Strain Improvement of Yeast for Ethanol Production Using a Combined Treatment of Electric Field and Chemical Mutagen N-Methyl-N'-nitro-N-nitrosoguanidine

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Abstract The feasibility of using combined treatments of electric field and chemical mutagen N-methyl-N'-nitro-N-nitroso-guanidine (NTG) for the strain improvement of *Saccharomyces* sp. in ethanol production was examined. The treatment of electric field alone resulted in no effect on the lethality of yeast cells under the conditions of this study. However, when the electric field was applied together to the treatment of yeast cells with NTG, the electric field increased the lethal effect and auxotrophic mutation rate of NTG. The combined treatment of electric field and NTG also increased the chances of ·obtaining superior yeast strains for the ethanol production from tapioca. A higher number of improved clones was obtained by the combined treatments of electric field and NTG than by the NTG treatment alone. The best clone, NF 30-9, which was also obtained by the combined treatment, produced 11.07% (w/v) ethanol from tapioca slurry containing 25% (w/v) reducing sugar while the parental strain produced 9.77% (w/v).

Key words: Strain improvement, yeast, ethanol, electric field, NTG

Mutation is one of the ways of genetic manipulation to improve the yield of a certain useful product formed by industrial microorganisms. Chemical agents such as base analogs and alkylating agents or physical agents such as UV and X-rays have been used to increase mutation frequences. An electric field is one of the physical stresses and there have been many studies on the lethal effects of electric fields on microorganisms [4, 5, 10, 14]. An analysis of the effect of electric fields revealed that they cause reversible loss of permeability [6, 8]; this

phenomenon has been utilized for the introduction of genetic materials into microbial cells [3, 12]. Previously, we have shown that the treatment of low voltage electric field increased the lethal effect and mutation rate of chemical mutagen NTG in the spores of *Streptomyces coelicolor* [13].

Ethanol has been considered as the most suitable substitute for petroleum. The production of a high concentration of ethanol from an elevated level of substrate is desirable since the size of the fermentor and the distillation cost for the recovery of ethanol can be reduced [7]. However, it was reported that the fermentation of high levels of substrate by conventional yeast strains resulted in the significant reduction of ethanol yield [7].

The objective of the present investigation is to examine the feasibility of using combined treatments of electric field and chemical mutagen in improving yeast strains for the production of higher concentrations of ethanol from an elevated level of tapioca substrate.

MATERIALS AND METHODS

Yeast Strains
Haploid yeast *Saccharomyces diastaticus* (ATCC 28339) and polyploid distiller's yeast *Saccharomyces* sp. KL were used. The KL strain forms spores and is confirmed as a polyploid. The KL strain has been used for ethanol production from tapioca slurry containing 18% (w/v) reducing sugar for several years.

Media and Culture
YPD medium (1% Difco yeast extract, 2% Difco peptone, 2% dextrose) was used as a complete medium for the yeast cell cultures. Minimal SD medium was composed of 0.67% Difco yeast nitrogen base without amino acid and 2% dextrose. Media were solidified with

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2% (w/v) agar. YPD25 containing 25% (w/v) glucose were used as a fermentation medium for the primary and secondary screenings of improved yeast clones. The temperature for yeast growth was 30°C. When the cells had to be grown aerobically, the culture flasks were incubated in a rotary shaking incubator (200 rpm).

Tapioca Fermentation

Ground tapioca with a smaller particle size than 20 mesh was mixed well with water and α-amylase (Termamyl 120 L, Novo Co. Denmark) [0.1% (v/w) of dried tapioca] and liquefied at 100°C for 2 h. After liquefaction, the mixture was cooled to 60°C and the pH of the mixture was adjusted to 4.0~4.5 with 0.1 N H_2SO_4, and then glucoamylase (Kleistase TIOS, Daiwa Kasei, Osaka, Japan) [30 sp/g of dried tapioca] was added to the mixture. This mixture was saccharified at 60°C for 2 h and autoclaved for sterilization. After cooling, the mixture was used for ethanol fermentation. Half loopful of activated cells (10^8 cells/ml) were inoculated into 5 ml tapioca-slurry containing 25% (w/v) reducing sugar, and the fermentation was carried out at 33°C for 5 days.

Fermentation Analysis

The amount of ethanol produced were assayed by using the method of Bernet and Gutman [1] with modification [9] which was based on the enzymatic reaction of alcohol dehydrogenase and β-NAD. For the assay of residual sugar content in the fermented medium, the colorimetric method using 3,5-dinitrosalicylic acid reagent [2] was employed with using glucose as a standard.

Electric Equipment

The cell suspensions with or without 0.06 mg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were exposed to AC 38 V in 1.5 ml Eppendorf tubes (Fig. 1) [13]. Two electrodes of stainless pin (1.5 cm apart from each other) were connected to AC 38 V, which was converted from a DC 9 V-battery by using a DC-to-AC converter and a step-up transformer.

Survival Rate

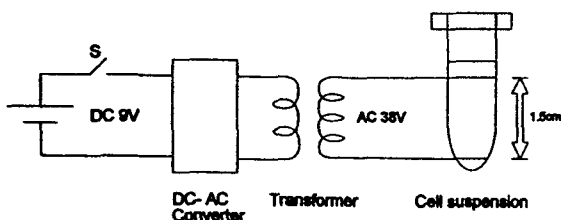*Saccharomyces* sp. KL was activated in a 50 ml Erlenmeyer flask containing 10 ml YPD at 30°C for 1

day. One hundred microliter of the activated cells was mixed with 900 μl of sterile D.W. and 64 μl of NTG (1 mg/ml) in an Eppendorf tube. After the suspension was subjected to the electric field for an interval of 20 min, the suspension was diluted into 5% $Na_2S_2O_3$ to terminate the reaction with NTG. Then, 0.1 ml of the diluted suspension was spread onto YPD agar plates. After 2 days of incubation of the plates at 30°C, the number of colonies was counted to determine the survival rate.

Mutation Rate

The surviving colonies after the exposure of the cells to the electric field or electric field plus(+)NTG were collected and spread onto YPD agar plates. The colonies that appeared on the YPD agar plates were replica-plated onto minimal SD agar plates. The percentage of auxotroph formed was calculated using the following equation and expressed as the mutation rate.

Mutation rate (%)

$$= \frac{\text{The number of colonies not grown on SD agar plate}}{\text{The number of colonies appeared on YPD agar plate}} \times 100$$

RESULTS AND DISCUSSION

Survival and Mutation Rate

The survival rates of haploid yeast cells subjected to electric field (EF), NTG, and NTG + EF were examined and the results are shown in Fig. 2. The treatment of electric field alone resulted in no effect on the viability of yeast cells under the conditions of this study. However, when the EF was applied together with NTG, there was a definite synergistic effect of EF in decreasing

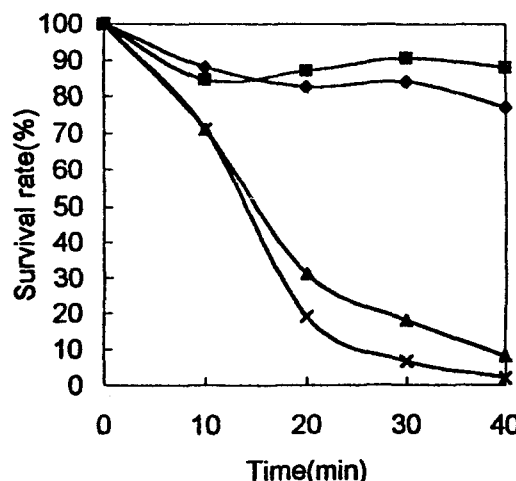Fig. 1. Electric circuit used for electric treatment. S, Switch.

Fig. 2. The survival rate of haploid yeast treated with EF (electric field), NTG, or NTG plus EF during various treatment time.
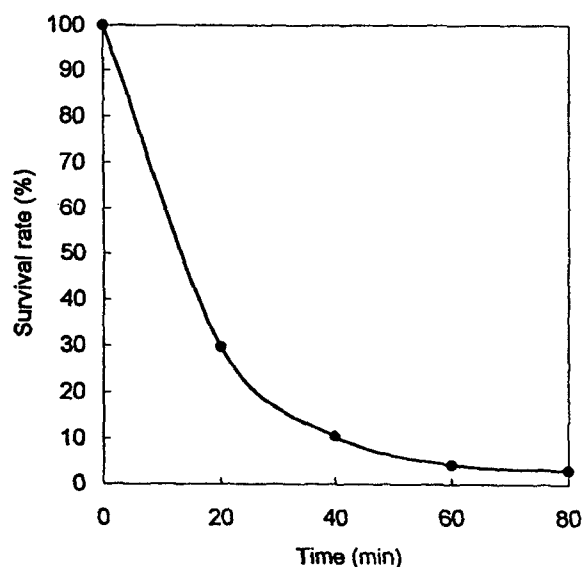◆ No treatment, ■ EF, ▲ NTG, ✕ NTG + EF.

Fig. 3. The survival rate of polyploid yeast KL treated with the combination of NTG and electric field.

the survival rate of the cells by NTG. When the cells were exposed to the combination of NTG and EF, the cells were killed more rapidly than the cells exposed to NTG alone; the survival rate decreased from 9 to 2% after 40 min of treatment. The synergistic effect of EF in decreasing the survival rate of the cells by NTG was also reported in *Streptomyces* spore; the survival rate decreased from 72 to 48% after 180 min of treatment [13].

Although an electric field has been reported to kill bacteria and yeast cells [5, 14], no effect on the viability of yeast cells was observed even after the 40 min of treatment in this study. This is due to the fact that the strength of the electric field used in this study was much weaker than those used by others [5, 14]. Figure 3 shows the survival rate of polyploid distiller's yeast KL subjected to the treatment of NTG + EF. When the survival rate of the haploid yeast was compared with that of the polyploid KL, the lethal effect of NTG + EF was greater in the haploid cells than in the polyploid (Figs. 2 and 3). A longer treatment time was required to kill the polyploid than the haploid cells; it took at least 80 min to get the survival rate of less than 3% for polyploid

Table 1. The mutation rate of haploid yeast treated by NTG or NTG plus electric field after different treatment times[a].

Mutagen	Mutation rate (%)	
	20 min	40 min
NTG	0.7	2.4
NTG + EF[b]	2.2	4.8

[a]Haploid yeast cell suspension (1×10^8 cells/ml) was treated with NTG or NTG plus electric field for different times and then each auxotrophic mutation rate was determined. [b]EF, electric field.

cells while it took 40 min for haploid cells.

The mutation rate of haploid yeast by NTG or NTG + EF was examined and the results are shown in Table 1. The mutation rate was more than twice for the treatment of NTG + EF than it was for the treatment of NTG alone. The increase in the mutation rate by NTG + EF compared to that by NTG only was also observed in *Streptomyces*; the mutation rate increased from 1.8 to 13.6% after 120 min of treatment [13].

These results show that the electric field treatment increased the lethal effect and mutation rate of NTG in yeast cells. It has been reported that the electric field treatment caused reversible loss of permeability of the cell membrane [6, 8]. Zimmermann [15] reported that the reversible or irreversible breakdown of the cell membrane occurred depending on the field strength. Yonemoto *et al.* [14] observed that the yeast cells treated with the electric field was stained better with red dye phloxine B compared to untreated cells. Therefore, even though the electric field used in this experiment did not kill the yeast cells, it seemed to have rendered the yeast cells more permeable to NTG and had higher lethal and mutational effects.

Primary Screening and Selection

The primary screening and selection was performed based on the fact that the less residual sugar content in the fermented broth means that more sugar is used by yeast cells, increasing the chance to produce more ethanol. Our preliminary experiment also showed that, generally, the fermented broth containing more ethanol had less residual sugar content. From the colonies survived after the treatment of cells with NTG or NTG + EF, 80 randomly picked colonies were tested for their residual sugar content after fermentation in YPD25 media and screened for clones showing less residual sugar than the original KL strain. As shown in Table 2, at all treatment times, more clones showing less residual sugar content were obtained with the treatment of NTG +

Table 2. The number of clones showing less residual sugar content in the fermented broth than the original KL strain during various treatment times with NTG or NTG combined with electric field[a].

Treatment	The number of clones[b] showing less residual sugar			
	10 min	20 min	30 min	40 min
NTG	0	0	6	2
NTG + EF[c]	6	4	7	5

[a]Ten randomly picked colonies survived after treatment with NTG or NTG + EF for different times were inoculated into 1 ml YPD25, incubated at 33°C for 4 days, and the residual sugar content in the fermented broth was determined. [b]The number of clones showing less residual sugar content among the randomly picked 10 clones for each group of treatment time. [c]EF, electric field.

EF than with the treatment of NTG alone. From NTG treatment and from NTG + EF treatment, 8 and 22 clones were obtained, respectively. Therefore, a total of 30 clones was selected from the initial 80 clones, according to less residual sugar content in the fermented broth, as the result of the primary screening and selection.

Secondary Screening and Selection

The 30 clones selected from the primary screening were tested for their ethanol production using YPD25 and the results are shown in Table 3. Here, we can see that generally the fermented sample containing more ethanol showed less residual sugar content. Four clones from 8 of the NTG-treated clones and 13 clones from 22 of the NTG + EF-treated clones, and therefore a total of 17 clones which produced more ethanol than the untreated

Table 3. Ethanol fermentation[a] of various mutant clones selected from the primary screening.

Treatment	Clone	Ethanol (%, w/v)	Residual glucose (%, w/v)
None	KL	4.98	7.61
NTG	N30[b]-2	4.85	6.73
	N30-4	5.23	5.88
	N30-5	4.68	7.77
	N30-6	5.74	ND
	N30-8	4.36	8.97
	N30-9	3.66	8.44
	N40-3	7.93	3.25
	N40-4	6.38	5.22
NTG + EF	NE10[c]-2	5.15	6.79
	NE10-3	7.02	5.66
	NE10-4	5.43	6.17
	NE10-5	7.52	4.15
	NE10-9	6.83	3.06
	NE10-10	4.21	7.83
	NE20-1	6.19	6.43
	NE20-3	4.35	8.90
	NE20-6	7.07	4.17
	NE20-10	5.42	7.57
	NE30-1	4.52	7.87
	NE30-2	4.53	7.96
	NE30-4	4.95	7.59
	NE30-6	4.17	8.60
	NE30-8	4.93	7.83
	NE30-9	6.91	4.08
	NE30-10	7.04	4.63
	NE40-3	5.31	7.57
	NE40-4	5.00	7.31
	NE40-5	5.62	7.01
	NE40-8	3.67	8.77
	NE40-9	4.35	8.86

[a] One loopful of activated cells of each clone was inoculated into 10 ml YPD25 broth and the fermentation was carried out at 33°C for 5 days. [b] The number written right next to the N represents the time (min) of NTG treatment. [c] The number written right next to the NE represents the time (min) of NTG + EF treatment.

control KL were selected as the result of the secondary screening.

Tertiary Screening and Selection

The 17 clones selected from the secondary screening were tested for their ethanol production from tapioca slurry and the results are shown in Table 4. The number of clones which produced more ethanol than the untreated control KL was 7 in total, which were N40-4, NF10-3, NF20-10, NF30-9, NF30-10, NF40-3, and NF 40-4. Among the 7 selected clones, 6 clones were from the treatment of NTG + EF, whereas only one clone was obtained by the treatment with NTG alone. The highest concentration of ethanol [11.07% (w/v)] was produced by NF30-9 which was obtained by the combined treatment of NTG + EF, while the untreated control KL produced 9.77% (w/v).

The ethanol productions by KL and its mutant clones from tapioca were more efficient than those from YPD as shown in Table 3 and 4. This seems to be due to the fact that KL has been used by a distillery to produce ethanol from tapioca for several years and well adapted to tapioca. At any rate, the simultaneous treatment of NTG and EF increased the chance to obtain superior clones both from YPD and tapioca.

In conclusion, the combined treatment of NTG and electric field, compared to the single NTG treatment, increased the lethal and the mutation rates of yeast cells, and increased the chance to obtain more clones of improved yeast for the production of a high

Table 4. Ethanol production from tapioca slurry[a] by various mutant clones selected from the secondary screening.

Treatment	Clone	Ethanol (%, w/v)
None	KL	9.77
NTG	N30-4	9.43
	N30-6	9.77
	N40-3	8.05
	N40-4	10.14
NTG + EF	NE10-2	9.13
	NE10-3	9.92
	NE10-4	9.47
	NE10-5	9.39
	NE10-9	8.13
	NE20-1	9.28
	NE20-6	9.69
	NE20-10	10.44
	NE30-9	11.07
	NE30-10	10.70
	NE40-3	9.92
	NE40-4	10.58
	NE40-5	9.60

[a] Half loopful of activated cells of each strain was inoculated into 50 ml tapioca slurry containing 25% (w/v) reducing sugar and the fermentation was carried out at 33°C for 4 days.

concentration of ethanol from elevated concentrations of tapioca slurry. The best clone, NF30-9, which produced 11.07% (w/v) from tapioca slurry containing 25% (w/v) reducing sugar was obtained while the original strain KL produced .9.77% (w/v) under the same conditions.

Acknowledgments

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