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Strain Improvement of *Aspergillus niger* GSICC 60108 for Increased Cellulase Production by Irradiation of Electron and $^{12}C^{6+}$- Ion Beams

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The *Aspergillus niger* strain GSICC 60108 was subjected to mutation involving irradiation with an electron beam and subsequently with a $^{12}C^{6+}$-ion beam. Successive mutants showed clearance zone on the cellulose-Congo red medium, 2-fold increase of the soluble protein production and multi-fold enhancement of cellulase activities. The characteristics of cellulases in the conditions of this solid-state fermentation were evaluated, and the optimum temperature, pH and culture time for maximum cellulase production by the selected mutant were 30 °C, 4.5 and 5 days. We obtained significantly higher hydrolysis yield of sawdust using the improved cellulases (60.1%) than that using parent cellulases (26.3%). Furthermore, sequence determination of the celllase gene revealed some mutation sites existed, suggesting that some amino acid changes in the protein caused by base mutations could lead to the enhanced cellulase activity and production. These results suggested that the compound mutagenesis with electron and $^{12}C^{6+}$-ion beams could be developed as a convenient and effective tool for breeding high-yield strains of cellulase producing.

Key words: *Aspergillus niger*; Electron and $^{12}C^{6+}$-ion beams; Cellulase; Mutation.

Cellulose is the most abundant renewable natural biological resource in the biosphere . Degradation of this biopolymer to monomeric sugars has been receiving a great attention in recent years (Adsul *et al*. 2007). Cellulases are regarded as one of the key elements in effective bioconversion of cellulosic biomass to fermentable sugars. Among microorganisms, fungi have been extensively studied particularly due to its highly intrinsic ability to secrete extra-cellular cellulases, which act synergistically during conversion of cellulose to glucose (Eveleigh 1987). In the fungal family, *Trichoderma reesei* is regarded as the most efficient producer of cellulase. However, this fungus does not excrete sufficient amount of β-glucosidase for efficient enzymatic hydrolysis, for which *Aspergillus* strains are known to be good producers (Damisa *et al*. 2011; Sorensen *et al*. 2011). The characteristics of *Aspergillus niger* make it ideal for use in industry, such as high-potency fermentation capabilities, high-level secretory production of proteins, ability to utilize an

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enormous variety of organic substrates and suppress development of other microorganisms and exceptionally high sporulation capacity (De Vries and Visser 2001). Additionally, the filamentous fungus *Aspergillus niger* is non-toxigenic and non-pathogenic for production of cellulases and has a long history of safe use in the industrial scale enzyme production.

However, the production of cellulase is a major factor inhibiting its use in the hydrolysis of cellulosic material (Liu *et al.* 2008). The efficiency of mutation induction and selection of current methods is still low and ineffective, requiring screening of large population for low-rate mutants which is laborious and costly (Chand *et al.* 2005; Gokhale *et al.* 1988; Najafi *et al.* 2011). Therefore, new effective methods with higher mutation efficiency are extremely desired (Adsul et al. 2007). Electron and heavy ion beams have attracted increased attention because some studies have reported the complex biological effects of electron and ion implantation, such as low germination and survival rates and obvious genetic variations in plants and microorganisms (Hu *et al*. 2013; Yoshihara *et al*. 2013; Zhang *et al*. 2012). In comparison with the mutation from other inducing methods, they are characterized as broad mutation spectrum and high mutation frequency (Okamura *et al.* 2003; Yamaguchi *et al.* 2003; Zhou *et al.* 2006). The mutant with the broadest mutations spectrum was expected by combined use of these two beams.

The purpose of this work was to isolate cellulase hyper-producing mutants for the development of safe and more economical production of cellulolytic enzymes (Pinelo *et al.* 2008). The mutation effects of electron and heavy ion beams were investigated by determination of the mutation sites of cellulase genes. To our knowledge, it is the first time to study the mutation breeding of *Aspergillus niger* with the electron beam first and then with the heavy ion beam. Furthermore, this study indicates the bioconversion of cellulosic components into fermentable sugars will be facilitated through the utilization of cellulases produced by the selected mutants and the compound mutagenesis with electron and $^{12}C^{6+}$-ion beams provides a new alternative way for effective and efficient induction of mutants.

MATERIALS AND METHODS

Materials and apparatus

Aspergillus niger GSICC 60108 for this experiment was purchased from Gansu Culture Collection of Industrial Microorganisms, P. R. China. The strain was cultured on a potato dextrose agar slant (PDA: potato leachate, 1000 mL; glucose, 20.0 g; agar, 20.0 g and pH 5.5-5.7) at 30 °C for 3-5 days (Li *et al.* 2010). Then, the spores that are full of the PDA slant were harvested by washing with sterile normal saline, suspended in sterile normal saline and then diluted to 10^8 spores/mL based on the counting under a light microscope. Afterwards, we sterilely suctioned 1 mL of spore suspension in a 35 mm disposable petri dish for the irradiation at room temperature.

The modified Mandels' medium was chosen as the basal medium contained (g/L) : KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; urea, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.00156; $ZnSO_4$, 0.0014; $CoCl_2$, 0.0002 and then mixed with 0.5% PDA liquid medium. Moreover, the medium used for screening consisted of (g/L) : microcrystalline cellulose (MCC), 5; agar, 15; $(NH_4)_2SO_4$, 5; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 1; NaCl, 0.1; $CaCl_2$, 0.1; yeast extract, 0.2; Congo red, 0.002 and pH 6.4-6.6. Additionally, the fermentation medium used for cell wall degradation contained (g/L) : wheat bran (WB), 5; peptone 0.3; basal medium, 10 mL. After inoculation, all of the culture materials were maintained at the temperature of 30 °C for 3-5 days.

WB and potato were purchased from Yiwu Commercial Plaza. *p*-nitrophenyl-β- D-glucopyranoside, carboxymethylcellulose (CMC) and MCC were obtained from Sigma-Aldrich co. St. Louis, USA. Yeast extract and peptone were from Oxoid Ltd., Basingstoke, Hampshire, England and double-distilled water was used in all experiments. All other reagents were of analytical grade and were obtained locally.

We performed electron-beam irradiation using a high power low-energy DG-2.5 accelerator at room temperature in the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, P. R. China). A $^{12}C^{6+}$-ion beam of 270 MeV/u was supplied by the Heavy Ion Research Facility in Lanzhou at the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, P. R.

China). The further exposure of the fungal strain was carried out at the terminal of the facility, which has a vertical beam line.

Irradiation with electron and $^{12}C^{6+}$-ion beams

Using the electron accelerator, the spore solution of the parent fungal strain *Aspergillus niger* GSICC 60108 was irradiated at the dose range from 5 Gy to 2000 Gy. Then, we exposed the spores of selected mutants with a $^{12}C^{6+}$-ion beam at 5 Gy-500 Gy; the dose rates for these two beams were adjusted to be approximately 4 Gy/min. After the radiations, all the cultures were sealed, diluted 5 times with 30% glycerol-physiological saline and then stored at 4 °C for the further study. The controls were treated similarly but irradiation.

Mutant screening

A kind of very simple and highly efficient method was used to preliminary screen for hypercellulase-producing mutants. The MCC screening medium was inoculated with the mutated spore suspensions and cultured at 30 °C for 3-5 days. We then estimated the cellulase activities by the ratio of hydrolysis halo diameter to colony diameter (H/C). Subsequently, the strains with higher ratios (H/C>1.5) were selected, cultured in a 96-well microplate containing the PDA medium for the further assay of enzyme activities and secondary screening of mutants. Although the standard filter paper assay is widely used to determine total cellulase activity, it is not suitable for the high-throughput determination of enzyme activity. By referring to the related literature, we developed a microplate-based method for assaying large sample numbers to screen the mutants. The reaction volume was reduced by 25 times from the 1.5 mL used in the IUPAC method and the absorbance was recorded with a Multiskan MK3 microplate reader (Thermo Labsystems) using a test wavelength of 540 nm (Xiao *et al.* 2004).

The mutants obtained by the above-mentioned methods were studied their hereditary stability for cellulase production for 9 generations. After an entire life cycle in one fermentation medium, each mutant was transferred into a new culture dish; the growth, morphological characteristics and cellulase production will be closely monitored and recorded.

Optimization of culture conditions for enzyme production

The culture was grown in a 250 mL Erlenmeyer flask that contained 100 mL of fermentation medium. We inoculated the flasks with spores (10^8 conidia/mL) from 7 days old culture grown on the PDA slants and incubated in an incubator shaker (200 rpm, IS-RDV1, Crystal Technology & Industries, Inc., America) at 30 °C up to 2 days. The culture was obtained from fermentation medium inoculated with the selected mutants and then centrifuged at 5000 rpm for 10 min. The resulted supernatant used as enzyme source was collected and stored at 4 °C.

Culture time, pH and temperature are important parameters for enzyme production by the *Aspergillus niger* mutant. The optimum culture times were investigated by measuring the cellulases activity at different culture times (12 h-204 h). Moreover, we determined the most suitable pH value of the fermentation medium by adjusting the pH of the culture medium at different levels in the range of pH 2 to 10. In order to determine the optimum temperature for cellulase production, fermentation was performed at 5 °C interval in the range of 20 °C to 80 °C.

Various carbon sources such as MCC, CMC and WB were examined by comparing the enzyme activities measured. The broth was distributed into different flasks and 5 g/L of each carbon source was then added before the inoculation. Followed by the inoculation with spores, the flasks were incubated for 5 days at 30 °C.

Thermal stability was investigated by determining the residual filter paper activity (FPA) after incubating each enzyme solution at various temperatures (20 °C-80 °C) for 100 min, respectively.

Analytical methods

FPA was measured with the standard procedure recommended by the Commission on Biotechnology, International Union of Pure and Applied Chemistry (Ghose 1987). Monitoring of total reducing sugars was determined colorimetrically by the dinitrosalicylic acid (DNS) method using glucose as a standard (Schwartz 1977). Moreover, exoglucanase and β-glucosidase activities were estimated using regenerated amorphous cellulose and *p*-nitrophenyl-β-D-glucopyranoside as substrates, respectively (Kovács *et al.* 2009).

Gene sequence of cellulases

Genomic DNA were extracted from normal

wild *Aspergillus niger* and its mutants using an E.Z.N.A.® SP Fungal DNA Kit, according to the manufacturer's instructions. The full-length genes encoding cellulases were amplified with pairs of primers which were designed based on the sequences of 5' and 3' ends of the target genes (The primers used for amplification are shown in Table 1). The reaction mixture for polymerase chain reaction (PCR) contained 50 ng of chromosomal DNA as a template, 80 ng of each of the primers used, 2.5 unit (0.5 µl) of DNA polymerase (Takara) in 10 mmol/L Tris-HCl buffer (pH 8.5) containing 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 1% (w/v) Triton X-100 and 0.2 mmol/L dNTPs in a total volume of 50 µl. Subsequently, we performed the amplification reaction under the following conditions: 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C (30 cycles), a final cycle of 10 min at 72 °C and then 4 °C forever. The PCR products with the expected sizes were purified and recovered by a DNA gel extraction kit (Takara) and inserted into the pMD18-T vector (Takara). The resulting plasmids were used to transform the *Escherichia coli* strain JM-109 and followed by sequencing.

Purifying the main components of cellulase complex

All purification steps were performed at 4 °C unless otherwise specified. A crude cellulase solution from the fermentation medium inoculated with *Aspergillus niger* CIA 915 was obtained by centrifuging at 5000 rpm for 10 min. Then, the crude extracts were concentrated 10 fold by ultrafiltration using a diether sulphone membrane (Millipore) of 10 KD cutoff and precipitated with ammonium sulfate at 80% saturation.

Enzyme purification was carried out by anion exchange chromatography, hydrophobic interaction chromatography (HIC), cation exchange chromatography and gel filtration. Firstly, the crude enzyme preparation was applied to a Sephadex DEAE A-50 previously equilibrated with 25 mmol/L Tris-HCl buffer (pH 6.8) containing 1mmol/L dithiothreitol. Elution was achieved with a linear gradient of 0.15 mol/L-0.5 mol/L of NaCl in the equilibration buffer at a flow rate of 0.5 mL/min.

Eluted fractions from the column were analyzed for endoglucanase, exoglucanase and β-glucosidase activities. Subsequently, the fraction mainly showed β-glucosidase activity was processed on a HIC column (eluted using a linear $(NH_4)_2SO_4$ gradient (pH 5, 0-1 mol/L) at a flow rate of 0.5 mL/min) and a step of Sephadex G-75 filtration (eluted with three bed volume of 50 mmol/L NaAC (pH 5) at a flow rate of 0.3 mL/min). The proteins with endoglucanase activity were loaded on an anion exchange column (eluted with a linear NaCl gradient (0-0.5 mol/L) at a flow rate of 1 mL/min) and a 10 KD ultrafilter membrane. Furthermore, the purified product with exoglucanase activity further separated by a cation exchange column (eluted with a linear NaCl gradient (0-0.5 mol/L) at a flow rate of 1 mL/min) and a HIC column (eluted using a linear $(NH_4)_2AC$ gradient (pH 5, 0-1 mol/L) at a flow rate of 0.5 mL/min) (Zhou *et al*. 2008). Additionally, the protein concentrations of each fraction were measured by the Bio-Rad protein estimation kit using bovine serum albumin as a standard.

Hydrolysis of cellulosic biomass

Sawdust was obtained in a local company (Lanzhou, P. R. China). It was selected as a substrate for saccharification experiment because of its local and abundant availability. After sieving with a 40 mesh screen, the powder was dried in an oven at 60 °C to a constant weight. Then, we suspended 50 g of the dried powder in 500 mL of 4% sodium hydroxide (w/v) solution and allowed to react at 85 °C for 3 h, with solid to liquid ratio as 1 : 5. Furthermore, the sawdust powder was collected by filtration, neutralised with 1 mol/L hydrochloric acid, washed thoroughly with distilled water and dried in the oven at 60 °C to maintain a constant weight.

The pretreated sawdust powder was treated by the purified cellulases from the cultured mutant of *Aspergillus niger* in the fermentation medium. Briefly, 50 g of dried powder was suspended in enzyme liquid to 200 mg/mL containing respectively endoglucanase, exoglucanase and β-glucosidase at the dosage of 150 U/g substrate. After adjusting to pH 6.5, the solution was incubated at 30 °C for 8 h, with continuously stirring. The samples were analyzed for the reducing sugars after suitable time intervals and we calculated the saccharification values by using the formula as follows.

$$\text{Saccharification (\%)} = \frac{(\text{Grams glucose formed} \times 0.9 \times 100)}{(\text{Grams cellulose added})}$$

Statistical analysis

All treatments in the experiments were

replicated a minimum of three times and all experiments were repeated twice with similar results. Mean values of all data were obtained from triplicate experiment. Significance of differences was evaluated at the $p < 0.05$ level.

RESULTS

Mutagenesis and screening of mutants

The lethality rates of spores exposed to electron and heavy ion beams responding under varying times are illustrated in Fig. 1. The mortality was 100% when *Aspergillus niger* GSICC 60108 spores were respectively exposed to electron beam with 1000 Gy dose, or to heavy ion beam with 150 Gy dose. Fungi in general, especially melanized ones, are highly radioresistant when subjected to high doses of ionizing radiation under experimental conditions (Dadachova and Casadevall 2008). It is reported that the melanin produced by *Aspergillus niger* was defensive against the irradiation (Grossi *et al.* 1998). The current perception of melanin radioprotective properties is that it quenches the cytotoxic short-lived free radicals and thus prevents DNA damage (Dadachova and Casadevall 2008).

The screening of mutants was a two-step process which included preliminary and secondary screening. For the preliminary screening, we isolated the strains by their ability to grow on MCC as the carbon source and colonies with an increased halo diameter were picked up as the putative secretion mutants. Around the cellulase-positive colony, a clear halo would be developed after 30 °C incubation of irradiated spores on the screening medium. Subsequently, the enzyme activities of obtained colonies were detected by the modified microplate-based method.

After the irradiation by the electron beam, 10^3 of surviving *Aspergillus niger* spores at each dose of radiation were inoculated in a 100 mL fermentation medium for the determination of the FPA. It is found that *Aspergillus niger* spores at the dose of 750 Gy gave the highest FPA. Therefore, the spores at this dose were the focus of screening. From the remaining spores at the above dose, we then respectively selected 210 *Aspergillus niger* colonies on the basis of zone of cellulose hydrolysis in the preliminary screening medium. Each of them had a H/C value higher than the parent strain did.

The strain named *Aspergillus niger* EBA 105 showed the H/C value of 3.9, higher than that of the parent strain (1.1) and other mutants. The mutant was further assessed for FPA in a 96-well microplate and exhibited higher FPA (152.5 FPU/mL) than *Aspergillus niger* GSICC 60108 (42.3 FPU/mL) and other colonies. Additionally, it secreted higher levels of endoglucanase, exoglucanase and β-glucosidase than the parental strain (see Table 2). The further mutation of EBA105 led to isolation of 200 mutants capable of giving larger clearance zones on cellulose-Congo red agar media at the dose of 80 Gy. We finally obtained a mutant *Aspergillus niger* CIA 915 with the H/C value of 5.3 and FPA of 431.6 FPU/mL.

Mutation rates of electron and $^{12}C^{6+}$-ion beams

After the irradiation, we randomly selected 1000 colonies from the PDA plates and then measured the FPA by the microplate-based method. The positive or negative mutant was defined as its H/C value higher or lower than 10% of that of the parent strain. Table 3 and Table 4 contain the mutation rates of electron and $^{12}C^{6+}$-beams at different radiation doses, respectively.

In the Table 3, the highest positive mutation rate was found at the sublethal dose. With the irradiation dose increasing, the positive and nonsense mutation rates of *Aspergillus niger* GSICC 60108 spores increased and the negative

Table 1. The primer sequences for PCR amplification of cellulase genes.

Cellulase gene	Primer sequence
Endoglucanase	5'-TTCGTTGATCGAGCAGTCGTAGCG-3' (forward)
	5'-TGTGACGTCAGTCAGTGCGCT-3' (reverse)
Exoglucanase	5'-TTGCCACTGCCAATGCTCAGC-3' (forward)
	5'-GTCGGACCAGTCCAGCCCTGT-3' (reverse)
β-glucosidase	5'-ACGGTCACTTTGTGGCGCTC-3' (forward)
	5'-AGGGGCTCGACGTGTGGGTG-3' (reverse)

mutation rate showed steady downward trend. The "down-up-down" pattern tendency (also was called "saddle" shape) was not observed and differed from exponential relationships caused by microwave and ultraviolet irradiation (Yongquan *et al.* 2003). It is indicated that the interaction of electron beam and biological material is complicated. Despite the extensive researches were conducted, the underlying mechanisms have not yet been clarified.

The mutation rates induced by the $^{12}C^{6+}$-ion beam implantation are illustrated in the Table 4. The highest positive mutation ratesof *Aspergillus niger* EBA 105 spore was achieved when the implantation dose was 80 Gy. The implantation dose of wide mutation spectrum fit the "saddle" model. We found that the positive mutation rates increased with the irradiation dose increasing. However, they tended asymptotically towards the maximum value, then, decreased rapidly with the continuously increased irradiation dose. Perhaps the reason for this may be due to the occurrence of reversible mutation after the low-dose ion implantation. Furthermore, the ionic etching and dilapidation of biomembrane by the high-dose ion implantation would lead to higher total mutation rates and lower positive mutation rates (Wang *et al.* 2007). Additionally, the positive mutation rate induced by the $^{12}C^{6+}$-ion beam implantation was much higher than that resulted from the electron beam and we found the electron beam has demonstrated much greater deactivation effect of the spores. One the one hand, the strains were first subjected to electron beam and subsequently with $^{12}C^{6+}$-ion beam, so it is an expected result that the second mutagenesis treatment on already once mutagenized spores is more severe. On the other hand, the relative biological effectiveness of heavy ion radiation is higher than that of electron beam. The mutation rates have been investigated using visible known *Arabidopsis* mutant phenotypes, indicating that the mutation frequencies induced by carbon ions were 20 fold higher than by electron. Novel mutants and their responsible genes, such as ultraviolet light, gamma-ray and X-ray resistant organisms, could be induced by ion beams (Tanaka *et al.* 2010).

Cellulase activities and hereditary stability of the mutant

We selected the mutated strain colony

Table 2. Enzyme activities and purification steps of cellulases isolated from *Aspergillus niger* strains

Fraction	Strain NO.	Protein concentration (mg/mL)	FPA (IU/mL)	Exoglucanase (IU/mL)	Endoglucanase (IU/mL)	β-glucosidase (IU/mL)	Yield (%)
Crude enzyme solution	GSICC 60108	11.3±0.2	8.3±0.3	9.1±0.3	28.2±1.3	13.5±0.5	100
	EBA 105	16.4±0.7	22.5±0.8	29.6±1.2	91.4±4.3	35.3±1.5	100
	CIA 915	22.8±0.4	73.6±3.7	106.2±4.5	310.8±11.5	95.9±4.3	100
HIC and Sephadex G-75 columns	CIA 915	1.4±0.1	25.8±1.2	0	0	121.5±4.7	43.5±2.1
Anion exchange columnand ultrafilter membrane	CIA 915	108.6±2.4	2006.7±36.7	0	1893.8±53.2	0	36.6±1.2
Cation exchange and HIC columns	CIA 915	76.5±2.1	1413.5±18.8	847.5±28.2	0	0	23.4±1.4

Aspergillus niger CIA 915 with the highest H/C ratio (H/C>1.5) and enzyme activities by the compound mutagenesis. The data of enzyme activities we summarized were in Table 5. After a studied period of 9 generations, the results indicate that cellulase production had remained fairly stable with variation coefficients not exceeding 5% and the growth and morphological change had not been observed (data not shown). Thus, we could take the mutant as a steady and inheritable one.

Cellulase production with optimized conditions

The statistic results showed that there were extremely significant differences of cellulase activities between different culture conditions such as culture time, temperature and pH value ($P < 0.01$).

Fig. 2 summarizes the optimum pH, temperature and culture time of *Aspergillus niger* CIA 915. We found the cellulase enzymes excreted by the fungus where was cultured in the fermentation medium, exhibited extremely significant maximum activity at the optimum conditions. The curves follow bell-shaped profiles, suggesting that the culture time, temperature and pH would be 5 days, 30 °C and 4.5.

In studying the effect of temperature on the enzyme activity, the enzyme was found to retain more than 80% of their maximal activity for 100 min at 55 °C and retained no more than 60% of the maximal activities after incubating at 95 °C for 20 min. To see the relationship between temperature and enzyme activity we put the details in Fig. 3.

The fungal strain used in this study showed differential cellulase production and growth according to the medium used. Cellulases were produced in the presence of a variety of carbohydrates such as MCC, CMC and WB. Table 5 shows the specific cellulase activity values of culture supernatants of *Aspergillus niger* CIA 915 fermented in basal media containing different carbon sources. In the Table 5, the enzyme activities of endoglucanase, exoglucanase and β-glucosidase

Table 3. The mutation rates of *Aspergillus niger* GSICC 60108 spores mutated by electron beam irradiation

Irradiation dose of electron beam (Gy)	100	200	300	500	750
Positive mutation rate (%)	2	5	10	8	12
Negative mutation rate (%)	65	55	43	36	31
Nonsense mutation rate (%)	33	40	47	56	57

Table 4. The mutation rates of *Aspergillus niger* EBA 105 spores mutated by $^{12}C^{6+}$-beam irradiation

Irradiation dose of $^{12}C^{6+}$-beam (Gy)	10	20	30	40	50	60	70	80	90	100	125
Positive mutation rate (%)	2	5	4	6	8	10	9	11	7	10	9
Negative mutation rate (%)	12	20	18	16	21	27	19	17	18	25	22
Nonsense mutation rate (%)	86	75	78	78	71	63	72	72	75	65	69

Table 5. Cellulase activities of medium supernatant of *Aspergillus niger* strains fermented in basal media containing different carbon sources

Strain No.	Carbon source	FPA (FPU/mL)	Endoglucanase (IU/mL)	Exoglucanase (IU/mL)	β-glucosidase (IU/mL)
GSICC 60108	CMC	4.6±0.2	18.2±0.8	3.4±0.2	6.3±0.1
	MCC	6.1±0.1	21.3±0.7	6.5±0.2	11.5±0.4
	WB	8.3±0.3	28.2±1.3	9.1±0.3	13.5±0.5
CIA 915	CMC	30.5±1.2	223.7±9.2	65.9±2.7	59.3±2.6
	MCC	61.4±2.3	281.3±8.4	82.5±3.5	74.1±2.8
	WB	73.6±3.7	310.8±11.5	106.2±4.5	95.9±4.3

of *Aspergillus niger* CIA 915 were respectively higher than those of the parental strain. It is found that the maximum enzyme activity was obtained using WB as the carbon source and the cellulase activity increased greatly after the irradiation. In the presence of WB, the most significant induction of cellulase activity was observed, whereas the other carbon sources gave moderate enzyme production extracellularly. This might be some disaccharides and oligosaccharides liberated mostly during the hydrolysis of WB and induced the cellulase activity. Additionally, we also found some mutants exhibited cellulase activity regardless of the substrate used. This could be attributed to the induction mechanism might be blocked after the irradiation. These data reported here indicate that the enzyme activities were improved greatly after the compound irradiation and the optimization of carbon source.

In some yeast strains with biodegrading different media. We found that the fungal strain showed better growth on the medium containing natural cellulose, WB.

The total amount of the filamentous fungus cellulases was affected by the carbon source presented in the medium and regulated directly and/or indirectly by the carbon metabolism regulators (Aro *et al.* 2005). For example, the transcriptional regulator XInR that was identified in *Aspergillus niger* as the transcriptional regulator of xylanase-encoding genes controls the transcription of about 20-30 genes encoding hemicellulases and cellulases (Stricker *et al.* 2008). It appeared to be an important regulator in the intracellular carbon metabolism. The XlnR-binding motif was found in the upstream regulatory regions abilities, cellobiose was found to be the most effective for the production of endoglucanase among the carbon sources. This view has been conformed scientifically by the literature (Thongekkaew *et al.* 2008). In our experiments, the medium containing MCC and CMC yielded higher amounts of cellulase compared to the medium containing easily metabolizable carbon source such as glucose. The observations in the present investigation suggest that the low levels of cellulase are produced in the presence of easily metabolizable carbohydrate. Though, the CMC and MCC, induced higher level of cellulase in *Aspergillus niger* CIA 915. The maximal inducibility of cellulase in the fungus was achieved by using the substrate of WB which can be ascribed to the heterogeneous nature and structural complexity. Furthermore, the differences in the complexity of the carbon sources could also account for the disparity in the growth of the organism in the

Table 6. Distribution of the mutation sites of cellulase-encoding genes in *Aspergillus niger* CIA 915

Cellulase gene	Position	Mutation type	Sequence context	Putative amino acid change
Endoglucanase	209	GT→CA	GGT GGT GAG GGT GCA GAG	Glycine→Alanine
	336	CG→AT	GTA TCG TATG TC GCG TAT	Alanine→Serine
	886	T→C	AGC TCG GGA AGC CCG GGA	Serine→Proline
Exoglucanase	591	A→T	AGC CAA GTA AGC CAT GTA	Glutamine→Histidine
	683	TT→AA	AAG CTT ATA AAG CAA ATA	Leucine→Glutamine
â-glucosidase	522	G→A	TTT AGG CTT TTT AGA CTT	Arginine→Arginine
	1286	G→C	GTG GCC CGT GTC GGC CGT	Glycine→Alanine
	1541	A→T	CCT TAT GGT CCT TTT GGT	Tyrosine→Phenylalanine
	2178	CCC→AAA	CGC CCC AGG CGA AAC AGG	Arginine→Arginine Proline→Asparagine

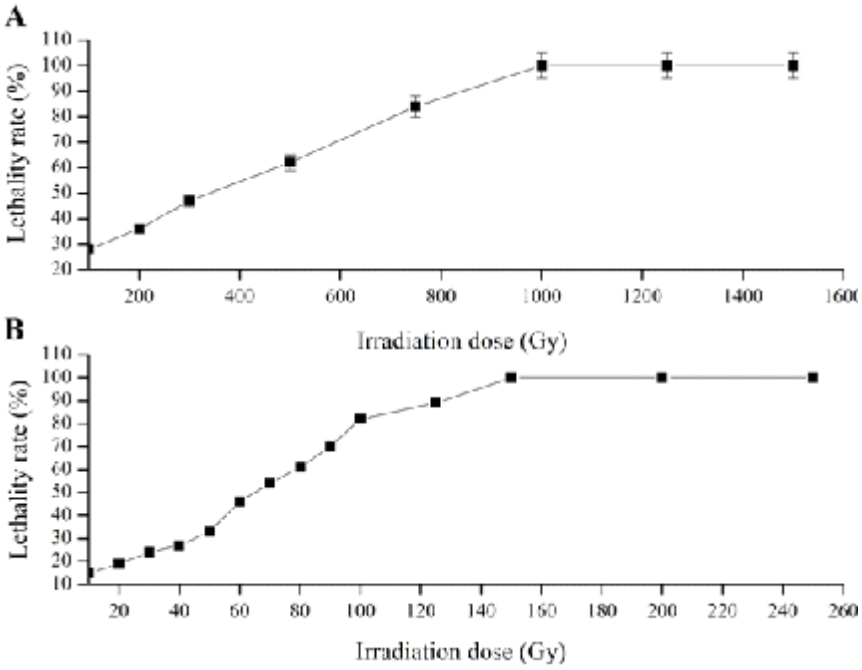

Fig. 1. The lethality rates of spores exposed to electron (A) and $^{12}C^{6+}$-ion (B) beams with varied irradiation doses

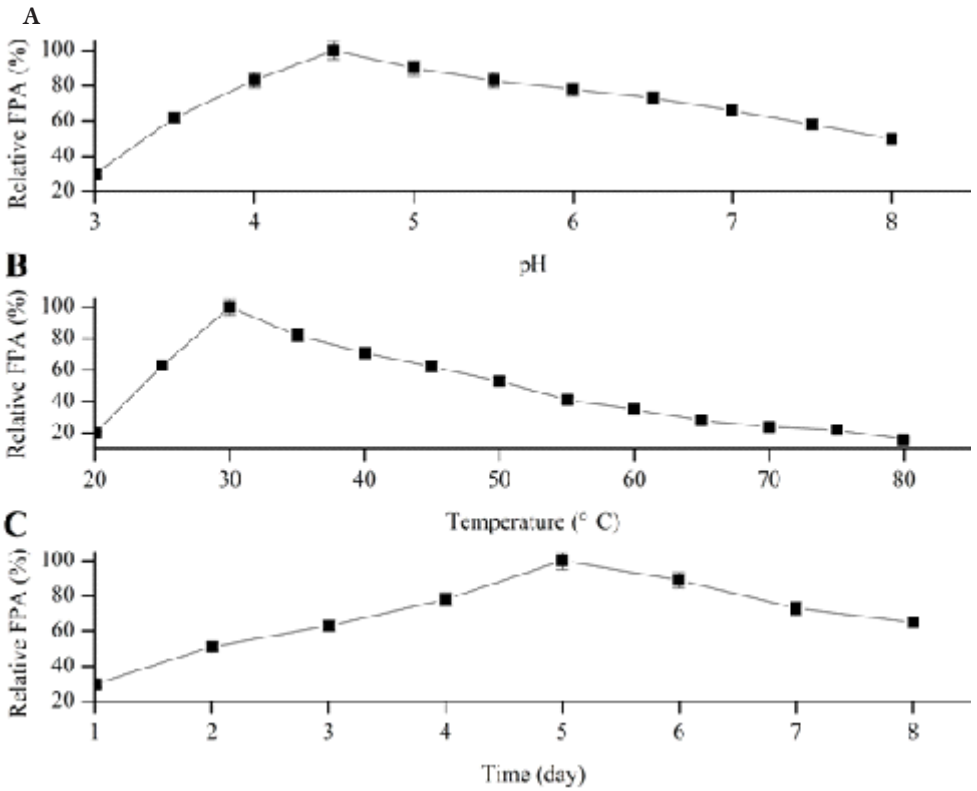

Fig. 2. Effects of pH, temperature and culture time on FPA of *Aspergillus niger* CIA 915. A, effect of pH; B, effect of temperature; C, effect of culture time

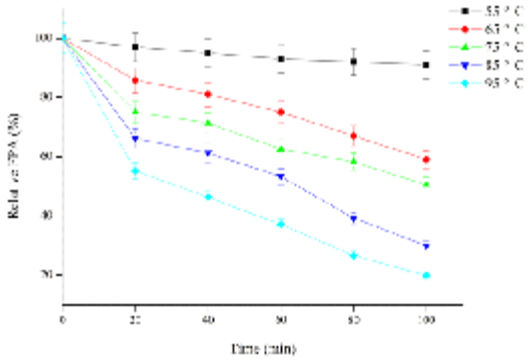

Fig. 3. Thermotolerance of cellulases from *Aspergillus niger* CIA 915

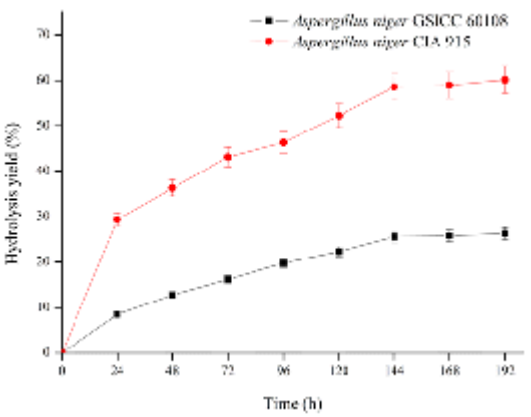

Fig. 4. The hydrolysis of sawdust using enzyme preparations from parent and mutant strains

of all these genes in *Aspergillus niger* that were controlled by XlnR, except for the gene encoding cellobiohydrolase B (Van Peij *et al.* 1998). In addition, some genes involved in carbon catabolism in *Aspergillus niger* have been cloned and sequenced (Drysdale *et al.* 1993).

Determination of mutation sites of cellulase genes

In order to further understand the reason for enhanced cellulase activities, the cellulase genes of the wild strain and mutants were cloned by PCR with primers based on the cellulase coding DNA reference sequences from Genbank. The cellulase genes, including the full-length open reading frame, were sequenced and characterized. After the electron irradiation, we did not find any change of the cellulase genes. Instead, we only found that there were some mutation sites in genes encoding endoglucanase, exoglucanase and β-glucosidase after the $^{12}C^{6+}$-ion irradiation. The distribution and relative information of all mutations of *Aspergillus niger* CIA 915 were summarized in Table 6, which included position, mutation type, sequence context and putative amino acid change. These results indicated that the characteristics of ion beam for mutation induction are high mutation frequency and broad spectrum and therefore, efficient induction of novel mutants.

As shown in the Table 6, the majority of the observed mutations were single base substitutions, while the rest were base-pair and multi-base substitutions in turn. On the other hand, no large and irreparable DNA damage was determined, for which the possible reason was this mutation type might result in production of nonsense, negative or lethal mutation. The mutants had higher activities towards cellulose than the wild type, especially the $^{12}C^{6+}$-ion induced mutants. It is suggested that these mutation sites may be the key regulatory sites for controlling cellulase activities. Furthermore, we conclude that the putative amino acids with more reactive groups and higher polarity were occurred after the $^{12}C^{6+}$-ion irradiation, especially for endoglucanase and exoglucanase. The mutated amino acid residues might affect the protein folding and stability or favored of forming new conformations. Ultimately, these mutations allowed for functional expression and increased the cellulase activities (Ni *et al.* 2010). Many previous studies have used rational design, for instance, site-directed mutagenesis and DNA shuffling, to create strains with higher enzyme activities (Boer *et al.* 2007; Wang *et al.* 2005). Enzymatic protein properties, such as charge, hydrophobicity and percentage of aromatic amino acids can be manipulated to alter their structures and biological functions. Additionally, continuing studies will yield further insight into the relationship between the mutation sites and cellulase properties.

Purification of cellulases

The development of the methodology for purifying the cellulase components from *Aspergillus niger* prompted us to apply these simple methods. After continous culture for 5 days, the components of the cellulolytic system from *Aspergillus niger* CIA 915 were prepared according to the method above. Then, crude protein samples obtained as a cell-free supernatant were

precipitated using ammonium sulfate to 80% saturation and applied to the resin. The major active peaks corresponded to adsorption onto the Sephadex DEAE A-50 column and elution from the column at 0.15 mol/L-0.5 mol/L NaCl solution. Subsequently, we collected the active fractions, concentrated and further purified with HIC and ion-exchange columns. The purified cellulase proteins exhibited multi-fold higher activity than the crude enzyme solution and appeared as single and relatively clear bands on the gel. A summary of the purification is presented in the Table 2.

Saccharification

Cellulases produced by both parent and mutant strains were evaluated for their efficiency of bioconversion or saccharification. The effect of hydrolysis time on reducing sugar production was examined and summarized in Fig. 4. We observed that the saccharification with the cellulase system produced by the mutant strain (60.1%) was greater than that with the cellulases from parent strain (26.3%), which could be due to the improvement of the strain with the irradiations.

DISCUSSION

Mutagenic effects of electron and $^{12}C^{6+}$-ion beams

Ionizing radiation has long been used as mutagens in the fields of genetics and breeding. It has a complementary role or even an advantage in terms of generating varying types of alleles and acquiring mutations with high frequencies. Electron and heavy ion beams can frequently cause DNA alterations such as inversion, translocation, large deletion and point mutation, which result in producing characteristic mutants. But these two beams may cause different types of injuries for microorganisms. We found that the lethality rates of the spores irradiated by electron beam were higher than that treated by the heavy ion beam. It is possible to extrapolate that the resulting injuries may be not only the nucleic acids but also the microbial cell walls, membranes and enzymatic or transport system and so on. The process of induction of mutations after exposure to ionizing radiation is much more complex than that associated with most other mutagens. However, the mutation types are highly dependent on the target cell, the genetic locus studied, and, in some cases, the dose, dose rate and quality of radiation exposure (Schwartz *et al.* 2000). Interestingly, the samples irradiated by the heavy ion beam did not show significant changes in morphology and growth kinetics, but our DNA sequence analysis of mutants further showed that ion beam irradiation frequently caused point mutations. These data suggest that the principal targets of the ion radiation may be the nucleic acids.

It is reasonable to assume that the mutation strategy should include attenuating the strains with electron beam firstly and then creating the novel mutant by the heavy ion irradiation. The effect of the electron radiation was mainly inactivation of the target strains, while the heavy ion radiation was found to induce the point-like mutation. The greatest mutagenic effect was produced following the synergistic use of electron beam with the heavy ion beam. These synergies achieved in the irradiation of electron and heavy ion beams may be due to interaction between these two beams, producing microbicidal and mutagenic radicals or molecules.

For the radiation, the track structure of energy deposition plays an important role in the biological effects caused by charged particles and different biological responses are caused by different particle species even if the linear energy transfer values are similar (Suzuki *et al.* 2003). All biological differences between high and low linear energy transfer radiations arise owing to differences in their ionization tracks (Narang *et al.* 2009). Actually, the relative biological effectiveness (lethality and mutagenicity) depends on the quality of radiation, the dose, dose-rate effects and the biological endpoints studied; the mutation could be due to some physiological and/or irradiation conditions, causing different types of DNA damage generated or different repair modes (Yatagai 2004). Therefore, it is important to provide a quantification of the role played by track structure of charged particle in modulating the induction of important radiobiological damage and producing the novel mutant (Ballarini *et al.* 2008).

Mutant screening method

The screening strategy is a critical step for finding the desired mutants from a large mutant library. Moreover, efficient screening method is a prerequisite. We performed a typical facilitated screening method on solid agar that relied on product solubilization followed by an enzymatic

reaction that gave rise to a zone to identify. However, plate-screening method using Congo red is not quantitative or sensitive enough due to poor correlation between enzyme activity and halo size. So the secondary screening is necessary to validate primary screen findings. Furthermore, this method is tedious and zones of hydrolysis are sometimes not easily discernable (Maki *et al.* 2009). In view of these facts, some improved high-throughput mutant screening methods have been studied and published (Decker *et al.* 2003; Fia *et al.* 2005; Kasana *et al.* 2008). The next step for the research team will be improving the current method or designing a new one to screen or select cellulase mutants more efficiently and effectively.

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REFERENCES

1. Adsul, M.G., Bastawde, K.B., Varma, A.J., Gokhale, D.V. Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. *Bioresour. Technol.*, 2007; **98**(7): 1467-1473.
2. Aro, N., Pakula, T., Penttila, M. Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol. Rev.*, 2005; **29**(4): 719-739.
3. Ballarini, F., Alloni, D., Facoetti, A., Ottolenghi, A. Heavy-ion effects: from track structure to DNA and chromosome damage. *New J. Phys.*, 2008; **10**(7): 1-17.
4. Boer, H., Simolin, H., Cottaz, S., Söderlund, H., Koivula, A. Heterologous expression and site-directed mutagenesis studies of two *Trichoderma harzianum* chitinases, Chit33 and Chit42, in *Escherichia coli*. *Protein Expr. Purif.*, 2007; **51**(2): 216-226.
5. Chand, P., Aruna, A., Maqsood, A.M., Rao, L.V. Novel mutation method for increased cellulase production. *J. Appl. Microbiol.*, 2005; **98**(2): 318-323.
6. Dadachova, E., Casadevall, A. Ionizing radiation: how fungi cope, adapt, and exploit with the help of melanin. *Curr. Opin. Microbiol.*, 2008; **11**(6): 525-531.
7. Damisa, D., Ameh, J.B., Egbe, N.E.L. Cellulase production by native *Aspergillus niger* obtained from soil environments. *World Journal of Ferm. Tech. & Bioeng.*, 2011; **1**(3): 62-70.
8. De Vries, R.P., Visser, J. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Mol. Biol. Rev.*, 2001; **65**(4): 497-522
9. Decker, S.R., Adney, W.S., Jennings, E., Vinzant, T.B., Himmel, M.E. Automated filter paper assay for determination of cellulase activity. *Appl. Biochem. Biotechnol.*, 2003; **107**(1): 689-703.
10. Drysdale, M.R., Kolze, S.E., Kelly, J.M. The *Aspergillus niger* carbon catabolite repressor encoding gene, creA. *Gene*, 1993; **130**(2): 241-245.
11. Eveleigh, D.E. Cellulase: a perspective. *Philos. Trans. R. Soc. London, Ser. A*, 1987; **321**(1561): 435-447.
12. Fia, G., Giovani, G., Rosi, I. Study of beta-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. *J. Appl. Microbiol.*, 2005; **99**(3): 509-517.
13. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.*, 1987; **59**: 257-268.
14. Gokhale, D.V., Puntambekar, U.S., Deobagkar, D.N., Peberdy, J.F. Production of cellulolytic enzymes by mutants of *Aspergillus niger* NCIM 1207. *Enzyme. Microb. Technol.*, 1988; **10**(7): 442-445.
15. Grossi, G.F., Durante, M., Gialanella, G., Pugliese, M., Mosse, I. Effects of melanin on high- and low- linear energy transfer (LET) radiation response of human epithelial cells. *Radiat. Environ. Biophys.*, 1998; **37**(1): 63-67.
16. Hu, G., Fan, Y., Zhang, L., Yuan, C., Wang, J., Li, W., Hu, Q., Li, F. Enhanced lipid productivity and photosynthesis efficiency in a *Desmodesmus* sp. mutant induced by heavy carbon ions. *PLoS One*, 2013; **8**(4): 1-8.
17. Kasana, R., Salwan, R., Dhar, H., Dutt, S., Gulati, A. A rapid and easy method for the

detection of microbial cellulases on agar plates using Gram's iodine. *Curr. Microbiol.*, 2008; **57**(5): 503-507.

18. Kovács, K., Szakacs, G., Zacchi, G. Comparative enzymatic hydrolysis of pretreated spruce by supernatants, whole fermentation broths and washed mycelia of *Trichoderma reesei* and *Trichoderma atroviride*. *Bioresour. Technol.*, 2009; **100**(3): 1350-1357.
19. Li, X., Yang, H., Roy, B., Park, E.Y., Jiang, L., Wang, D., Miao, Y. Enhanced cellulase production of the *Trichoderma viride* mutated by microwave and ultraviolet. *Microbiol. Res.*, 2010; **165**(3): 190-198.
20. Liu, Y., Xuan, S., Long, C., Long, M., Hu, Z. Screening, Identifying of cellulose-decomposing strain L-06 and its enzyme-producing Conditions. *Chin. J. Biotechnol.*, 2008; **24**(6): 1112-1116.
21. Maki, M., Leung, K.T., Qin, W. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int. J. Biol. Sci.*, 2009; **5**(5): 500-516.
22. Najafi, N., Ahmadi, A.-R., Hosseini, R., Golkhoo, S. Gamma irradiation as a useful tool for the isolation of astaxanthin-overproducing mutant strains of *Phaffia rhodozyma*. *Can. J. Microbiol.*, 2011; **57**(9): 730-734.
23. Narang, H., Bhat, N., Gupta, S., Santra, S., Choudhary, R., Kailash, S., Krishna, M. Differential activation of mitogen-activated protein kinases following high and low LET radiation in murine macrophage cell line. *Mol. Cell. Biochem.*, 2009; **324**(1): 85-91.
24. Ni, J., Takehara, M., Watanabe, H. Identification of activity related amino acid mutations of a GH9 termite cellulase. *Bioresour. Technol.*, 2010; **101**(16): 6438-6443.
25. Okamura, M., Yasuno, N., Ohtsuka, M., Tanaka, A., Shikazono, N., Hase, Y. Wide variety of flower-color and -shape mutants regenerated from leaf cultures irradiated with ion beams. *Nucl. Instrum. Methods Phys. Res. Sect. B*, 2003; **206**: 574-578.
26. Pinelo, M., Zornoza, B., Meyer, A.S. Selective release of phenols from apple skin: Mass transfer kinetics during solvent and enzyme-assisted extraction. *Sep. Purif. Technol.*, 2008; **63**(3): 620-627.
27. Schwartz, D.P. Methods for the isolation and characterization of constituents of natural products : XXI. Use of a celite-potassium methylate column for rapid preparation of methyl esters from microgram amounts of glycerides. *Microchem. J.*, 1977; **22**(4): 457-462.
28. Schwartz, J.L., Jordan, R., Sun, J., Ma, H., Hsie, A.W. Dose-dependent changes in the spectrum of mutations induced by ionizing radiation. *Radiat. Res.*, 2000; **153**(3): 312-317.
29. Sorensen, A., Luebeck, P.S., Luebeck, M., Teller, P.J., Ahring, B.K. Beta-glucosidases from a new *Aspergillus* species can substitute commercial beta-glucosidases for saccharification of lignocellulosic biomass. *Can. J. Microbiol.*, 2011; **57**(8): 638-650.
30. Stricker, A.R., Mach, R.L., de Graaff, L.H. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). *Appl. Microbiol. Biotechnol.*, 2008; **78**(2): 211-220.
31. Suzuki, M., Tsuruoka, C., Kanai, T., Kato, T., Yatagai, F., Watanabe, M. Qualitative and quantitative difference in mutation induction between carbon- and neon-ion beams in normal human cells. *Biol. Sci. Space*, 2003; **17**(4): 302-306.
32. Tanaka, A., Shikazono, N., Hase, Y. Studies on biological effects of ion beams on lethality, molecular nature of mutation, mutation rate, and spectrum of mutation phenotype for mutation breeding in higher plants. *J. Radiat. Res.*, 2010; **51**(3): 223-233.
33. Thongekkaew, J., Ikeda, H., Masaki, K., Iefuji, H. An acidic and thermostable carboxymethyl cellulase from the yeast *Cryptococcus* sp. S-2: Purification, characterization and improvement of its recombinant enzyme production by high cell-density fermentation of *Pichia pastoris*. *Protein Expr. Purif.*, 2008; **60**(2): 140-146.
34. Van Peij, N.N.M.E., Gielkens, M.M.C., De Vries, R.P., Visser, J., De Graaff, L.H. The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. *Appl. Environ. Microbiol.*, 1998; **64**(10): 3615-3619.
35. Wang, L.S., Cai, K.Z., Cheng, M.J., Chen, L.J., Liu, X.L., Zhang, S.Q., Yu, Z.L. Damaging effect of low energy N^+ implantation on *Aspergillus niger* spores. *Plasma Sci. Technol.*, 2007; **9**(3): 307-311.
36. Wang, T., Liu, X., Yu, Q., Zhang, X., Qu, Y., Gao, P., Wang, T. Directed evolution for engineering pH profile of endoglucanase III from *Trichoderma reesei*. *Biomol. Eng.*, 2005; **22**(1-3): 89-94.
37. Xiao, Z., Storms, R., Tsang, A. Microplate-based filter paper assay to measure total cellulase activity. *Biotechnol. Bioeng.*, 2004; **88**(7): 832-837.
38. Yamaguchi, H., Nagatomi, S., Morishita, T., Degi, K., Tanaka, A., Shikazono, N., Hase, Y.

Mutation induced with ion beam irradiation in rose. *Nucl. Instrum. Methods Phys. Res. Sect. B*, 2003; **206**: 561-564.

39. Yatagai, F. Mutations induced by heavy charged particles. *Biol. Sci. Space*, 2004; **18**(4): 224-234.
40. Yongquan, L., Shifei, C., Peilin, C. Mutagenesis and screening of high yield xylanase production strain of *Aspergillus usamii* by microwave irradiation. *Chin. J. Chem. Eng.*, 2003; **11**(05): 594-597.
41. Yoshihara, R., Nozawa, S., Hase, Y., Narumi, I., Hidema, J., Sakamoto, A.N. Mutational effects of gamma-rays and carbon ion beams on *Arabidopsis* seedlings. *J. Radiat. Res.*, 2013; **54**(6): 1050-1056.
42. Zhang, Q., Fu, Y., Wang, Y., Han, J., Lv, J., Wang, S. Improved ethanol production of a newly isolated thermotolerant *Saccharomyces cerevisiae* strain after high-energy-pulse-electron beam. *J. Appl. Microbiol.*, 2012; **112**(2): 280-288.
43. Zhou, J., Wang, Y.H., Chu, J., Zhuang, Y.P., Zhang, S.L., Yin, P. Identification and purification of the main components of cellulases from a mutant strain of *Trichoderma viride* T 100-14. *Bioresour. Technol.*, 2008; **99**(15): 6826-6833.
44. Zhou, L., Li, W., Yu, L., Li, P., Li, Q., Ma, S., Dong, X., Zhou, G., Leloup, C. Linear energy transfer dependence of the effects of carbon ion beams on adventitious shoot regeneration from *in vitro* leaf explants of *Saintpaulia ionahta*. *Int. J. Radiat. Biol.*, 2006; **82**(7): 473-481.