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Breeding of L(+)-Lactic Acid Producing Strain by Low-Energy Ion Implantation

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Abstract In order to obtain an industrial strain with higher L(+)-lactic acid yield, the wild type strain *Rhizopus oryzae* PW352 was mutated by means of Nitrogen ions implantation (15 Kev, 7.8×10^{14}- 2.08×10^{15} ions/cm^2) and two mutants RE3303 and RF9052 were isolated. After 36 h shake-flask cultivation, the concentration of L(+)-lactic acid reached 131- 136 g/l, the conversion rate of glucose was as high as 86%- 90% and the productivity was 3.61 g/l·h. It was almost a 75% increase in lactic acid production compared with the wild type strain. Maximum fermentation temperature of RF9052 was increased to 45°C from original 36°C. At the same time, the preferred range of fermentation temperature of RF9052 was broadened compared with PW352.

Key words: Ion implantation, L(+)-lactic acid, *Rhizopus oryzae*

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. The Nitrogen ions are the main composition of DNA, which caused higher mutation, so it was popularly used as implanted ions. In the 1980's, Chinese researchers started to explore the interaction of low energy heavy ions and complicated organisms. The mutagenicity of low energy ions was firstly reported in 1986 in rice. Since then, a damage mechanism including energy absorption, mass deposition, and charge exchange has been proposed [9, 10, 11]. Accumulating evidences [4, 5, 12] have indicated that those three factors may play essential roles in bio-effect of low-energy ions. Study of low energy ions has led to new opportunities in breeding [2, 6], gene transfer [8], and cell modification [10]. In recent years, the ion beam implantation technique has been attracting considerable attention.

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L(+)-lactic acid is mostly used in food industry as a preservative or flavorenhancing additive. Various lactic acid salts are being used in pharmaceutical industry for their therapeutic functions [7]. Particularly, L(+)-lactic acid can be used to synthesize poly lactic acid (PLA), which is proposed to be used in manufacturing new biodegradable plastic [1]. PLA is one of the most promising polymers which will play an important role in solving the world-wide environmental problems. However, whether PLA can replace commercial plastic depends on whether L(+)-lactic acid can be produced at a lower cost than that of petrochemical derivatives. *Rhizopus oryzae* is a common fungus used in industry for production of high optically pure L(+)-lactic acid. The low energy nitrogen ion beam has been characterized as an efficient physical mutagen in microbial breeding [2, 6, 10]. In order to obtain industrial strain with higher L(+)-lactic acid yield, the wild type strain *Rhizopus oryzae* PW352 was mutated by means of nitrogen ions implantation. In this paper, the parameter of N^+ implantation and properties of the mutant *Rhizopus oryzae* RE3303 and RF9052 were studied.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

The strains *Rhizopus oryzae* PW352, RE3303, RF9052 were cultivated on 3xPDA medium which contains the following constituents (m/v): 6.0% glucose, 60% potato extract, 1.0% $CaCO_3$ and 2.0% agar. The composition of the seeding medium was as follows (g/l): glucose, 100; $(NH_4)_2SO_4$, 2; $ZnSO_4{\cdot}7H_2O$, 0.15; KH_2PO_4, 0.5, $MgSO_4{\cdot}$ $7H_2O$, 0.75; $CaCO_3$, 10 (sterilized alone). The main culture medium consisted of the following constituents: (g/l) glucose, 150; $(NH_4)_2SO_4$, 3; $ZnSO_4{\cdot}$

$7H_2O$, 0.15; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.75; $CaCO_3$, 90 (sterilized alone).

Spores were incubated on the 3xPDA slants at 36°C for 4 d. Then, the new spores were picked up with a sterilized inoculating loop and suspended in distilled water. A 250 ml flask containing 50 ml of the seeding medium was inoculated with spores to a final concentration of 10^6/ml and was cultured in a rotary shaker (200 rpm) at 36°C for 12 h. Finally, three milliliter of inocula were inoculated into a 250 ml flask containing 50 ml of the main medium and cultured for 36 h. The agitation rate and culture temperature were 200 rpm and 36°C, respectively.

Ion Implantation

Implantation sources were produced by an ion beam bioengineering instrument (Patent No. ZL93103361.6, Zeng-liang Yu *et al.* 2000. P.R.C.) devised by ASIPP (Chinese Academy of Sciences, Institute of plasma physics).

A spore solution (0.1 ml) of spore solution with 10^2 dilution was spread on the Petri plate. Then it was implanted with nitrogen ion beam under dry vacuum station. At the same time, in order to evaluate the effects of vacuum on mutation, the spores of control group without N^+ beam implantation were also placed into the target chamber.

The sample was washed with distilled water, and then 0.05 ml of washed sample was smeared for each plate. After incubation at 36°C for 24 h, the colonies in the plate were transferred to 3xPDA slants. After incubation in flasks, the colonies with high L(+)-lactic acid yield were screened.

Analytical Methods

The concentration of lactic acid was tested by EDTA titration method and L(+)-lactic acid was quantified by a biosensor which is specifically sensitive to L(+)-lactic acid. The optical purity of lactic acid was measured by HPLC using a μBondpak C18 column (Waters, Millipore Corp., MA, U.S.A.). The mobile phase composition was $CH_3OH:H_2O:H_3PO_4$ (10:90:0.3, V:V:V) and the flow rate was 0.8 ml/min. The detection was made in a UV range at 210 nm at the temperature of 25°C. The standard L(+)-lactic acid was purchased from Singma (St. Louis, U.S.A.). The residual sugar was quantified by Fehling's reaction.

Results and Disscussion

Parameter Determination of N^+ Implantation

For ion implantation, mutation frequency and screen efficiency are closely related to energy and the dose of ions. It has been reported that the highest positive mutation rate was obtained when the lethal rate of the microorganism ranges from 70–80% [3].

In Fig. 1, the shape of survival rate curve with different energy of N^+ implantation in dose of 1.04×10^{15} ions/cm^2 was shown. Under the lower energy, the survival rate decreased slowly. When the energy exceeds 6 keV, it decreased sharply. When energy surpassed 10 keV, the rate of decrease slowed down. In order to obtain the highest positive mutation, 15 keV was chosen as the optimum energy according to the theory stated in the reference [3].

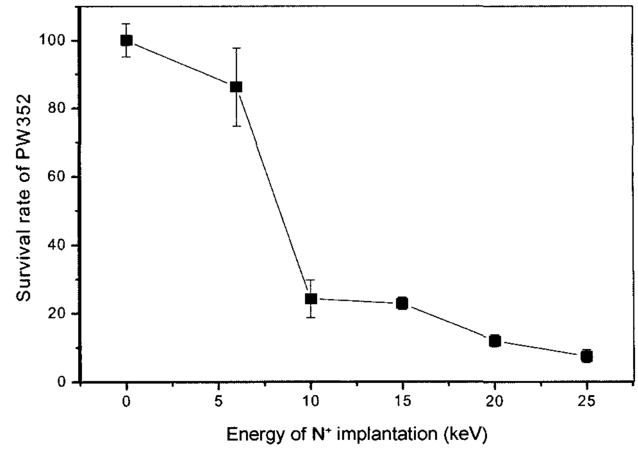

Fig. 1. Effect of N^+ energy on survival rate of PW352.

The survival rate was related to the dose of N^+ implantation, and showed a characteristic curve shaped like a 'saddle' (Fig. 2). 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–$12 \times 2.6 \times 10^{13}$ ions/cm^2), then increased in a short dose range (12–$30 \times 2.6 \times 10^{13}$ ions/cm^2), and finally decreased when dose surpassed $50 \times 2.6 \times 10^{13}$ ions/cm^2. The shape trend of dose ranging from $12 \times 2.6 \times 10^{13}$ ions/cm^2 to $80 \times 2.6 \times 10^{13}$ ions/cm^2 looks like a "saddle", so we used 'saddle' curve to denote the abnormal radiation damages induced by low energy ions.

In dose ranging between $30 \times 2.6 \times 10^{13}$ and $80 \times 2.6 \times 10^{13}$ ions/cm^2, the positive mutation was higher than the negative one, and the range of dose was located right in the

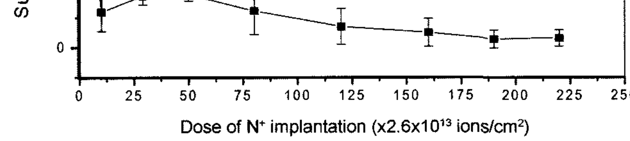

Fig. 2. Effect of N+ implantation dose on survival rate of PW352.

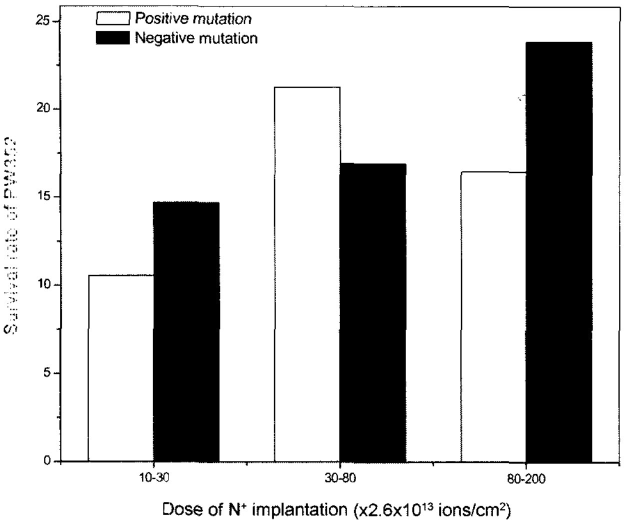

Fig. 3. Relationship between N^+ implantation dose and mutation rate of PW352.

① 10–30, stochastic choosing 218 colonies, 30–80 choosing 207 colonies and 80–200 choosing 176 colonies. ② Regard the positive and negative mutation higher and lower respectively than the contrast by 10% as the standard.

saddle (Fig. 3). At dose ranging from $10\times2.6\times10^{13}$ ions/cm^2 to $30\times2.6\times10^{13}$ ions/cm^2, a lower mutation was obtained. In comparision, at a dose ranging from $80\times2.6\times10^{13}$ ions/cm^2 to $200\times2.6\times10^{13}$ ions/cm^2, mutation yield was higher than at the dose of $30\text{-}80\times2.6\times10^{13}$ ions/cm^2, but at range of $80\text{-}200\times2.6\times10^{13}$ ions/cm^2, the positive mutation was lower than the negative mutation, and lower than the positive mutation in that of $30\text{-}80\times2.6\times10^{13}$ ions/cm^2.

The Directive Breeding and Cultivation of L(+)-lactic Acid Producing Strain

Nitrogen ions (15 keV, 7.8×10^{14}–2.08×10^{15} ions/cm^2) were implanted to the original strain. Fig. 4 showed the flow of mutation and the isolation procedure.

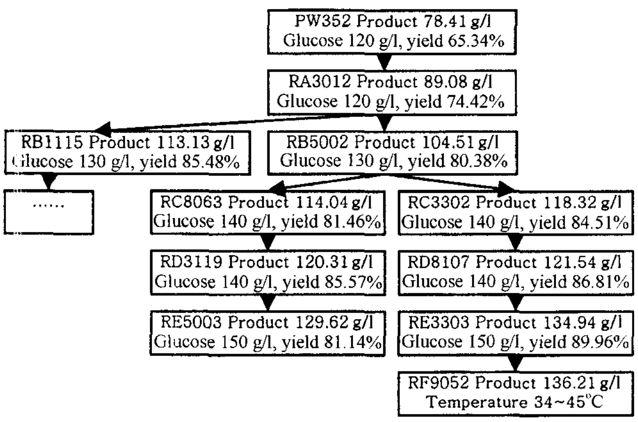

Fig. 4. Flow of mutation and isolation procedure of L(+)-lactic acid producing strains.

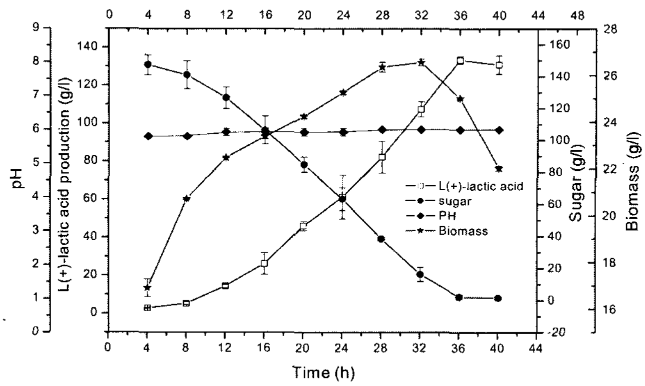

Fig. 5. The curves of the fermentation of L(+)-lactic acid producing strain RE3303.

Properties of the Mutant *Rhizopus oryzae* RE3303 and RF9052

Figure 5 shows that L(+)-lactic acid yield increased with growing mycelia and arrived at the maximum level in 36 h. The production of L(+)-lactic acid was related to the consumption of glucose. After 36 h shake-flask cultivation, the concentration of L(+)-lactic acid reached 131–136 g/l. The HPLC chromatogram of fermentation broth at different time points was similar to the standard L(+)-lactic acid (Fig. 6), and there were no miscellaneous peaks of other organic acids.

The maximum fermentation temperature of RF9052 was increased to 45°C from original 36°C (Fig. 7), and the preferred range of fermentation temperature of RF9052 was broadened compared with that of PW352. Hence the strain will have more extensive applications in industry.

Ion beam implantation, as a new mutation method, has been characterized by higher mutation frequency and

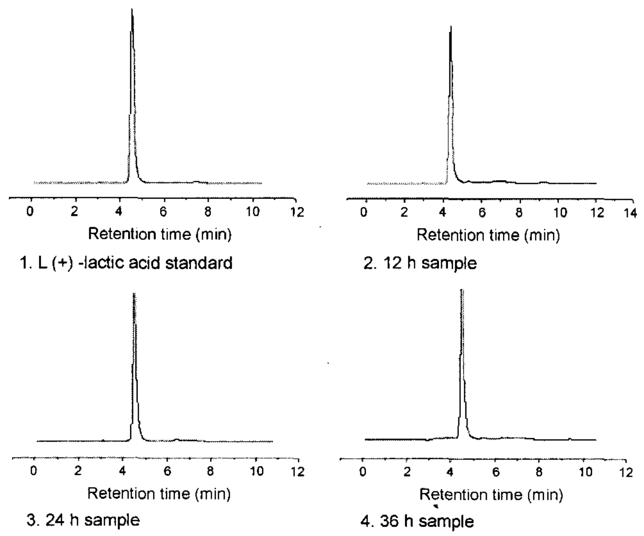

Fig. 6. HPLC chromatogram of fermentation broth of RE3303.

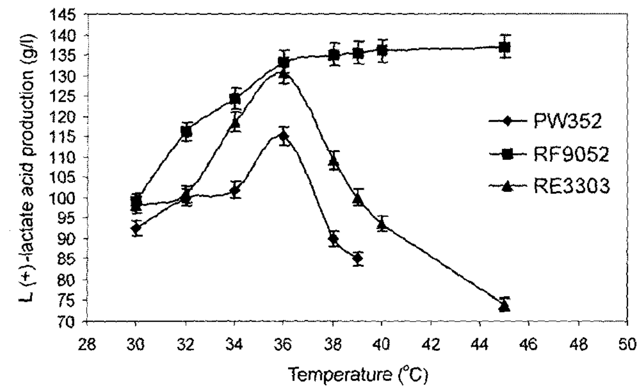

Fig. 7. The effect of temperature on the fermentation of lactate acid.

wider mutation spectra. In the studies of microbial breeding, ion energy was generally in the range of 10–50 keV, and the dose was 10^{14}- 10^{17} ions/cm^2. The survival rate with a low-energy ion beam showed a characteristic curve like 'saddle', and there were the abnormal radiation damages induced by low energy ions. In this article, the optimum energy (15 keV) and dose (7.8x10^{14}-2.08x10^{15} ions/cm^2) were chosen. With the optimum energy and dose, mutant strains RE3303 and RF9052 were screened. After 36 h shake-flask cultivation, the concentration of L(+)-lactic acid reached 131-136 g/l, the conversion rate of glucose was as high as 86%-90% and the productivity was 3.61 g/l·h. It was almost a 75% increase in lactic acid production compared with the wild type strain. Maximum fermentation temperature of RF9052 was increased to 45°C from original 36°C, hence the preferred range of fermentation temperature of RF9052 was broadened compared with that of PW352.

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