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ORIGINAL PAPER

Mutation breeding of chitosanase-producing strain *Bacillus* sp. S65 by low-energy ion implantation

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Abstract In order to obtain an industrial strain with higher chitosanase yield, the wild strain *Bacillus* sp. S65 cells were mutated by a novel mutagen, nitrogen ion beam, with energy of 15 keV and dose ranging from 2.6×10^{14} to 5.2×10^{15} ions/cm^2. One mutant, s65F5 with high yield of chitosanase was isolated. Results showed that the production of chitosanase of s65F5 was dramatically increased from 4.1 U/ml in s65 to 25 U/ml by ion beam implantation, while the fermentation time was shortened from 72 to 56 h, both of which greatly increased efficiency and reduced the cost of industrial production. Besides, the mutagenic effects of low-energy ion beam on survival rate showed characteristic down–up–down pattern, which was different from the traditional mutagens such as UV and γ-ray and the possible mutation mechanism was discussed.

Keywords Low energy N^+ ion beam implantation · Mutation screen · Chitosanase · Chitooligomers

Introduction

Hermann Muller, the father of radiation genetics, performed his pioneering work in 1927, which proved that X-ray radiation could increase mutation rate [14]. After the ground breaking work of Muller, a great number of studies on the high-energy physical radiation have been carried out using a variety of mutagenic sources, such as γ-rays, ultraviolet, alpha particles, beta particles, neutron, etc. Low-energy heavy ions are the atoms deprived of some or all electrons, whose energy are lower than 100 keV and the protonic number is more than 2. Due to the short penetrating ranges of low-energy ions [3], their mutation effect was questioned and ignored. In the mid-1980s, the mutation effect induced by low-energy ion implantation was observed in rice seeds in our lab. A program of mutation breeding for rice by low-energy ion beam then was performed in Institute of Plasma Physics, collaborating with Institute of Rice of China in 1986[36]. Through exploring the mutagenic effect for years, it was found that this approach possesses a higher mutation rate and a wider mutational spectrum with a higher survival rate [34]. Since then, a lot of scientists devoted themselves to this interdisciplinary field of ion beam biotechnology. Researchers in the laboratory of Ion Beam Bioengineering, Chinese Academy of Sciences, are engaged in research on interactions between low-energy ions and the organism. Ample proof demonstrated that ion beam mutation had a wide range of biological effects, including extracellular and intracellular DNA damage [4, 39], amino acid and nucleotide decomposition in aqueous solution [21], inactivation of viruses, microbes [5] and aberrant chromosomes in wheat [30]. Experimental results also showed that ion beams had a very high linear energy transfer (LET) and relative biological effectiveness (RBE) compared with γ-ray or high energy particles. Furthermore its mutation efficiency was higher than the above-mentioned mutagens. Therefore, ion beam has been widely used in breeding of crops and microorganisms [35]. Recent advances

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have opened new areas of study in the field of life sciences, such as the role of low-energy ions in the chemical origin and evolution of life, the health risk of low-dose radiation in the environment, and the application of ion beam to genetic modification.

Chitosan, a D-glucosamine polymer, is totally or partially deacetylated derivative of chitin. Even chitosan has very strong functional properties in many areas, such as medicine, agriculture, and environmental treatment [2, 10, 15, 22, 29], its high molecular weight and high viscosity may restrict the use in vivo. In addition, there is no doubt that such properties will influence absorption in the human intestine.

Chitosan oligomers possess additional functional properties such as antifungal [7, 11], antimicrobial [1, 9, 28], antitumour [25, 27], and immuno-enhancing, weight losing effects [24, 26]. Moreover, they have lower viscosities, lower molecular weights and shorter chain lengths, and are soluble in neutral aqueous solutions. Therefore, converting chitosan to water-soluble oligosaccharides has attracted an increasing attention, especially in functional food and medicine industries.

Traditionally, chitosan oligosaccharides were processed by chemical methods in industries. There are many problems existing in chemical processes, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides [16], high cost in separation and also environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost and reproducibility, chitosanase hydrolysis becomes more and more popular in recent years. However, the utility of chitosanase in such hydrolysis is limited because of its cost and unavailability in bulk quantity [33].

In the present study, we aim to mutate and screen a potential industrial chitosanase high-yield strain with this novel mutagen, ion beam implantation.

Materials and methods

Microbe

Bacillus sp. S65 was isolated from soil on the bank of a lake and stored in our lab [23].

Ion beam implanting equipment

The implantation sources were produced by an ion beam bioengineering instrument (Patent No. ZL93103361.6, Zengliang Yu et al. 2000, People's Republic of China, Fig. 1) designed by ASIPP (Chinese Academy of Sciences, Institute of Plasma Physics). In this machine, ions were produced by a radiofrequency ion source, electrostatically extracted and accelerated, focused, and finally transported to the target chamber where a special bio-sample holder was installed.

The pulse implantation technique was used with a pulse time of 5 s and an interval time of 30 s. The dose of each pulse to the sample was 2.6×10^{13} ions/cm^2. The pulse in current was 30 mA. During ion bombardment, the pressure in the target chamber was kept around 10^{-5} Pa by a turbomolecular pump, and the temperature of the target in such an environment was estimated to be around 0°C.

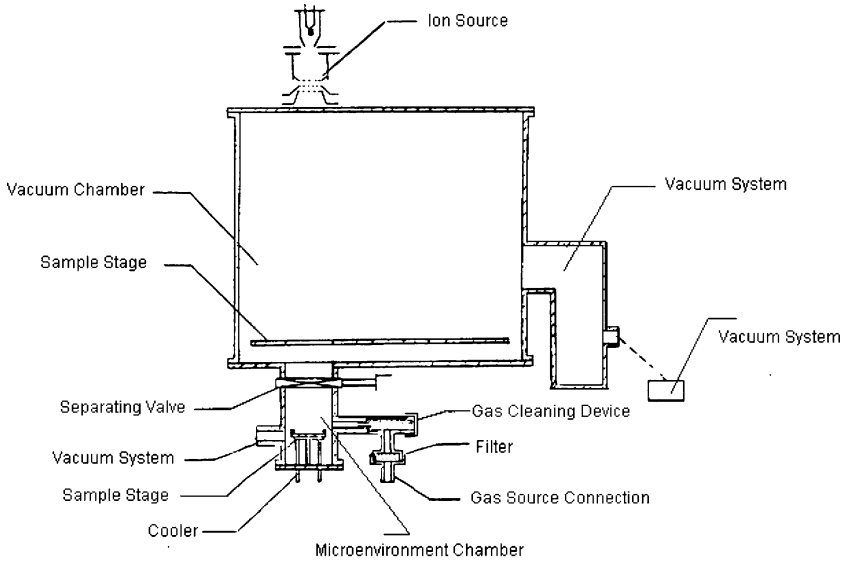

Fig. 1 Schematic diagram of the experimental system of ion beam implantation

Sample preparation and ion beam implantation protocols

Transfer one loop of cells of *Bacillus* sp. s65 into 100 ml seed medium [beef extract (3 g), peptone (5 g), NaCl (1 g), water(1 l)] in a 500-ml flask. For solid seed medium plate, 18 g agar is added. Incubate the culture for 24 h at 30°C with rotating speed of 120 rpm. The cells of *Bacillus* sp. s65 were sequentially diluted in sterilized water and 100 µl suspensions with appropriate dilution were spread as a single-cell layer on a sterilized Petri dish (90 mm) and desiccated by filtrated air on a clean bench. The dishes were then placed on the sample holder, which was designed specifically for ion implantation. The ion source was nitrogen ions (N^+), whose energy was chosen at 15 KeV.

The dose for implantation ranged from 2.6×10^{14} to 5.2×10^{15} ions/cm^2. The control samples were placed in the target chamber without ion implantation to test the desiccation and vacuum effect on survival fraction.

After implantation, the sample was immediately washed with sterilized water, and then 50 µl of washed sample was spread for each seed medium plate. After incubation at 29–30°C for 24 h, we counted the number of colonies to determine the surviving rate. For purpose of mutation screening, single colonies were cultured in seed medium slanted tubes.

Mutation screen

The nitrogen ion implanted colonies were cultivated in fermentation medium at 29–30°C for 5 days on a rotary shaker at 120 rpm. At 1-day intervals, the microorganisms were removed from the culture broths by centrifugation (12,000 rpm, 10 min) and the supernatants were used in a chitosanase assay. The positive mutants were defined as the chitosanase production was increased by more than 10% when compared with the original strains. The negative mutants were defined as the chitosanase production was decreased by more than 10% comparing with the original strains. The mutation rate was calculated as the number of either positive or negative mutants divided by the total number of screened mutants.

The composition of 1-l fermentation medium was glucose (10 g); yeast extract (5 g); Na_2HPO_4 (1.3 g), KH_2PO_4 (3.0 g), NaCl (0.5 g), NH_4Cl (1.0 g), $MgSO_4$ (0.24 g) and $CaCl_2$ (0.01 g).

Assay of chitosanase activity

Chitosanase activity was determined by measuring the reducing sugars liberated during the hydrolysis of the chitosan with 3,5-dinitrosalicylic acid method [13]. The assay was performed by mixing 2 ml 1% chitosan (predissolved in 200 ml of 1% acetic acid) pH 6.0, with 2 ml of suitably diluted enzyme. After incubation for 10 min at 65°C, individual hydrolysis reactions were terminated and analysed by adding 4 ml of dinitrosalicylic acid reagent. The mixture was boiled for 10 min, chilled and centrifuged to remove insoluble chitosan. The resulting adducts of reducing sugars were measured spectrophotometrically at 520 nm. One unit of chitosanase activity was defined as the amount of enzyme required to release 1 µmol of detectable reducing sugar in 1 min, with D-glucosamine used as the standard.

Results

Survival rate determination

The ion source in Fig. 1 can be varied depending on specific purposes and different ion species such as N^+, Ar^+, H^+, He^+ can be produced. In this study, N^+ was chosen as ion source and energy was fixed at 15 keV.

Nitrogen ions have a higher mutation frequency and a wider mutation spectra than other ions. It is the most popular ions used in ion beam implantation [32].

For ion beam implantation, desiccation and vacuum conditions are required. Only those cells which survive these treatments can receive at least one hit of ions and therefore can be screened as mutant based on phenotypic expression. The effect of desiccation and vacuum at 10^{-5} Pa on survival rate was investigated. Figure 2 demonstrates that after the desiccation with filtrated air on a clean lab bench, the survival rate of s65 decreased relative to the increase of vacuum time. In order to exclude the effect of desiccation and vacuum on survival rate, control samples were placed in target chamber without ion implantation for every treatment.

The dose of nitrogen ion radiation to *Bacillus* sp. S65 cells on survival rate is shown in Fig. 3. The survival rate was related to the dose of N^+ implantation, and showed a characteristic curve shaped like a "saddle". The reduction in survival rate did not follow the exponential law which is also called the log-linear model, but firstly decreased along with doses (0–2.08×10^{15} ions/cm^2), then increased in a short dose range (2.08×10^{15}–2.6×10^{15} ions/cm^2) and finally decreased when dose surpassed 2.6×10^{15} ions/cm^2. The down–up–down pattern (also called saddle shape) of survival due to ion implantation suggested that it had some obvious difference from the results of other traditional mutagens irradiation, such as UV and γ-ray. The mutational

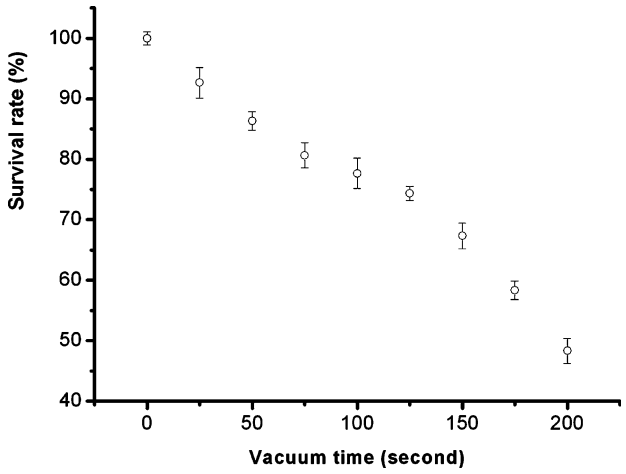

Fig. 2. Effect of vacuum on survival rate. Data were pooled from three independent experiments. Error bars indicate SEs of mean values

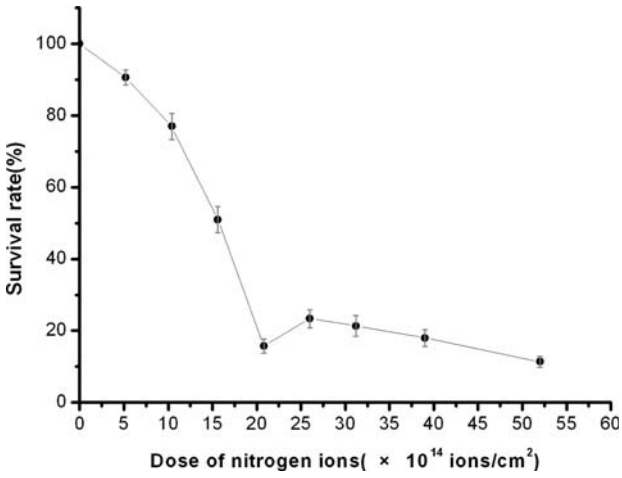

Fig. 3 Effect of dose of nitrogen ions on s65 survival rate. Data were pooled from three independent experiments. Error bars indicate SEs of mean values

mechanisms of low-energy ion radiation were not totally understood. Yu [36] proposed that the interaction between low-energy ions and the organisms was characterized by energy deposition, momentum transferring, mass deposition and charge neutralization and/or exchange. Some experiments have been done to test the above hypothesis [19, 20]. According to this hypothesis, at the first "down" period (0–2.08×10^{15} ions/cm^2), energy deposition causes a series of ionization, which causes the DNA breaks and the oxidation of the cell membrane. Besides, momentum transferring causes damage on the cell by etching of the cell wall, perforation of the membrane, destruction of the cell framework, etc. The degree of this damage to cell activity increases with increasing dose. When the dose increases to a certain value (2.08×10^{15} ions/cm^2 in this experiment), the collision cascade results in a large number of vacancies in the genetic substance. Part of the vacancies in single-strand DNA breaches induce an SOS reaction or other reparation and increase the survival rate of damaged cells. When the dose further increases (2.6×10^{15} ions/cm^2), the cells are subject to serious damages, leading to a large quantity of double-strand breaks, which accumulate to an unrecoverable level, and the cell survival rate decreases again. Because modest ion penetration can cause quality deposition, and because irradiation needs desiccation and vacuum conditions, the low-energy ion beam has different biological effects from the high-energy ionizing radiation.

Mutation induced by ion implantation

The mutation rates induced by nitrogen ion implantation are shown in Fig. 4. The mutation rate had correlation with implantation dose. In the range from

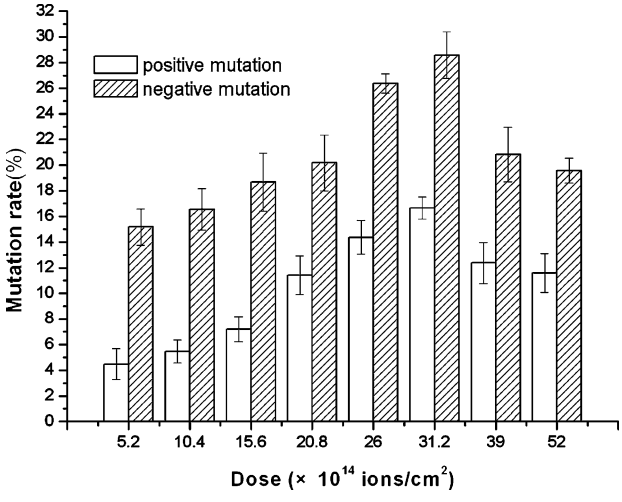

Fig. 4 Positive and negative mutation rate of *Bacillus* sp. s65 exposed to N^+ with 15 keV

2.6×10^{15} to 3.12×10^{15} ions/cm^2, the highest positive and negative mutation rates were achieved, which indicated the broad mutation spectrum. This dose range of broad mutation spectrum also fit the saddle part in the survival rate line well.

Mutation frequency and screen efficiency are closely related to energy and dose of ions. It has been reported that the highest positive mutation rate was obtained when the lethal rate of the microorganism ranges from 70 to 80% [40]. The results of mutation rate by N^+ in this research validate this viewpoint.

Screening mutated strains with high production

Some high yield mutants were obtained after *Bacillus* sp. s65 was implanted by 15 keV nitrogen ions at

3.12×10^{15} ions/cm^2. The mutant with the highest yield was continually implanted by nitrogen ions with energy 15 keV and dose of 3.12×10^{15} ions/cm^2. After six serial mutations, two mutants, s65F5 and S65F16, with high yields were obtained. The average yield of s65F5 was dramatically increased from 4.1 U/ml in wild type to 25.1 U/ml in the mutant. The enzyme activity remained stable during five generations of flask culture (data not shown). Figure 5 shows the fermentation characteristics of s65 and two mutants. From Fig. 5 we can demonstrate that the fermentation time of mutant s65F5 is shortened from 72 to 56 h, which provides great advantages in industrial production by increasing efficiency.

Discussion

Ion implantation is a material engineering process originated in the early 1960s and popularized in the 1980s as part of semiconductor device fabrication and in metal finishing (as well as various applications in materials science research). However, the biological effect of low-energy (< 100 keV) ion implantation was largely ignored due to its lower carrying energy and limited penetrating depth. In the mid-1980s, the biological effect induced by low-energy ion beam exposure of seeds was observed in our lab [37]. Since then ion beam implantation, as a new mutagenic source, has been widely used in improving crops and microorganisms and small biological molecules [8, 18, 38]. However, our basic understanding of low-energy ion beam exposure is still in the primary stages, and the biological effect of ion beam exposure, only recognized a few years ago, is very different from traditional irradiation effects.

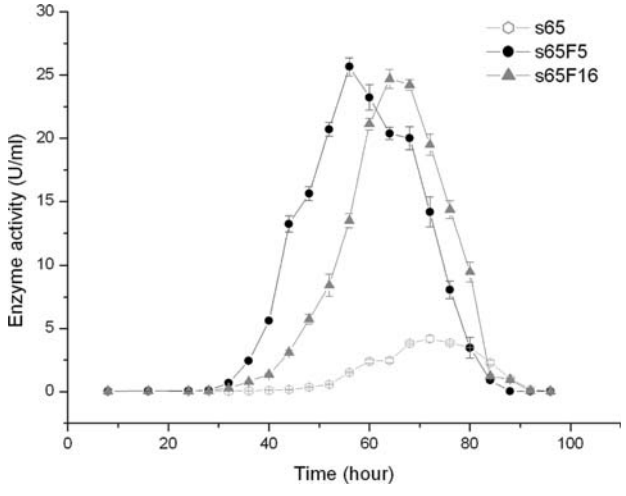

Fig. 5 Fermentation curve of chitosanase s65 and mutants s65F5, s65F16

In this research, the survival rate–dose relationship of s65 did not follow exponential law, but exhibited "down–up–down" pattern, which was different from the traditional mutagen irradiation, such as UV and γ-ray. This saddle type curve was also found by Shao and Yu [17]. The high radio-sensitivity and saddle-type survival curve could explain why we obtained a high mutation rate with low-energy ion exposure breeding. Yu [36] proposed a theory to explain the mechanism of ion beam mutation. According to his hypothesis, the interaction between low-energy ions and the organisms was characterized by energy deposition, momentum transferring, mass deposition and charge neutralization and/or exchange [8, 18, 19], whereas UV and other ionizing radiations into organisms only produced the effect of energy deposition. Shao reported that the mass deposition effects were the main reason for the specific saddle curve of low-energy ion beam exposure, since the interaction range of the 10–30 keV ion was less than 100 μm in a biological organism. By exchanging charges and the occurring substitute reactions forming new molecules, some implanted ions must be deposited in the organism to form new compounds which may compete with free OH radicals reacting with DNA and thereby decreasing the degree of DNA damage [19]. On the other hand, nuclear collision is also the main energy transfer mechanism leading to the decomposition of the bio-molecules as the electronic interaction in low-energy ion beam exposure, compared to other radiation sources where the electronic interaction is the dominant energy transfer mechanism. The nuclear collision process between a low-energy ion and a target molecule creates many types of active species and products which have been hypothesized and identified theoretically and experimentally. These may cause a new reconstituting process to increase the survival rate in high dose range in low-energy ion beam exposure.

Chitosanase is only a model enzyme which was mutated in our lab. In recent years, low-energy ion implantation has been applied to microbiology breeding area, such as vitamin C [32], L-lactic acid [6], xylanase [31], lipopeptide antibiotics [12], etc. According to the experimental results, we may deduce that there may be a new repair mechanism in s65 during ion beam exposure, which has not been discovered and distinguished from the repair of excision, recombination and SOS (error-prone repair). Although the mechanism of ion beam mutation is not elucidated, this research suggests that low-energy ion beam irradiation is a valuable mutagen source. It could be widely applied to the microbe breeding and could improve the selection efficiency.

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