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Effects of nitrogen ion irradiation on endoglucanase activity and gene mutation of *Bacillus subtilis* Bac01

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Abstract *Bacillus subtilis* Bac01 was mutated by 15 keV N^+ ions of 1.5×10^{16} cm^{-2}. The mutant strain Bac11 with high yield of endoglucanase was isolated using carboxymethylcellulose sodium and congo red indicative plates. It exhibited higher endoglucanase activitiy (381.89IU) than the original strain Bac01 (93.33IU). Two 1,500 bp endoglucanase gene fragments were obtained with PCR amplification from *B. subtilis* Bac01 and mutant strain Bac11. BLAST comparison result indicated that 10 nucleotides mutated. Bioinformatics methods were used to analyze the two predicted amino acid sequences, and it was found that 5 amino acid residues changed, being all in the cellulose-binding domain of endoglucanase.

Key words Ion irradiation, *Bacillus subtilis*, Endo-1,4-*β*-D-glucanase, Activity, Gene mutation

1 Introduction

Cellulose, the world's amplest renewable biomass resource, is degraded by microorganisms that produce cellulases for efficient hydrolysis of cellulose. This requires a synergism of three types of cellulases. Endoglucanases (endo-1,4-*β*-D-glucanases), being responsible mainly for hydrolyzing the internal non-crystalline cellulose by randomly cleaving *β*-1,4-glycosidic bonds and decreasing the length of the cellulose chains, produces plenty of low molecular weight cellulose with non-reducible ends. Cellobio-hydrolases (exo-1,4-*β*-glucanases) are responsible for hydrolyzing the end of linear cellulose molecules by cleaving *β*-1,4-glycosidic bonds and removing one cellobiose from the cellulose chains each time. And *β*-1,4-glucosidases convert cellobiose to glucose. Fungi secrete extracellular free cellulose to hydrolyze cellulose with mechanisms of hydrolase and oxidase. However, bacteria produce low level cellulase, which is mainly endoglucanase. Most of bacterial cellulases cannot be secreted extracellularly, neither can they hydrolyze crystalline cellulose. Further investigations are needed to improve the capability of bacteria to produce cellulases, consisting of the three essential components, for degrading natural cellulose more efficiently[1,2].

Since Hermann Muller[3] proved that X-ray irradiation increases mutation rate, radiation mutation has been applied to organism breeding. Various kinds of mutagenic source, i.e. X-rays, γ-rays, UV-rays, laser, neutrons, electrons etc, have been used in breeding various organisms[4]. In 1989, low energy ion beams were used for the first time for plant mutation[5], and this started a new interdisciplinary study, ion beam bioengineering, which has commanded increasing research interest[6], and ion beams have been widely applied in industries and agriculture[7-11].

In this work, N^+ ions were used for obtaining a mutant *Bacillus subtilis* strain of high endoglucanase yield, and the ion beam bioengineering effects on the endoglucanase activity and gene mutation of *B. subtilis* were discussed.

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2 Materials and methods

2.1 Bacterial strains and plasmid

Bacillus subtilis Bac01 was isolated and stored by the Center of Ion Beam Biotechnology, Xinjiang University. *Escherichia coli* TG-1 was provided by Ion Beam Bioengineering Key Lab, Institute of Plasma Physics, Chinese Academy of Sciences. Plasmid pGEM-T was from Promega.

2.2 Reagents

The reagents include carboxymethylcellulose sodium (CMC-Na), dinitrosalicylic acid (DNS), Congo red, ethidium bromide (EB), ampicillin (Amp), DNA polymerase (Takara, Dalian, China), DNA Gel Purification Kit, DNA and Plasmid Extraction Kit (Sangon, Shanghai, China). All chemicals are of reagent grade.

2.3 Media

CMC-Na medium: CMC-Na, 2.0 g/L; $(NH_4)_2SO_4$, 0.5g/L; K_2HPO_4, 0.05 g/L; $MgSO_4{\cdot}7H_2O$, 0.05 g/L; and agar 0.8 g/L.

CMC-Na liquid medium: CMC-Na, 2.0 g/L; $(NH_4)_2SO_4$, 0.5 g/L; K_2HPO_4, 0.05 g/L; and $MgSO_4{\cdot}7H_2O$, 0.05 g/L.

Luria-Bertani (LB) solid medium: peptone, 1.0g/L; yeast extract powder, 0.5 g/L; NaCl, 0.5 g/L; and agar 1.5 g/L.

LB liquid medium: peptone, 1.0 g/L; yeast extract powder, 0.5 g/L; and NaCl, 0.5 g/L.

2.4 Bacteria cell film preparation and ion beam irradiation

Bacteria cells from LB slant medium were diluted to 1.0×10^7 CFU/mL with sterile water, and 0.1 mL of the diluted Bacteria cell was spread in the middle of a sterile 90-mm petri dish with a sterile glass spatula and air dried under aseptic conditions.

The Bacteria cell film was placed on the aseptic sample holder in the target chamber of the IBB Device 1 ion implanter (Institute of Plasma Physics, CAS). The film was irradiated by 15 KeV N^+ ions of 1.5×10^{16} /cm^2 at 10^{-3} Pa. The control is a Bacteria cell film experienced the same vacuum conditions, but no N^+ ion irradiation.

2.5 Mutant strains screening

After the ion treatment, the cells were immediately placed in 2 mL sterile water, which were stirred for 2min with a sterile glass spatula. The eluent was collected and 0.1 mL was spread uniformly on CMC-Na medium plates using a sterile glass spatula. The plates were inverted and cultured for 48 h at 28–30°C. The control cultured with the same way.

The single survival colonies were numbered and inoculated on two petri dishes containing CMC-Na medium, and inverted and cultured for 48 h at 28–30°C. One plate was stained with 0.1% Congo red for 30 min and washed with 1.0 mol/L NaCl for 5 min. After selecting mutant strains with a larger transparent zone than control, the positive strains were inoculated on slant CMC-Na medium and cultured for 48 h at 28–30°C [12].

2.6 Preparation of crude endoglucanase enzyme complex and endoglucanase activity assay

Each positive strain was inoculated into CMC-Na liquid medium and incubated at 28–30°C on a rotary shaker at 200 r/min for 48 h. At the end of the incubation period, all culture broth were diluted to the same optical absorbance at 600 nm with CMC-Na medium, and then were placed on ice and disaggregated by ultrasonic cell disrupter system (Ningbo Rongshun Instrument, Zhejiang, China) at 20 kHz and 120 W. Lysates were centrifuged at 8,000 g for 15 min to remove the cell debris.

Enzyme activity was determined according to the rate of reduced sugar production from the substrate CMC-Na. The reduced sugar was measured and quantified by DNS method[13] with glucose as the standard. A standard enzyme activity assay was performed in a 2.0 mL reaction mixture containing 1.5mL 1% CMC-Na (w/v) in phosphate buffer (pH 6.6) and 0.5 mL properly diluted enzyme solutions (the control containing 0.5 mL distilled water). After incubation at 40℃ water bath for 30 min, the reaction mixture was mixed with a 2-fold volume of DNS reagent and boiled for 5 min, and was quickly cooled down with running water and fixed to a volume of 25mL with distilled water. The optical absorbance was recorded at 540 nm, using a UV-spectrophotometer

(Prism Spectrum Instruments Ltd., Shanghai, China). One unit of endoglucanase activity was defined as the amount of endoglucanase that released 1 μmol of reduced sugar equivalents from substrate per minute.

2.7 Cloning of endoglucanase gene, DNA sequencing and analysis

The genomic DNA of mutant strain and original were extracted using DNA Extraction Kit. The open reading frame of endoglucanase gene was amplified from mutant strain and original genomic DNA using a pair of primers: P_1, (5'-GATATGAAACGGTCAATCTC-3') and P_2, (5'-ACTAATTTGGTTCTGWTCCC-3').

Reactions were conducted in a reaction mixture of 50 μL, containing 100 ng of template DNA, 8 μL of 2.5 mmol/L dNTP, 1 μL of 20 μmol/L P_1, 1 μL of 20 μmol/L P_2, 5 μL of 10×buffer, and 0.5 μL of 5 U/μL LA Taq polymerase. The initial denaturation was 3min at 94°C, followed by 35 cycles of 50 s at 94°C, 50 s at 51°C, 80 s at 72°C. PCR products were loaded into agarose gel prepared with 1.0% agarose at 5 V/cm, stained with 0.25 μg/mL EB and photographed over UV light.

The PCR products were purified using the DNA Gel Purification Kit and cloned into pGEM-T Vector by T/A cloning, and then transformed into competent *Escherichia coli* (strain TG-l) using chemical transformation method. Based on blue/white selection, individual clones were picked to screen the positive clones with inserted DNA fragments. DNA sequencing was performed by the Invitrogen sequencing company (Shanghai, China). Similarity searches on nucleotide and amino acid sequences were carried out using BLAST at the NCBI GenBank database[14].

3 Results

3.1 Screening mutated strains Bac11 with high yield of endoglucanase

When CMC bond with Congo red, red color appears, and no decoloration by NaCl solution can be observed. The small molecular oligosaccharides, a product of CMC hydrolysis, however cannot bond with Congo red. Therefore, the colonies with the endoglucanase activity, which hydrolyze CMC, could be examined with a transparent zone after decoloring by NaCl solution.

The N^+ ion treated *B. subtilis* Bac01 cells with transparent zone larger than that of control was inoculated on CMC-Na slant medium and cultured at 28–30°C for 28 h. The mutant strain was transferred from slant medium into CMC-Na liquid medium and cultured on a rotary shaker at 200 r/min at 28–30°C for 48 h. After endoglucanase activity was assayed, the mutant strain Bac06 was obtained; comparing with the original strain, the endoglucanase activity of Bac06 increased by 189.27%. From the mutant strain Bac06 treated by N^+ ions at the same dose, mutant strain Bac09 was obtained, with an endoglucanase activity of 266.56% greater than Bac01. And from mutant strain Bac09 treated by N^+ ions at the same dose, mutant strain Bac11 was obtained, with an endoglucanase activity of 309.18% greater than Bac01(Fig.1).

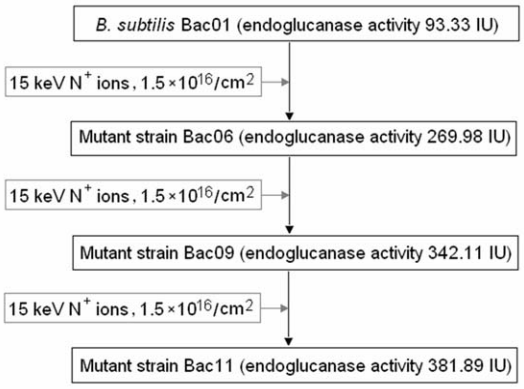

Fig.1 Pedigree chart of mutant strain Bac11with high yield of endoglucanase activity

The Bac01 treated with 15 keV N^+ ions of $1.5\times10^{16}/cm^2$, i.e. Bac06, showed a 2.89 times increase of endoglucanase activity. Although the biological effect is not so significant for further treatments of the N^+ ions, with an endoglucanase activity increase of 26.72% and 11.63% for mutant strain Bac09 and Bac11, respectively, the accumulative increase of endoglucanase activity of Bac11 is 4.09 times higher than Bac01.

3.2 BLAST comparison of the nucleotide sequence of endoglucanase

The P_1 and P_2 primers were designed according to the consensus sequence of endoglucanase from *B. subtilis*. A fragment of 1,500 bp was amplified by PCR using

P_1 and P_2 based on genomic DNA of *B. subtilis* Bac01 and mutant strain Bac11 as templates (Fig.2).

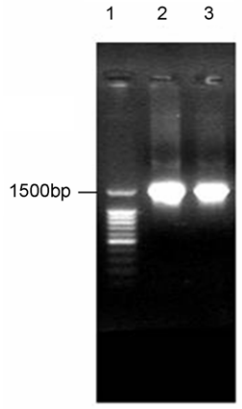

Fig.2 PCR amplification of endoglucanase gene with genomic DNA of *B. subtilis* Bac01 and mutant strain Bac11 as template. Lane 1: DNA Marker; Lane 2: *B. subtilis* Bac01 genomic DNA; Lane 3: the mutant strain Bac11 genomic DNA.

The PCR products were purified and cloned into pGEM-T vector by T/A cloning, and then transformed into competent *Escherichia coli* TG-l. Based on blue/white selection, the positive clones with inserted DNA fragments were obtained by PCR using P_1 and P_2. Two 1,500 bp fragments were amplified with primers P_1 and P_2 from *B. subtilis* Bac01 and mutant strain Bac11. Sequencing and BLAST comparison of the two nucleotide sequences revealed that 10 nucleotides have changed, which were induced by nitrogen ion irradiation The multiple sequence alignment was done using the multiple sequence alignment editor (Fig.3).

3.3 BLAST comparison of the amino acid sequence of endoglucanase

Based on the two 1,500 bp fragments of endoglucanase from *B. subtilis* Bac01 and mutant strain Bac11, the fragments contained one complete open reading frame (ORF) without intron, encoding for 499 amino acid polypeptide. Computer analysis suggested that the amino acid sequence from Met 1 to Arg 330 might be the catalytic domain of the endo-1,4-*β*-D-glucanase from *B. subtilis* Bac01 [15]. BLAST comparison of the two amino acid sequences revealed that 5 different amino acid residues had changed, which were all in the cellulose-binding domain (CBD) of endoglucanase. The multiple sequence alignment was done using the multiple sequence alignment editor (Fig.4).

4 Discussion

Bacillus subtilis was irradiated repeatedly by low-energy nitrogen ions and the mutant strain Bac11 was isolated, the endoglucanase activity of which was improved significantly comparing with that of the original one.

With DNA sequences analysis and comparison of endoglucanase between the original strain and mutant strain, it shows that 10 nucleotides of endoglucanase gene mutated, some of which could cause relatively changes in amino acid sequence. Amino acids are the framework of protein, and the change of some crucial amino acid residues could affect the structure and function of entire protein molecule. For endoglucanase gene of mutant strain Bac11, the mutation probably could change the catalytic activity and stability of enzyme molecule, which affects indirectly the activity of endoglucanase. In addition, ion irradiation may act on the regulatory region of endoglucanase gene and then affect the activity. The investigations on mutated endoglucanase and its regulatory region would explain further the internal mechanism about the difference in endoglucanase activity between original and mutant strains induced with ion irradiation.

Many researches have suggested a “down-up-down” survival-rate-to-dose relationship in many microbes irradiated by ions. This differs from the traditional mutagen irradiation with UV-ray and γ-ray[16]. A possible explanation is that the interaction between low-energy ions and complicated organisms is more complex than that of traditional mutagen and organisms. The mechanism for the interaction between low-energy ions and the organisms was characterized by energy deposition, momentum transferring, mass deposition and charge exchange and/or neutralization, whereas other ionizing radiations caused effect of energy deposition[17,18].

At the beginning of ion irradiation, most ions reach the cytoplasm and indirect induction of nucleolus damage, which can directly lead to death of cell. As the fluence of ion beam increases, more energy and mass deposition in the nucleolus is likely to be the main factor that caused cellar damage, as well as activated cell repair system. No other than the activation process could bring an increase of survival

rate. With the continual increase of ion beam fluence, non-repairable damage little by little appeared as a great deal of energy and mass deposited in the whole cell. As a result, the survival rate gradually decreased with the increase of ion beam fluence. The specific biological effects that possibly lead to mutation effects of ion irradiation were of remarkable mutagenic efficiency and broad mutation spectrum. The reason causing higher mutation rate and wider mutation spectrum may be that the multifactor effect leads to complex biological effects in the treated organism in the collision system[4], though the mechanism concerning mutation caused by ion beams is yet to be completely understood.

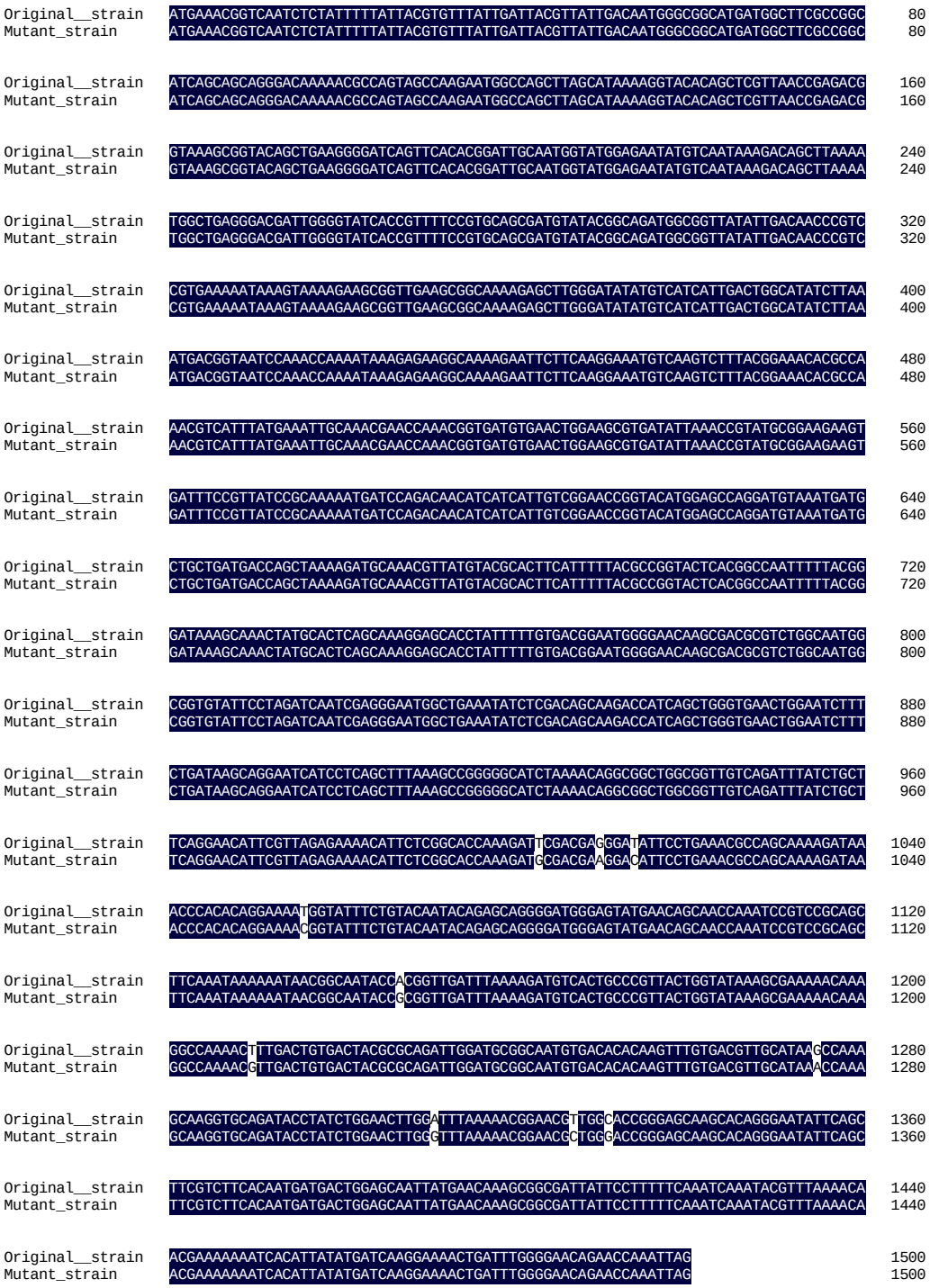

Fig.3 Multiple alignment of original strain Bac01and mutant strain Bac11 nucleotide sequences.

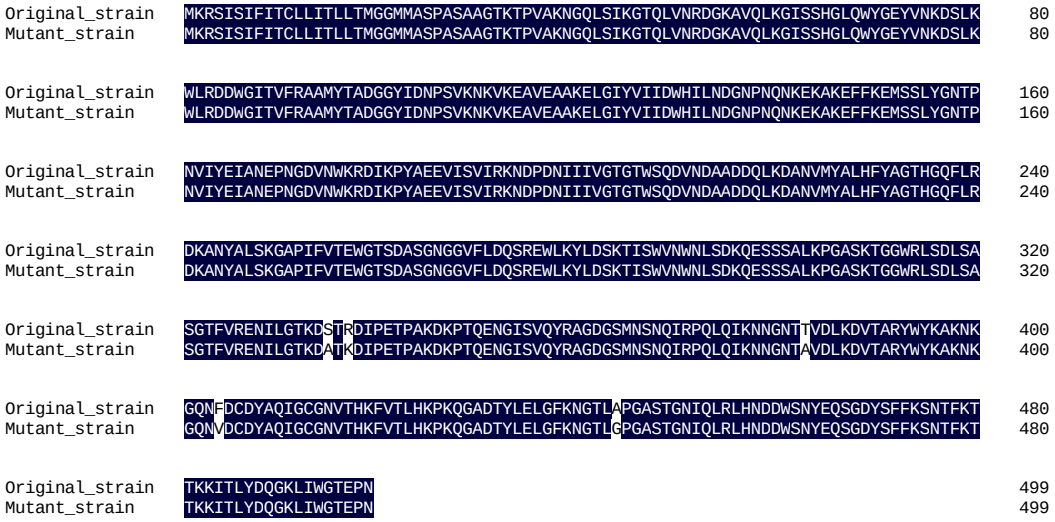

Fig. 4 Multiple alignment of original strain Bac01and mutant strain Bac11 amino acid sequences.

Typically, bacterial and fungal cellulases consist of at least a cellulose-binding domain and a catalytic domain. The domains act independently. CBDs endow the enzymes with high affinity for native and ordered cellulose, while the catalytic domain performs hydrolysis of substrates.

Even though there is general function similarity among all CBDs, affinity and reversibility of cellulose binding vary[19]. For example, activity of *Humicola grisea* cellobiohydrolase 1 (CBH1) on crystalline cellulose is high due to its high cellulose-binding capability and related directly to the composition and configuration of the three aromatic residues[20]. CBDs are classified into 54 families[15]. Computer analysis showed that endo-1,4-*β*-D-glucanase from *B. subtilis* Bac01contained a putative family 3 CBD and amino acid sequence of the catalytic domain is from Met 1 to Arg 330. Our BLAST comparison of the two amino acid sequences revealed 5 amino acids, being all in the CBD of endoglucanase. The relationship between the mutated amino acid residues and the mechanism of high enzyme activity need to be demonstrated further.

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