with a liner equation of $y = 3.6307x - 0.5544$ and the correlation coefficient of $R^2 = 0.9983$.

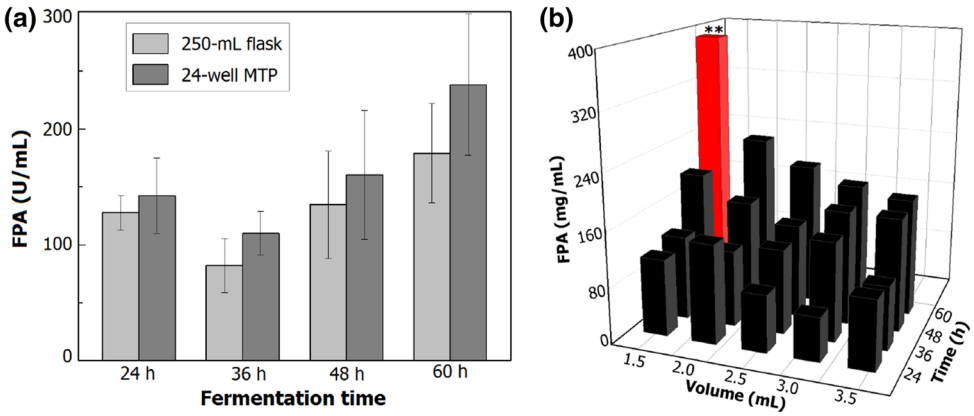


Fig. 3 Establishment of micro-cultivation technique. **a** The correlation determination of FPA between MTP fermentation and shaking flask fermentation; **b** the influence of loading volume and fermentation time on FPA

Table 2 RSD examination of standard 96-well MTP

1	2	3	4	5	6	7	8	9	10	11	12
0.9688	0.9615	0.8988	0.9751	0.9737	0.9471	0.8710	0.9321	0.9484	0.9688	0.9380	1.0049
0.9313	0.9758	0.9854	0.9542	0.9541	0.9920	0.9474	0.9286	0.9753	0.9811	0.9882	1.0258
0.9956	0.9620	0.9688	0.9737	0.9656	0.9708	0.9738	0.9803	0.9363	0.9596	0.9755	0.9748
0.9636	0.9730	0.9155	0.9626	0.9565	0.9990	0.9536	0.9805	0.9689	0.9797	1.0893	1.0003
0.8687	0.9778	0.8986	0.9936	0.9992	0.9492	0.9899	1.0156	1.0303	0.9821	0.9702	0.9767
1.0003	0.9743	0.9795	0.9889	0.9571	1.0043	1.0068	0.9538	1.0049	1.0348	0.8995	0.9698
0.9025	0.9647	0.9688	1.0010	0.9587	0.9942	1.1046	1.0169	1.0070	1.0648	1.0581	0.9745
0.9649	0.9295	1.0293	1.0171	0.9900	0.9923	0.9888	1.0197	1.0011	1.0593	1.0887	0.9832
		MAX = 1.1046		MIN = 0.8710		$X_{average}$ = 0.9783		SD = 0.0409		RSD = 4.19%	

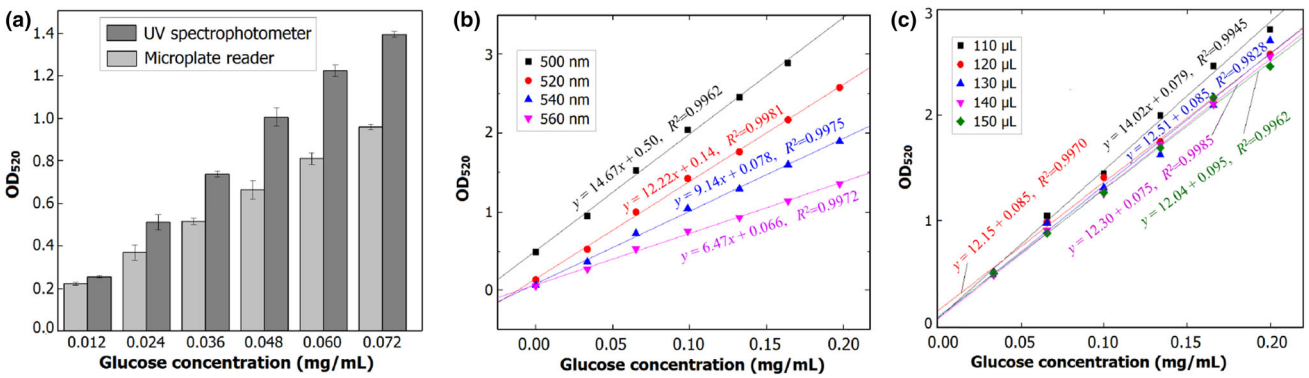


Fig. 4 Establishment of high-throughput assay technique. The correlation determination of reducing sugar content between micro-plate reader and ultraviolet spectrophotometer (**a**), and the optimization of detection wavelength (**b**) and DNS additive amount (**c**), using 96-well MTP

The two techniques supplemented each other to form a complete screening system to lay a foundation for large-scale non-rational strain selection.

3.2.3 Screening results after the progressive irradiation

The mutants screened out from the plate were filtered by the MTP fermentation. The primary screening results after MTP fermentation are given in Table 3. Seventeen mutants showed marked cellulase over-production records after the MTP fermentation. An increase in the irradiation dose led to increased number of positive mutants.

3.2.4 Screening results after the second screening by shaking flask fermentation

The seventeen mutants were re-screened by shaking flask fermentation. The *A. niger* H11201 showed higher

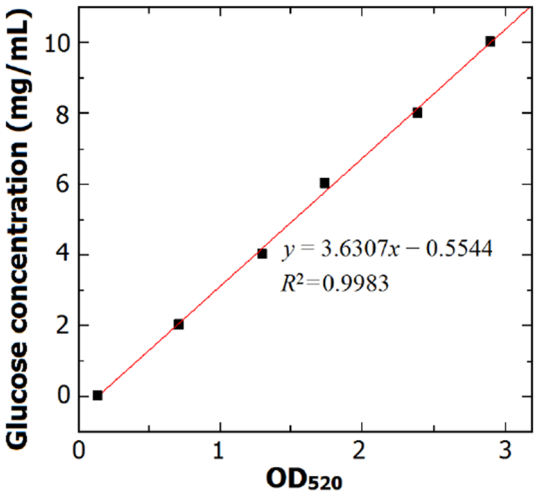


Fig. 5 Glucose standard curve

Table 3 Primary screening results after MTP fermentation

Irradiation dose (Gy)	FPA (U/mL)	Count
0	139.72 ± 26.90	1
40	281.99 ± 91.10	1
80	277.86 ± 19.14	1
120	236.91 ± 31.98	1
140	330.60 ± 36.34	1
160	218.55 ± 16.95	4
	228.14 ± 18.70	
	322.53 ± 13.84	
	313.46 ± 57.83	
200	209.28 ± 34.18	5
	259.10 ± 10.01	
	239.94 ± 23.32	
	239.03 ± 59.10	
	267.57 ± 40.42	
250	274.73 ± 69.66	4
	201.51 ± 6.72	
	225.01 ± 17.95	
	326.57 ± 7.30	

FPA and BGL activities than the initial strain *A. niger* H11 after the second screening with shaking flask fermentation.

As shown in Fig. 6a, after 96-h fermentation, the FPA of *A. niger* H11 and H11201 is 273.88 ± 31.40 and 379.99 ± 37.54 U/mL, respectively, while their BGL activity is 821.19 ± 49.60 and 1340.42 ± 122.71 U/mL, respectively. The FPA and BGL activity of *A. niger* H11201 are 38.74% and 63.23% higher than those of *A. niger* H11, respectively. Figure 6b shows the cellulase production of *A. niger* H11201 at different hours of fermentation. Both the FPA and the BGL activity reach the maximum after being fermented for 96 h. The mycelium of *A. niger* H11201 accumulated at the early stage of growth, and it is good for cellulase production after growing 96 h. The fermentation conditions might be changed along with the extension of fermentation time, and as a result, the FPA and BGL activity reduced at 120 h. The *A. niger* H11201 was passaged to nine generations, and the FPA detected in every generation did not differ significantly (Fig. 6c). Therefore, the *A. niger* H11201 is genetically stable.

4 Conclusion

The high-throughput screening method has been established through MTP fermentation and micro-plate detection. Seventeen mutants show higher cellulase activity compared with the initial strain after the high-throughput screening. We screen out the mutant H11201 with high cellulase activity after three months, the FPA and BGL activity have increased 38.74 and 63.23% separately compared with H11 after shaking flask fermentation, and it was genetically stable after being passaged to nine generations. It is proved that the high- throughput screening method can be used for the quick screening of *Aspergillus niger* with high cellulase activity after $^{12}C^{6+}$ ion beam irradiation.

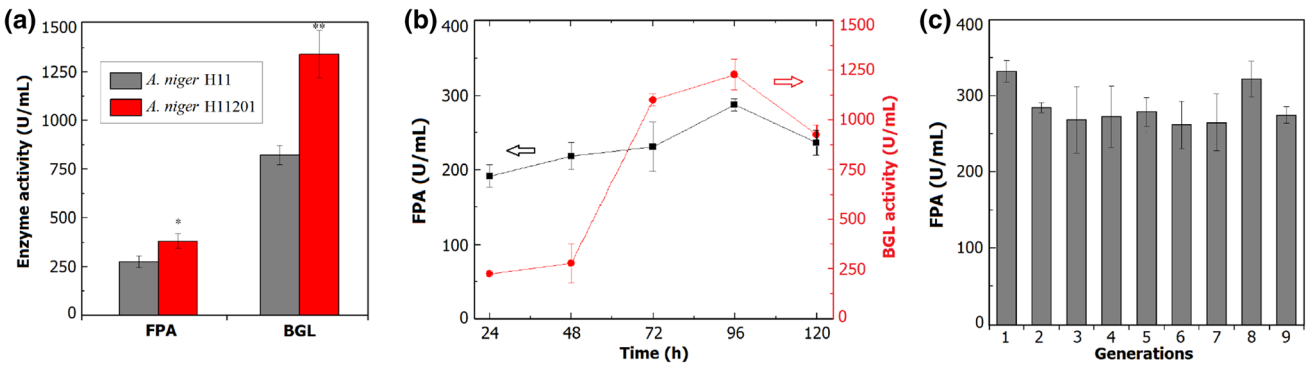


Fig. 6 Screening results by shaking flask fermentation. **a** The comparison of cellulase production between *A. niger* H11 and *A. niger* H11201; **b** time courses of cellulase production of *A. niger* H11201; **c** the genetic stability analysis of *A. niger* H11201

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