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Creation of an ethanol-tolerant *Saccharomyces cerevisiae* strain by 266 nm laser radiation and repetitive cultivation

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Laser radiation is an efficient approach for rapid improvement of industrial microbial phenotypes. To improve ethanol tolerance in *Saccharomyces cerevisiae* strains, a 266 nm laser radiation with the use of repetitive cultivation was explored in this work. After irradiated by 266 nm laser radiation and repetitive cultivation, a genetically stable SM4 strain was obtained. The SM4 strain could grow on YPD plate with extra 15% (v/v) ethanol. Moreover, the ethanol production performance of SM4 strain was 29.25% more than that of the wild type strain when they were cultivated in 5% (v/v) ethanol fermentation medium for 72 h. The DNA mutation was the possible characters for the phenotype of SM4 strain. Overall, the 266 nm laser radiation and repetitive cultivation approach might be a novel and useful for breeding fermentation microorganisms.

[**Key words:** *Saccharomyces cerevisiae*; Ethanol-tolerance; Ethanol fermentation; 266 nm laser radiation; Repetitive cultivation]

Bioethanol is derived mainly from grain or sugarcane. It is generally considered as a sustainable solution to many energy and environmental problems (1–4). The *Saccharomyces cerevisiae* strain is the most commonly used microorganism for industrial ethanol production (5). For breeding yeast strains with higher tolerance of several stresses (including heat, osmotic stress and ethanol stress), different strategies have been used (6,7). In addition to genetically engineered strains (6,8), several mutation strategies have been performed to obtain multiple-tolerance strains. The physical or chemical mutagenesis methods were used, including UV, X-radiation, γ-radiation and ethyl methane sulfonate (EMS) (9,10). Among all these methods, UV radiation mutagenesis was used most commonly (11). Recently, low-power laser irradiation technology was developed to breed the microbial strains (12). All these strategies have been successfully applied for the rapid improvement of industrial microbial phenotypes, i.e., osmotic pressure tolerance, thermotolerance, ethanol tolerance, and ethanol productivity.

The 266 nm laser is ultra compact, long lifetime, low cost and easy operating, which is widely used in UV curing, micro-electronics, CD carving, laser medical treatment, scientific experiment, and so on (13–15). Based on the previous researches, the laser irradiation, as a rational and directed approach, has been used to modulate key genes expression to increase glucose/ethanol conversion (16,17). Our previous study also reported that the high-energy-pulse-electron beam (HEPE) radiation has been successfully applied for the rapid improvement of industrially important microbial phenotypes (18–20). However, few studies were reported about the application of 266 nm laser radiation technology for breeding.

Protoplast is a naked cell whose wall has been removed by enzymes. Generally, fungal protoplast would be more sensitive to physical and chemical factors than their spores. In the present study, one of the aims was to optimize conditions for the protoplasts preparation. Then 266 nm laser mutagenesis was designed to attain high ethanol tolerance mutant strains for ethanol production. However, sometimes these approaches maybe led to unwanted side effects (e.g., reduced stress tolerance, fermentation rates) (7,21).

Recently, some researchers found the adaptation phenomena of yeast cells to the stress environment (i.e., high ethanol concentration) (22). So if yeast cells were exposed to a stepwise increased ethanol stress, the ethanol-tolerant strain might be obtained. However, few researches about the improvement of *S. cerevisiae* fermentation efficiency via repetitive cultivation was reported.

In this article, 266 nm laser radiation was applied on the *S. cerevisiae* protoplast and ethanol production was detected under extra 5% ethanol stress condition. Then the mutants go through repetitive cultivation with stepwise increased ethanol stress. The obtained mutant SM4 strain was investigated to determine the ethanol-tolerance and fermentation ability. Therefore, a combined approach with 266 nm radiation and repetitive cultivation is apparently useful for breeding yeast strains. Then DNA mutation and the size of the cell had been tested in the present work.

MATERIALS AND METHODS

Strain and growth conditions *S. cerevisiae* YE0 strain was used for all cultivations. The YM4 strain was a mutant strain derived from strain YE0 by 266 nm

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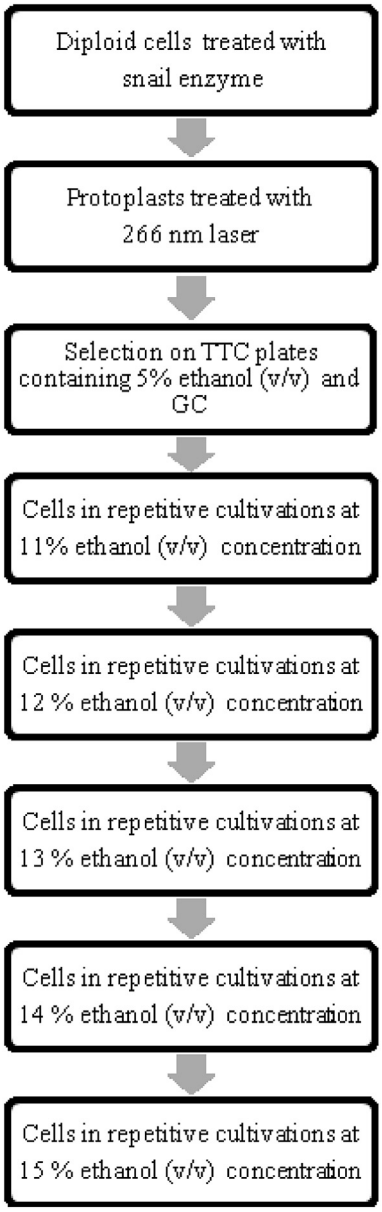

FIG. 1. Flow chart showing the procedure of novel 266 nm laser radiation and repetitive cultivation. The protoplasts of YE0 were irradiated by 266 nm laser. Then isolation of mutant strains by TTC and ferment ability. The resulting cells were cultivated to the YPD medium with extra ethanol, the concentration from 11% to 15% (v/v) via 11%, 12%, 13% and 14% (v/v) through the repetitive cultivation.

laser irradiation. The strain SM4 was the ethanol-adaptive strain screened from strain YM4 through repetitive cultivation. The ethanol-adaptive SM4 strain was using a strategy of 266 nm laser irradiation accompanied with the increasing ethanol stress.

The strains were grown in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose) at 30°C with shaking at 180 rpm. For fermentation, the strains were inoculated into 100 ml of 20% glucose YPD medium (YPD with 30% glucose) in shake flasks (500 ml) to an OD_{660} of approximately 10. Triphenyltetrazolium chloride (TTC) medium, which was used to detect the yield of ethanol, comprised two layers: the upper layer contains (w/v) 0.5% TTC and 1.5% agar and the bottom layer contained (w/v) 0.5% yeast extract, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $CaCl_2$, 0.02% $MgSO_4$ $7H_2O$, and 2% agar.

Preparation of protoplasts and 266 nm laser source mutagenesis TheYE0 strain was grown in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose) for 18 h at 30°C with shaking at 180 rpm. The YE0 strain cells were harvested by centrifugation at 4000 ×*g* at 4°C for 5 min, washed twice with PB buffer (0.01 M Tris−HCl, pH 6.8, 20 mM $MgCl_2$, and 0.5 M sucrose as a stabilizer, pH 7.0), and resuspended in 1% (w/v) β-mercaptoethanol and 2% (w/v) snail enzyme. The

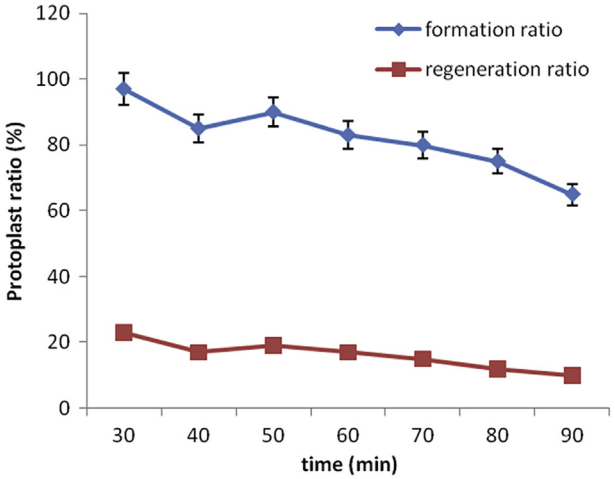

FIG. 2. The *S. cerevisiae* and protoplasts was observed by light microscope. Results of experimental conditions used for preparation and regeneration of protoplasts. Data are expressed as the mean values ± standard deviation of at least three independent experiments.

protoplasts were obtained by holding the suspension at 30°C for 60 min. The protoplast formation ratio and protoplast regeneration ratio were calculated as shown below:

$$\text{Protoplast formation ratio } (\%) = [(A - B)]/A \times 100\%$$

$$\text{Protoplast regeneration ratio } (\%) = [(C - B)]/[(A - B)] \times 100\%$$

where *A* is the total colony number on the YPD agar (the cells without enzyme-treated were spread on YPD agar); *B* is colony number on the RM agar (the cells with enzyme-treated were spread on the RM agar). The regeneration medium (RM) was YPD supplemented with KCl (0.6 M), $CaC1_2$ (25 mM) and agar (2%, w/v); *C* is the colony number on the YPD agar (the cells with enzyme-treated were spread on the YPD agar) (see Fig. 1).

Before yeast cells were exposed to 266 nm laser irradiation, a round of single factor tests were investigated to choose optimal conditions for protoplasts preparation and regeneration of *S. cerevisiae* YE0 strain: selecting 16 h yeast cells and incubating them in the PB buffer for 30 min followed by the addition of 1% (w/v) β-mercaptoethanol to digest the *S. cerevisiae* cell wall. Then the suspensions were shaken slowly with 2% (w/v) snail enzyme for 60 min at 30°C. The rates of protoplasts preparation and regeneration were 99.11% (Fig. 2) and 76.38% (Fig. 2), respectively.

The laser used was a Neodymium:Yttrium Aluminum Garnet, which has a power output of 25−30 mJ/pulse; it emitted light with a wavelength of 266 nm in a beam of diameter 6 mm. The prepared protoplasts were re-suspended in a tube with 5 mL PB buffer at a concentration of 1.0×10^7 cells mL^{-1}. Then a 100 μL protoplast suspension was transfused into the glass tubes. A laser beam (6-mm diameter) was adjusted to the height of a quartz tube containing the protoplast suspension to cover the entire solution. The suspension was irradiated by 266 nm laser in the dark for 0−300 s. The treated protoplasts were kept in the dark for 2 h to avoid photo-reactivation repair, and then spread on TTC agar plates. The plates were incubated at 30°C for 2 days. Non-irradiated protoplasts suspension was used as the control. The cell viability was calculated as a percentage of colony-forming units of the laser-treated sample compared with an untreated control for each culture of the strains.

Screening of mutants by TTC and ethanol fermentation The mutational protoplasts were centrifuged, washed and re-suspended in PB, and serial dilutions were plated on TTC agar for 2 days. The grown colonies were stained by a lay of TTC agar. The TTC agar-covered plate was further cultivated at 25°C for 24 h for color development. The colonies that showed dark red color were selected for ethanol production evaluation. TTC, which served as the color variation reagent, can react with yeast metabolites and turn on particular red color. The upper layer of the TTC medium was poured over the bottom TTC medium layer. The deeper color of the colonies implied that more ethanol was produced. So the colonies that successfully stained dark red were selected as mutative strains for further detection of the ethanol production.

The fermentation was performed in 500 mL shaken flask containing 300 g/L of glucose. Yeast cells were then harvested and inoculated in a fermentation medium (300 g/L glucose, 300 g/L total sugars at a concentration of 1×10^5 cells mL^{-1}). Yeast cells were pre-cultured in YPD for 20 h at 30°C, and 10 ml of 1 OD culture were transferred to a 500-ml flask. The flask was containing 100 ml YPD supplemented with 300 g/L glucose and extra 5% (v/v) ethanol. Then the fermentations were