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Research Article

Mutation Breeding of Salt-tolerant and Ethanol-producing Strain *S. cerevisiae* H058 by Low-energy Ion Implantation

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Abstract: To obtain an industrial strain with high ethanol fermentation efficiency under salted conditions, the wild strain H058 of *Saccharomyces cerevisiae* was mutated by means of nitrogen ions implantation. Mutagenic effects of strain H058 by low energy N^+ ion implantation were studied. A similar "saddle shape" survival curve due to ion beam irradiation appeared again in this study. By repeated screening, a high salt-tolerant and ethanol-producing strain M158 was obtained. Results showed that in medium contained 0, 1.5, 3.0, 4.5, 6.0% NaCl, M158 produced maximal ethanol of 98.3, 97.2, 96.4, 95.6 and 78.3 g/L at 54, 54, 54 and 72 h, respectively. However, the original strain H058 maximal ethanol of 95.2, 90.9, 84.8, 79.4 and 67.5 g/L at 60, 60, 66 and 72 h, respectively. In addition, the ethanol yield (g/g) in all of the NaCl concentrations for M158 is 0.492, 0.486, 0.482, 0.48 and 0.392 g/g, respectively, which were higher than those (0.476, 0.455, 0.424, 0.397 and 0.338 g/g, respectively) of the original strain H058. The higher production and shorter fermentation period suggest that strain M158 is a good salt-tolerant and ethanol-producing strain.

Keywords: Breeding, ethanol fermentation, ion implantation, salt-tolerant

INTRODUCTION

Ethanol production in certain area is encountered with a problem of high salinity water in the fermentation process which markedly affected to fermentation ability of yeast. Generally, *S. cerevisiae* has the ability to ferment ethanol with high efficiency in medium without sodium chloride. However, in high concentration of sodium chloride its ethanol fermentation is decreased while production of the other products such as glycerol, 2, 3-butanediol, acetoin, acetaldehyde and volatile acids are increased (Savitree *et al*., 1998; Limtong *et al*., 1986; Tajima and Yashizumi, 1975). High concentration of sodium chloride was found to inhibit the activity of various enzymes in glycolytic pathway (hexokinase, aldolase and thriose phosphate isomerase), therefore lower ethanol concentration was produced (Menggen and Liu, 2010). To solve the problem of low efficiency of ethanol producing in high concentration of sodium chloride, a salt tolerant yeast is required. Mutagenesis is one of the effective means to obtain high salt-tolerant and ethanol-producing strains.

Ion implantation biology is a new interdiscipline originated from China in the mid-eighties of last century. Since then, a damage mechanism including energy absorption, mass deposition and charge exchange has been proposed (Yu, 2000; Yu *et al*., 1989). Accumulating evidences (Wu and Yu, 2001; Wu *et al*., 1999) have indicated that those three factors may play essential roles in bio-effect of low-energy ions. Due to its high mutation rate and wide mutational spectrum with relatively low damage for the organisms being implanted (Yang *et al*., 2007), the low energy ion beam has been widely used for breeding of crops and microbes in agriculture and industry (Tanaka *et al*., 2002; Pang *et al*., 2009; Yu *et al*., 2009a; Zhang *et al*., 2010). In this study, a wild strain H058 of *S. cerevisiae* was mutagenized by means of nitrogen ion implantation for breeding of high salt-tolerant and ethanol-producing strains. The parameters of N^+ implantation were studied, ethanol productions and ethanol yields of wild type strain and mutant strain in different NaCl concentrations during the fermentation process were compared. A salt-tolerant strain for the production of ethanol was expected to be obtained.

MATERIALS AND METHODS

Microorganism and medium: *S. cerevisiae* H058 used in this study was obtained from Key Laboratory of Ion Beam Bio-engineering of Institute of Plasma Physics, Chinese Academy of Sciences. It was maintained on slants of the agar medium and kept at 4°C. The agar

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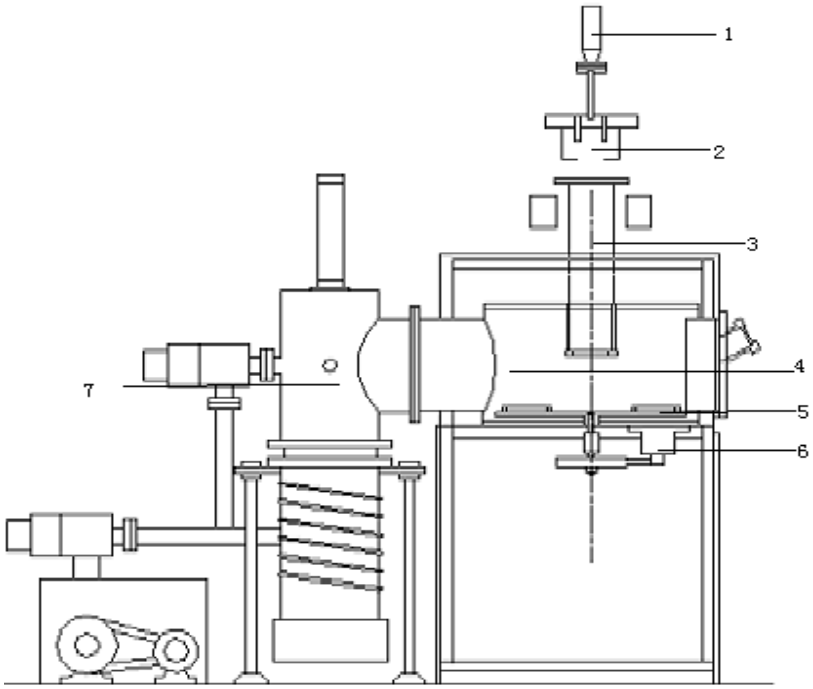

Fig. 1: Schematic diagram of the experimental system of ion beam implantation; 1. Gas source; 2. Ion source formation; 3. Ion beam; 4. Vacuum chamber; 5. Sample stage; 6. Target chamber; 7. Vacuum pump

medium contained 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar. The seed medium contained 5% glucose, 1% yeast extract and 2% peptone. The selective medium used for preliminary screening contained glucose 1%, peptone 0.2%, yeast extract 0.15%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.4%, NaCl 4.5%, agar 2% and adjusted pH to 5.0. The fermentation medium contained glucose 20%, peptone 2%, yeast extract 1% and adjusted pH to 5.0.

Ion beam implanting equipment: The implantation sources were produced by an ion beam bioengineering instrument (Fig. 1) designed by ASIPP (Chinese Academy of Sciences, Institute of Plasma Physics) (Yu *et al*., 2000). In this machine, ions were produced by a radio frequency ion source, electrostatic ally 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.

Ion beam irradiation: Transfer one loop of cells of *S. cerevisiae* H058 into 100 mL seed medium in a 250 mL flask. Incubate the culture for 24 h at 30°C with rotating speed of 120 rpm. The cells of *S. cerevisiae* H058 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 1.56×10^{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.

Mutation screen: After ion implantation, the cells of *S. cerevisiae* H058 were washed out from micropore membrane with sterilized physiological salt solution. The cell suspensions were serially diluted with the ratio of 1:10. Each dilution was spread over five to seven plates with the screen medium. The colonies appearing after 24 h of incubation at 30°C were recorded to determine the survival and the mutation rate and transferred to the slant agar medium for first screening. For the flask test, yeast cells grown 48 h on seed medium was inoculated to a 250 mL Erlenmeyer flask containing 100 mL seed medium on a shaker at about 200 rpm at 30°C. Ethanol fermentations were carried out at 30°C under anaerobic conditions for a period up to 72 h, with *S. cerevisiae* H058 suspension (2% v/v, approximately 1×10^8 CFU/mL) inoculated into 500 mL flasks containing a 400 mL fermentation medium contained 4.5% NaCl. The positive mutants were defined as the ethanol production was increased by

more than 5% when compared with the original strains. The negative mutants were defined as the ethanol production was decreased by more than 5% 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. All experiments were performed in triplicate and results represented the mean values of three independent experiments.

Ethanol fermentation of the mutant strain and H058 in salted medium: To investigate ethanol production, reducing sugar utilization in salted medium, the mutant and H058 were cultivated in fermentation medium contained 1.5, 3.0, 4.5 and 6.0% NaCl and the fermentation conditions were the same as those described upon.

Analytical methods: The reducing sugar and residual sugar were determined using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). The ethanol concentration was measured by using Shimadzu GC-2050 gas chromatography with cbp-20 capillary column and a flame ionization detector. The chromatogram was run at 180°C oven temperature and 90°C injection temperature using N_2 as a carrier gas and H_2 as a flaming gas (Yu *et al*., 2009b). The ethanol yield (g/g) was calculated as the actual ethanol produced and expressed as g ethanol per g total reducing sugar utilized.

RESULTS AND DISCUSSION

Determination of dosage in N^+ implantation: 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 (Xu *et al*., 2004).

The dose of nitrogen ion radiation to *S. cerevisiae* H058 cells on survival rate is shown in Fig. 2. 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 (0-5.2×10^{14} ions/cm^2), then increased in a short dose range (5.2×10^{14}-7.8×10^{14} ions/cm^2) and finally decreased when dose surpassed 7.8×10^{14} 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, diethyl sulfate (DES), ^{60}Co and γ-ray. The mutational mechanisms of low-energy ion radiation were not totally understood. Yu proposed that

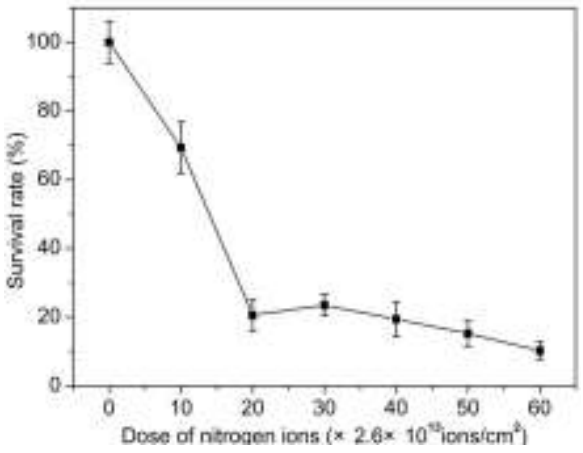

Fig. 2: Effect of dose of nitrogen ions on H058 survival rate. Data were pooled from three independent experiments. Error bars indicate SEs of mean values

the interaction between low-energy ions and the organisms was characterized by energy deposition, momentum transferring, mass deposition and charge neutralization and/or exchange (Yu *et al*., 1989). Some experiments have been done to test the above hypothesis (Shao and Yu, 1997; Shao *et al*., 1997). According to this hypothesis, at the first "down" period (0-5.2×10^{14} 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 to cell activity increases with increasing dose. When the dose increases to a certain value (5.2×10^{14}-7.8×10^{14} ions/cm^2), 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 (7.8×10^{14} 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. 3. At the beginning of ion implantation, with the fluence of nitrogen ions ranging from 0 to 7.8×10^{14} ions/cm^2, we observed comparably lower positive and negative mutation rates. For doses ranging between 7.8×10^{14} ions/cm^2 and 1.04×10^{15} ions/cm^2, the positive mutation was higher than the negative one and the

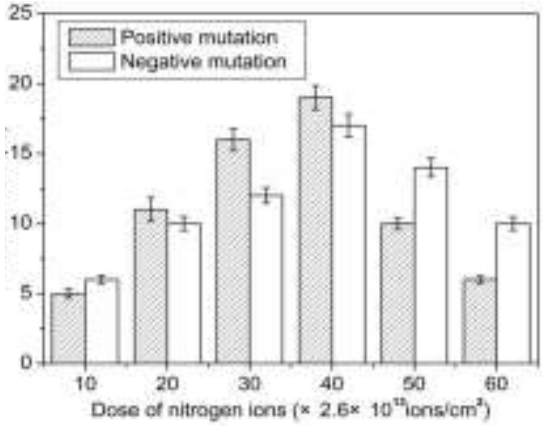

Fig. 3: Positive and negative mutation rate of *S. cerevisiae* H058 exposed to N^+ with 15 keV

range of dose was located right in the ''saddle''. This range of dose can help us improve the efficiency of mutant screening. As the flux of nitrogen ion implantation was continuously increased, a wide range of effect of fluence was observed, a tendency for gradual decrease of positive mutation was observed with continuously increasing negative mutation rate as the cellular damage became more and more serious. Energy absorption, mass deposition and charge exchange of energetic ions possibly lead to mutation effects of ion implantation with remarkable mutagenic efficiency and broad spectrum (Yu *et al*., 1989). 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% (Zhu and Wang, 1992). The results of mutation rate by N^+ in this research validate this viewpoint.

The directive breeding and cultivation of salt-tolerant and ethanol-producing strain: Some high yield mutants were obtained after *S. cerevisiae* H058 was implanted by 15 keV nitrogen ions at 7.8×10^{14}-1.04×10^{15} ions/cm^2. The mutant with the highest yield was continually implanted by nitrogen ions with energy 15 keV and dose of 7.8×10^{14}-1.04×10^{15} ions/cm^2. After six serial mutations and by repeated screening, one mutant, M158, with highest ethanol production of 95.7 g/L and yield of 0.48 g/g was obtained. Figure 4 showed the flow of mutation and the isolation procedure.

Fermentation properties of the original strain M518 and the mutant strain H058: Figure 5 and 6 showed batch ethanol fermentation of the original strain H058 and the mutant strain M158 under different NaCl concentrations. The difference of ethanol production by the H058 and M158 were not significant in low NaCl concentrations. However, both were decreased with the NaCl concentrations increased, but the M158 appeared

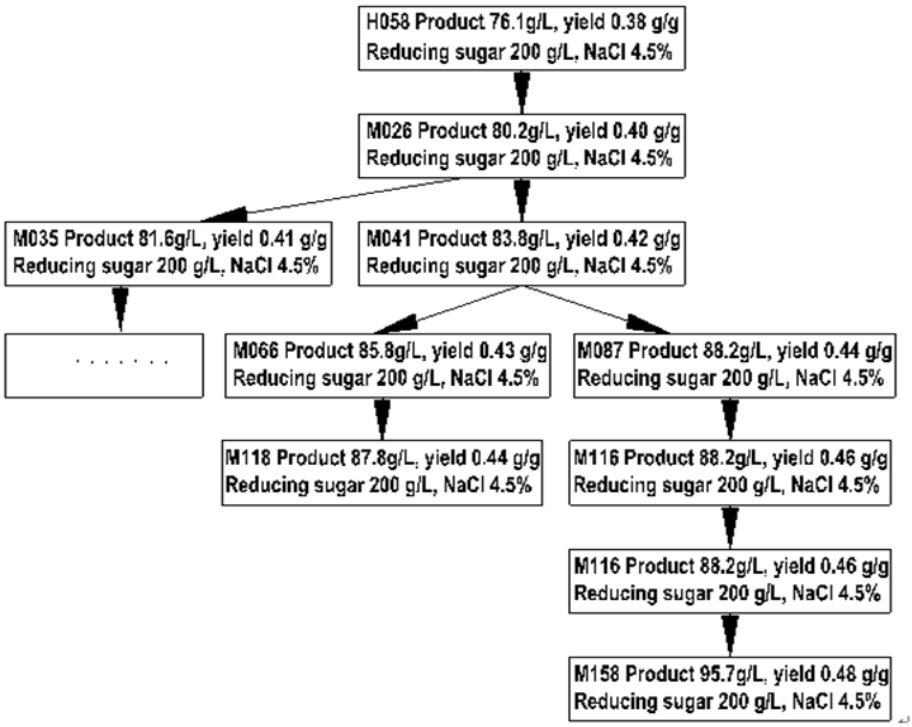

Fig. 4: Flow of mutation and isolation procedure of ethanol producing strains

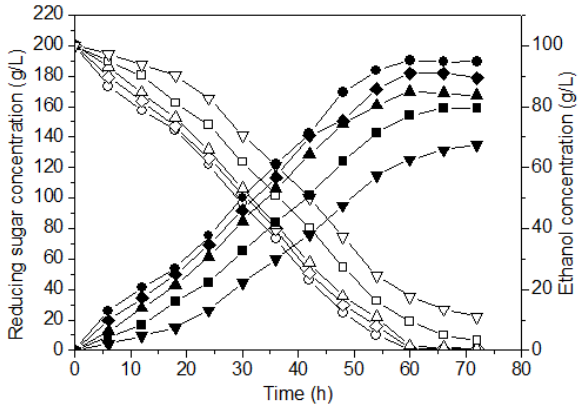

Fig. 5: Ethanol fermentation of the original strain *S. cerevisiae* H058 under different NaCl concentrations

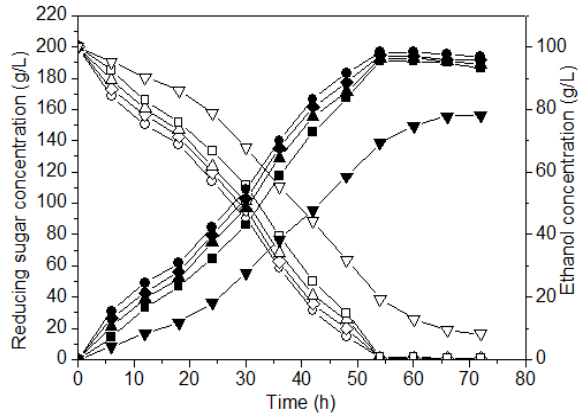

Fig. 6: Ethanol fermentation of the mutant *S. cerevisiae* strain M158 under different NaCl concentrations

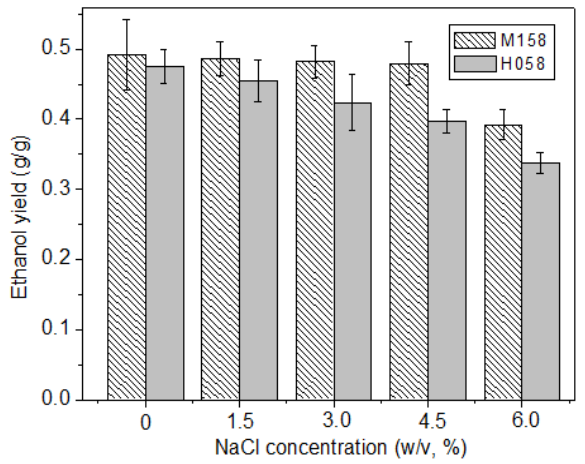

Fig. 7: Effect of NaCl concentration on ethanol yields of the original strain *S. cerevisiae* H058 and the mutant strain *S. cerevisiae* M158

to be less sensitive to higher NaCl concentrations. As illustrated by Fig. 6, in medium contained 0, 1.5, 3.0, 4.5, 6.0% NaCl, M158 produced maximal ethanol of 98.3, 97.2, 96.4, 95.6 and 78.3 g/L at 54, 54, 54 and 72 h, respectively. However, in the above mentioned four salted medium, H058 maximal ethanol of 95.2, 90.9, 84.8, 79.4 and 67.5 g/L at 60, 60, 66 and 72 h, respectively (Fig. 5). The higher production and shorter fermentation period suggest that strain M158 is a good salt-tolerant and ethanol-producing strain mutated by N^+ low-energy ion implantation method.

Figure 7 showed that the ethanol production yields per total reducing sugar were affected by different NaCl concentrations in the process of ethanol production by the mutant strain M158 and the original strain H058. The ethanol yields of M158 and H058 at low NaCl concentrations appeared to be less sensitive to NaCl. At high NaCl concentrations, ethanol yields decreased with NaCl concentrations increased, this might due to adverse effects of high osmotic pressure at high NaCl concentrations. Presence of NaCl and other dissolved nutrients have contributed to high osmotic pressure development causing inhibition on metabolism of the yeast cells. As illustrated by Fig. 7, the ethanol yield per total reducing sugar (g/g) in all of the NaCl concentrations for M1 58 is 0.492, 0.486, 0.482, 0.48 and 0.392 g/g, respectively, which were higher than those (0.476, 0.455, 0.424, 0.397 and 0.338 g/g, respectively) of the original strain H058. These results demonstrated that the mutant strain M158 exhibited higher fermentation efficiency than original strain in high NaCl concentrations. Savitree *et al.* (1998) constructed a high ethanol fermenting halotolerant hybrid RM11 by intergeneric protoplast fusion of *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* (Savitree *et al*., 1998), they reported that in 18% glucose broth containing 3.0% NaCl, the RM11 produced the highest ethanol yield (0.44 g/g) when compared to the other fusants and both parental strains. However, the yield was lower than that (0.482 g/g) of strain M158 obtained from our experiment under the same NaCl concentrations. 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.

CONCLUSION

This study was performed to obtain a salt-tolerant and ethanol-producing strain by low-energy ion implantation. With the proper energy and dose of the ion beam, the mutant *S. cerevisiae* M158 was screened as a promising microbial producer which effectively produced ethanol under high NaCl conditions. In medium contained 0, 1.5, 3.0, 4.5, 6.0% NaCl, M158 produced maximal ethanol of 98.3, 97.2, 96.4, 95.6 and 78.3 g/L at 54, 54, 54 and 72 h, respectively. However, the original strain H058 maximal ethanol of 95.2, 90.9, 84.8, 79.4 and 67.5 g/L at 60, 60, 66 and 72 h, respectively. In addition, the ethanol yield per total reducing sugar (g/g) in all of the NaCl concentrations for M158 is 0.492, 0.486, 0.482, 0.480 and 0.392 g/g, respectively, which were higher than those (0.476,

0.455, 0.424, 0.397 and 0.338 g/g, respectively) of the original strain H058. The higher production and shorter fermentation period suggest that strain M158 is a good salt-tolerant and ethanol-producing strain mutated by N^+ low-energy ion implantation method, which has the potential for biotechnology and industrial application.

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