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Breeding of D(–)-Lactic Acid High Producing Strain by Low-energy Ion Implantation and Preliminary Analysis of Related Metabolism

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Abstract The low-energy nitrogen ion beam implantation technique was used in the breeding of mutant D(–)-lactic-acid-producing strains. The wild strain *Sporolactobacillus* sp. DX12 was mutated by an N^+ ion beam with energy of 10keV and doses ranging from 0.4×10^{15} to 6.60×10^{15} ions/cm^2. Combined with an efficient screening method, an efficient mutant Y2-8 was selected after two times N^+ ion beam implantation. By using the mutant Y2-8, 121.6g/l of D-lactic acid was produced with the molar yields of 162.1% to the glucose. The yield of D-lactic acid by strain Y2-8 was 198.8% higher than the wild strain. Determination of anaerobic metabolism by Biolog MT2 was used to analyze the activities of the concerned enzymes in the lactic acid metabolic pathway. The results showed that the activities of the key enzymes responded on the substrates such as 6-phosphofructokinase, pyruvate kinase, and D-lactate dehydrogenase were considerably higher in the mutants than the wild strain. These might be affected by ion beam implantation.

Keywords Nitrogen ion beam implantation · D(–)-Lactic-acid-producing strain · Mutation · Breeding · Metabolic influence

Introduction

For centuries, lactic acid has traditionally been used in the food, textile, chemical, and pharmaceutical industries. In recent years, poly-L-lactic acid (PLLA) has attracted much interest as a renewable alternative to conventional petroleum-based plastics. Its properties make it useful for many applications such as biodegradable packaging and agricultural mulch film [1]. However, thermal stability of PLA is not sufficiently high to some

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applications. A polymer blend of PLLA and poly-D-lactic acid (PDLA) gives a racemic crystal called a stereo-complex; this blended PLLA/PDLA polymer has a melting temperature (T_m) of 225°C, 50°C higher than that of pure PLLA (177°C) due to the unusually strong interaction between PLLA and PDLA chains [2, 3]. The production of D-lactic acid through fermentation has been reported in several articles [4–6], but their yields were quite low compared with L-lactic acid. Therefore, the efficient production of D-lactic acid would be a breakthrough, allowing the large-scale application of poly lactic acid with a high melting point.

Low-energy ion implantation, a new mutagenesis technique, was used in the breeding of rice seeds for the first time by Yu et al. [7]. From then on, this method has been widely applied in the breeding of crops, plants, and microbes [8–10]. When low-energy ions are implanted into organic cells, some important biomolecules such as amino acids and nucleotides are decomposed, and DNA can be destroyed [11]. This means that ion beam mutation has a wide range of biological effects. Experimental results showed that low-energy ion implantation has many advantages, such as a low injury rate, higher mutation rate, and wider spectrum of mutation than traditional mutation methods [12]. The mechanism of low-energy ion implantation acting on microbes has not yet been clarified.

In this work, we screened an efficient mutant for D-lactic acid production through ion beam implantation and conducted a pilot study on the influence of N^+ ion implantation on the metabolism concerned with the lactic acid fermentation pathway of the cells.

Materials and Methods

Microorganism and Media

Sporolactobacillus sp. DX12 was isolated from soil and stored in our lab [13]. The agar medium contained (per liter): glucose 20g, yeast extract 2g, peptone 2g, KH_2PO_4 1g, corn steep liquor 2ml, sodium acetate 2g, $MgSO_4$ 0.2g, $MnSO_4$ 10mg, and $FeSO_4$ 10mg. The selection agar plates had the same compositions except that 100g/l (150g/l for second screen) of glucose and 0.1g/l bromocresol green (pH3.6 yellow −pH5.2 blue) were added [14]. The seed culture medium was composed of (g/l): glucose 20, yeast extract 2, peptone 2, corn steep liquor 5ml, $MgSO_4$ 0.2, $MnSO_4$ 0.01, $FeSO_4$ 0.01, and $CaCO_3$ 20, and the fermentation medium was composed of (g/l): glucose 100, (150g/l for second screen), yeast extract 3, corn steep liquor 20ml, $MgSO_4$ 0.5, $MnSO_4$ 0.01, $FeSO_4$ 0.01, and $CaCO_3$ 100.

Ion Implantation Equipment

The implantation sources were produced by an ion beam implanting instrument LZD-900 designed by Chinese Southwestern Institute of Physics.

Procedure of Ion Implantation on the Cells of *Sporolactobacillus* sp. DX12

Active cells were harvested from the culture after 48h anaerobic incubation at 37°C in agar medium. The fresh cultured cells of DX12 were diluted in sterilized physiological salt solution, and 80μl suspension was spread on an empty sterilized Petri dish (75mm) and desiccated by filtrated air to make a dry membrane of cells. Then, the dishes were put into the sample holder and implanted by nitrogen ion beam with energy of 10keV. The dose for implantation ranged from 0.40×10^{15} to 6.60×10^{15} ions/cm^2.

Selection of Efficient D-Lactic-Acid-Producing Mutants

After ion implantation, the DX12 cells were washed out from the plates with sterilized physiological salt solution. The suspension was spread over three to five selection agar plates to isolate high-yield D-lactic-acid-producing mutants. The colonies showing large halos (yellow) on the agar medium were selected. For the flask test, the mutants selected after 48h culture on selection agar plates (without bromocresol) were inoculated to a 150-ml flask containing 60ml seed culture medium and were shaken anaerobically at 170rpm at 37°C with liquid sealing by liquid paraffin for 24h. A 250-ml flask containing 120ml of the fermentation medium was inoculated with 10% of the above inoculums and cultured in a rotary shaker at 150rpm at 37°C for 7days.

Analysis of Concentration and Optical Purity of Produced Lactic Acid

After the cells and $CaCO_3$ had been removed by centrifugation, fermentation fluid was put through cation exchange resin to change calcium lactate into lactic acid eluted with distilled water for further detection. The concentration of lactic acid in the fermentation supernatant was measured by high-performance liquid chromatography (HPLC) using a C18 column (Allteck) under the following conditions: mobile phase, 2.5mM $NH_4H_2PO_4$; flow rate, 0.5ml/min; temperature, 30.0°C. The detection wavelength was UV 230mm. The optical purity of the produced lactic acid was measured by HPLC with a UV detector (254nm) under the following conditions: ligand exchange column, Sumichiral CA 5000 (150 × 4.6mm, Sumika Chemical Analysis Service, Osaka, Japan); mobile phase, 1mM $CuSO_4$; flow rate, 1.0ml/min and temperature, 35.0°C.

Cell Growth

Growth was determined by measurement of optical density (OD) at 660nm. As it is difficult to measure growth by OD in the presence of calcium carbonate, the sample of broth was settled down for a steady few minutes. The most of calcium carbonate precipitated with few cells because the sediment speed of calcium carbonate is faster than the cells'. Then, the upper layer of the sample broth was diluted to measure the optical densities at 660nm as the rough cell growth. It was confirmed that the OD 660nm of cell growth showed an approximate direct proportional relation to the protein concentration of the broth.

Metabolic Activity Assay Using Biolog Microstation System

Biolog Inc. (Biolog, Inc., Hayward, CA, USA) provides a rapid convenient technique to conduct simultaneous testing of different bacterial phenotypes using cell metabolism as the reporter system [15, 16]. The commercially available Biolog microplates contain a certain amount of tetrazolium violet, different substrates, and the necessary buffer. Tetrazolium violet is used as a redox dye to colorimetrically detect (at 590nm) the metabolic activity of cells for the substrates. The more metabolic activity the cells have, the more purple color will accumulate due to a reduction of the dye [17, 18]. In this research, the Biolog MT2 MicroPlate [19] containing a tetrazolium redox dye and a necessary buffer without substrates was used to analyze the metabolic activities of the mutant and wild strain. The substrates related to the metabolic pathway of lactic acid such as glucose, fructose-6-phosphate, glycerophosphate disodium, and sodium pyruvate were selected and added to the different wells of MT2 plate to assay the relevant metabolic activity of the D-lactic acid fermentation pathway.

Following the established Biolog procedures [15], suspensions of the wild strain and mutant were adjusted to give 60% transmittance with the Biolog turbidimeter. Subsequently, 15μl of different substrates and 135μl of the cell suspension were added into the MT2 MicroPlates. The microplates were incubated in an anaerobic culture box (Bug box dual gas, Ruskinn, UK) at 37°C, and the reduction of tetrazolium violet was determined at 6-h intervals.

Results and Discussion

Survival Rate Curve and Choice of Mutant Dose

The ion source can be varied depending in the specific purpose, and different ion species such as N^+, C^{2+}, and Ti^{2+} can be produced. In this study, N^+ was chosen as the ion source, and the energy was fixed at 10keV. Figure 1 shows the effect of the dose of nitrogen ion beam irradiation to DX12 cells on the survival rate. The survival rate was related to the dose of N^+ implantation and showed a characteristic curve shaped like a saddle. The survival rate decreased as the dose was increased (0.6×10^{15} to 2.6×10^{15} ions/cm^2), but when the dose was increased within a short range (3.2×10^{15} to 4.8×10^{15} ions/cm^2), the survival rate showed a temporary increase and then decreased as the dose continued to increase. The falling, rising, and then falling survival pattern was called "saddle curve" [20].

The mechanisms of low-energy ion irradiation are not totally understood. Low dosage ions only incur damage and etching of the cell surface; thus, the survival rate is higher. With the increase in dosage, the superficial injury to the cell becomes serious, and massive free OH radicals and radials cause the survival rate to drop. When they drop to a certain extent (3.2×10^{15}–4.8×10^{15} ions/cm^2), some kind of internal repair mechanism of the cell such as SOS (error-prone repair) is activated, and the survival rate have a temporary rise. But when the dose is further increased, the cell damage becomes irreparable. According to some reports, the "saddle" region of the survival curve, in which the survival rate ranges from 10% to 30%, is the region prone to mutate [9, 20], and doses ranging from 2.4×10^{15} to 5.2×10^{15} ions/cm^2 were decided as the appropriate dose for further mutation.

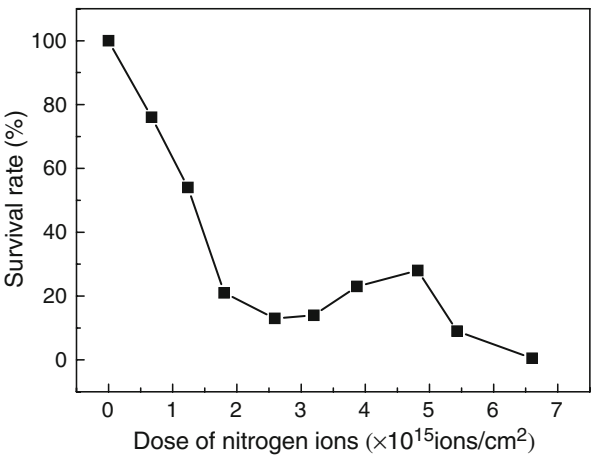

Fig. 1 The survival rate curve of nitrogen ion beam irradiated *Sporolactobacillus* sp. DX12 cells. The survival rate can be calculated using the number of clones scored in control plates without ion implantation as 100%. The averages for two independent experiments are presented

Table 1 The D-lactic acid yield and growth of mutants after first N^+ implantation.

Strain	Wild	Y1-2	Y1-3	Y1-7	Y1-9	Y1-13	Y1-14	Y1-16	Y1-18
Growth (OD_{660})	4.83	4.77	4.09	5.27	5.19	5.87	5.30	5.36	5.90
D-LA (g/l)	40.7[a]	86.8	81.8	88.4	80.4	88.2	89.2	87.3	87.3

The averages for three independent experiments are presented.

[a] The wild strain could not resist more than 60 g/l glucose.

Screening Using Agar Plates Containing High Concentrations of Glucose and Bromocresol Green

Combining the selection on selection agar plates, the mutants were isolated after DX12 cells had been implanted by N^+ ions (2.49×10^{15} to 4.82×10^{15} ions/cm^2). Twenty colonies showing comparatively large yellow halos were selected out. They were inoculated and fermented in flasks as described above. It was found that the D-lactic acid yields of all mutants were higher than that of the wild strain. Table 1 shows the growth and lactic acid yield of eight mutants, which produced more than 80g/l of D-lactic acid (D-LA) from 100g/l glucose. The yields were almost two times greater than the wild strain, which could only produce 40.7g/l D-lactic acid from 60g/l glucose (the wild strain could not resist more than 60g/l glucose [13]). Mutant Y1-14 produced 89g/l D-lactic acid and was used in a second mutation also by N^+ ion implantation.

Mutant Y1-14 was further mutated by N^+ ion beam implantation with the dose of 3.80×10^{15}–5.40×10^{15} ions/cm^2 according to the survival rate curve. The glucose concentration of the selection agar plates and fermentation medium were increased to 150g/l to screen more efficient mutants. Ten colonies were selected out and detected through flask batch fermentation. These mutants showed higher producibility than strain Y1-14 (Table 2), and the mutant Y2-8 was confirmed to produce 143.6g/l D-lactic acid. This was equivalent to a 191.5% molar yield to the glucose.

Mutant Y2-8 was generated and inoculated to flask fermentations to test their stability in producing lactic acid (Table 3). It can be seen from the results that the D-lactic acid producibility of the high-yield mutant fell during the initial generation and then remained relatively stable in subsequent generations. Average yield of D-lactic acid during two to four generations was 121.6g/l.

Detection of Enantiomeric Purity of Produced Lactic Acid

The enantiomeric purity of the product by mutant Y2-8 was 99.08% of D-type, higher than the parent strain (96%). This means that a better quality of product was obtained using *Sporolactobacillus* sp. mutant Y2-8.

In recent studies, a high productivity (120g/l) of D-lactic acid was obtained by genetically engineered *Corynebacterium glutamicum*, but it was fermented using nutrient-

Table 2 The D-lactic acid yield and growth of mutants after second N^+ implantation.

Strain	Y2-1	Y2-3	Y2-4	Y2-5	Y2-6	Y2-7	Y2-8	Y2-9
Growth (OD_{660})	7.77	9.71	9.41	6.11	8.31	6.20	11.24	7.84
D-LA (g/l)	115.2	120.6	124.2	96.3	111.7	106.2	143.6	100.3

The averages for three independent experiments are presented.

Table 3 Stability of mutant Y2-8 during generation.

Regeneration times	1	2	3	4
Growth (OD_{660})	11.24	9.02	8.88	9.67
D-LA (g/l)	143.6	121.3	119.8	123.6

rich medium with the supplement of biotin, thiamine, and some antibiotics [21]. *Lactobacillus delbrueckii* produced D-lactic acid from sugarcane juice or sugar beet juice, but the optical purity is relatively low [22]. In this work, high productivity (121.6g/l) of D-lactic acid was obtained by mutant Y2-8 with related longer fermentation times. Future study will focus on breeding more efficient mutants through further ion beam implantation to short fermentation period.

Analysis of Metabolic Activity Related with D-Lactic Acid Fermentation for Mutant and Wild Strain

Typical homolactic metabolic pathway including glucose becomes pyruvate through the glycolytic flux and pyruvate toward D-lactic acid production through D-lactate dehydrogenase (LDHD) under anaerobic conditions (see Fig. 2). Within the pathway, glucokinase (GK), 6-phosphofructokinase (6-PFK), pyruvate kinase (PYK), and LDHD are the key enzymes [23]. In this research, the Biolog approach was used to analyze the metabolic activities of the concerned substrates in mutant Y2-8 and the wild strain.

As Fig. 3 shows, the amounts of reduced tetrazolium increased rapidly in the metabolic analysis of mutant Y2-8 using four kinds of substrates related with the key enzymes of

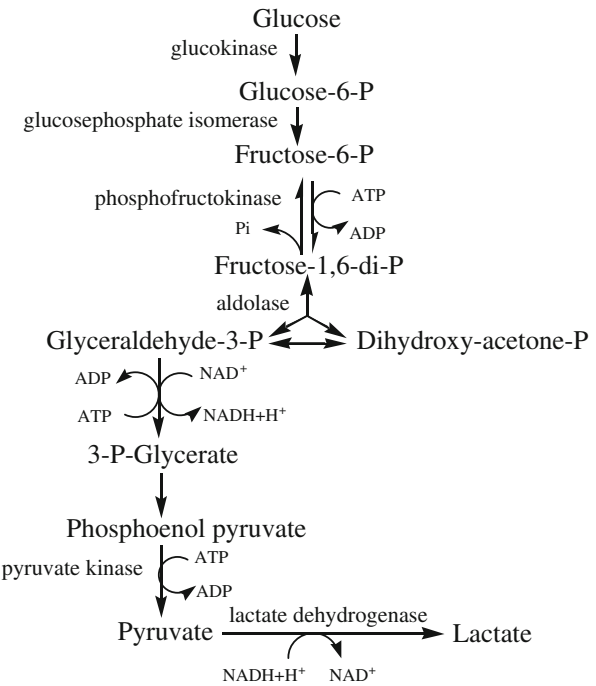

Fig. 2 The homolactic metabolic pathway of lactic acid fermentation. Within the pathway, glucokinase (*GK*), 6-phosphofructokinase (*6-PFK*), pyruvate kinase (*PYK*), and D-lactate dehydrogenase (*LDHD*) are the key enzymes

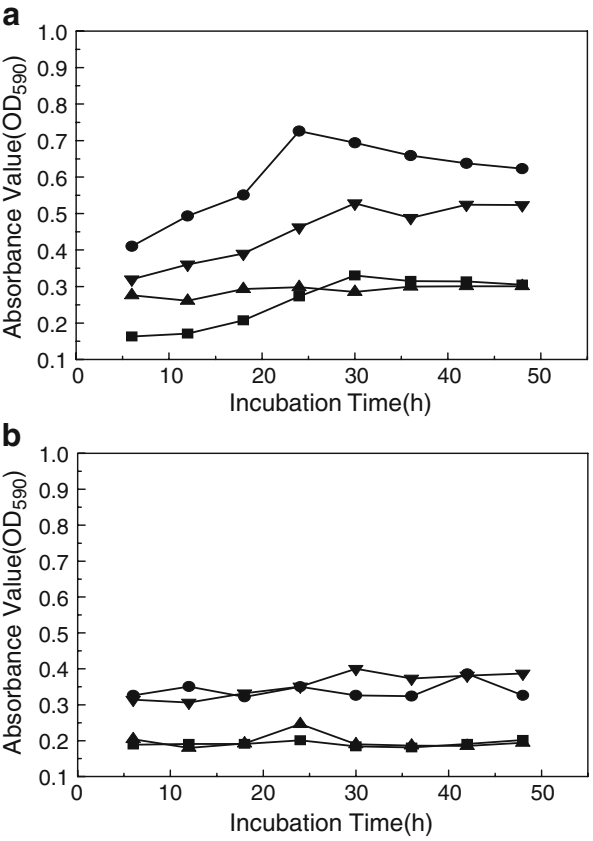

Fig. 3 The metabolic activities of mutant Y2-8 (**a**) and the wild strain (**b**) on the substrates related to the key enzymes of lactate fermentation. The MT2 microplate with the addition of the substrates was incubated anaerobically. The averages for two independent experiments are presented. The reduction of tetrazolium violet is expressed by the change of the absorbance value at 590 nm. D-Glucose (*filled square*), 6-p-fructose (*filled circle*), glycerophosphate (*filled triangle*), pyruvate (*filled inverted triangle*)

lactate fermentation, while the amounts of reduced tetrazolium stayed at low levels according to the metabolic analysis of the wild strain. The metabolic activity of mutant Y2-8 using glycerophosphate and pyruvate as substrate were 50% higher than the wild strain. These results suggest that the activities of the related enzymes, especially lactate dehydrogenase, in mutant Y2-8 was obviously higher than in the wild strain DX12. Glucokinase responding on glucose was a key enzyme that turns glucose into an Embden–Meyerhof–Parnas pathway. Another key enzyme responding on fructose-6-phosphate was 6-phosphofructokinase. The activity levels of mutant Y2-8 metabolizing D-glucose and fructose-6-phosphate were 40% and 100% higher than that of the wild strain. All of the metabolic speeds of the tested substrates concerned with the key enzyme of lactic acid formation were significantly faster in the mutant than in the wild strain. These results suggested that the enzymes concerned with the lactic acid fermentation pathway were activated through the mutation by N^+ ion beam implantation.

There are only a few of research on mechanisms of mutation by ion beam implantation. One important biological effect is the mutation of DNA. DNA mutation can affect the activities of enzymes and cause the increase or decrease of some metabolites. The decomposition of amino acids and nucleotides induced the change of DNA structure or even the break of DNA strand [24, 25]. The effects of ion beam implantation on metabolic ability related to the main key enzymes of D-lactic acid fermentation were elucidated in this

study; continuing studies will yield further insight into the differences of transcription regulation in the key enzyme of lactic acid formation between the mutants with the wild strain.

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