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Isolation of a Novel Mutant Strain of *Saccharomyces cerevisiae* by an Ethyl Methane Sulfonate-Induced Mutagenesis Approach as a High Producer of Bioethanol

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In order to obtain mutant strains showing higher bioethanol production than wild-type strains, a commercial *Saccharomyces cerevisiae* type was subjected to mutagenesis using ethyl methane sulfonate (EMS). After adding EMS to a shaken yeast suspension, the viability of yeast cells was assessed by diluted sample inoculation to solid yeast-extract peptone glucose (YEPG) medium at 15-min intervals. At 45 min, the viability of yeast cells was estimated to be about 40%. Mutagenized cells were recovered from YEPG broth after incubation at 30°C for 18 h. After this period, EMS-treated yeast cells were grown on solid aerobic low-peptone (ALP) medium containing 2–12% (v/v) ethanol. All plates were incubated at 30°C for 2–6 d in order to form colonies. The mutant strains that tolerated high concentrations of ethanol were selected for bioethanol production in microfuge tubes containing fermentation medium. Formation of bioethanol in small tubes was detected by the distillation-colorimetric method. In addition, trehalose content and invertase activity were determined in each mutant strain. Among many isolated mutant strains, there were six isolated colonies that grew on ALP medium supplemented with 10% (v/v) ethanol and one of them produced bioethanol 17.3% more than the wild type.

[**Key words:** strain isolation, ethyl methane sulfonate, mutagenesis, bioethanol, *Saccharomyces cerevisiae*]

Saccharomyces cerevisiae is the most important yeast strain because of its many biotechnological applications (1). The important features of this yeast are as follows: (i) its genomic DNA does not contain any intron, (ii) its complete genome sequence has been determined, and (iii) finally, its fermentation technology is quite distinct (2). In addition to its applications in bioethanol and bakers' yeast productions in a large scale, it is a suitable host for preparation of recombinant proteins, too (3, 4). Nowadays, biological or biotechnological processes (5) produce nearly 80% ethanol as clean fuel. The total world production of ethanol is estimated to be 30×10^9 *l* in 2002, which will be expected to reach 30×10^{11} *l* in 2010 because of the high global demand for ethanol (6). Many studies on bioethanol production and improvement of different strains of *S. cerevisiae* have been performed. The highest bioethanol production was reported in a thermotolerant strain of this yeast, which was about 7.5% (w/v) in the presence of 150 g·l^{-1} glucose (7). In two other reports, bioethanol productions are 2.2% and 6.6% (w/v) in a starch–glucose mixture (8) and 15% YEPG (9), respectively. A fluccolating strain of *S. cerevisiae* produces 4.5% (w/v) bioethanol in beet molasses (10). Another strain of this yeast can produce 4.02% (w/v) bioethanol in the presence of 95.8 g·l^{-1} glucose (11). On the other hand, a recombinant strain of *S. cerevisiae* produces a maximum of 0.7% (w/v) bioethanol in a xylose-glucose mixture as the substrate (12).

Nevertheless, ethanol is a chemical component that affects the cell structure and physiology of all microorganisms (2). Ethanol disrupts the rigidity of cytoplasmic membranes (13), inhibits endocytosis (14), blocks proton motive forces of inner mitochondrion membranes, inactivates several important enzymes such as protein kinase A and alcohol dehydrogenase (ADH), and prevents elements translocation across biomembranes in cells (15). From these observations, if a yeast strain has the ability to tolerate and grow under high concentrations of exogenous ethanol, it should have specific mechanism(s) for its survival (3). In bioethanol production proceses, there are very important factors that affect production efficiency such as the tolerance of the yeast cell wall to exogenous ethanol, the content of unsaturated fatty acids in the cytoplasmic membrane, the amount of stored trehalose in the cytoplasm, and the activity of various enzymes for example invertase (saccharase) and ADH (2, 16, 17). Accumulated trehalose in cells, a dimer of D-glucose with the

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α–α bond, is a critical factor for higher resistance of yeast strains to stress conditions such as high ethanol concentration and high osmotic pressure. Trehalose preserves the integrity of biological membranes, stabilizes proteins in their native state, and suppresses the aggregation of denatured proteins (14). Invertase is an extracellular enzyme in *S. cerevisiae* that is able to convert sucrose to its subunits, *i.e.*, glucose and fructose. This enzyme has an important role in bioethanol production, particularly in the presence of high-sucrose substrates such as molasses (18). The activity of this enzyme depends mainly on the type of substrate and yeast strain employed, and can regulate the osmolality and fermentation rate in molasses (19). Yeast invertase has an important role in ethanol tolerance of yeast cells (15).

Nowadays, yeast cell mutants are extensively applied in molecular and cellular studies (6, 16). In order to change the genome structure in different microorganisms, various methods have already been applied, such as random and site-directed mutagenesis (20, 21). Ultraviolet ray, transposons, and ethyl methane sulfonate (EMS) are used to induce random mutagenesis (16, 22–24). In addition, transposons are used to identify genes responsible for ethanol tolerance and cell wall biosynthesis in *S. cerevisiae* (13, 24). EMS is an alkylating agent that induces point mutagenesis by A-T transition to G-C (22). This mutagenic agent has been used to increase glucose oxidase activity and citric acid production in *Aspergillus niger* (16, 23) and to improve recombinant strains of *S. cerevisiae* (13). In addition, mutant strains of *S. cerevisiae* that have resistance to antifungal drugs have been isolated using EMS (25).

Although there are numerous studies on the application of EMS to induce mutagenesis in other microorganisms, to the best of our knowledge, we are the first to use this potent mutagenic agent for selection of mutant strains of *S. cerevisiae* resistant to exogenous ethanol. In addition, capacity for bioethanol production, trehalose content, and invertase activity were determined in all mutant strains that were produced by this approach.

MATERIALS AND METHODS

Microorganism and storage *S. cerevisiae* was obtained from S. I. Lesaffre Company in France. This yeast was stored on slants of yeast-extract chloramphenicol agar (0.5% yeast extract, 2% glucose, 1.49% agar-agar, and 0.01% chloramphenicol) at 4°C (2). After mutagenesis, yeast cells were grown on solid YEPG (1% yeast-extract, 1% peptone, 2% glucose, and 1.5% agar-agar). Cell viability was estimated by the standard plate counting method (5). Solid ALP (1.5% agar, 0.1% $(NH_4)_2SO_4$, 0.05% pancreatic digest of casein, 0.05% yeast extract, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, and 0.02% phenol red) medium containing 2–12% (v/v) ethanol was used to isolate strains resistant to exogenous ethanol. Plates with inoculated ALP medium were sealed tightly with parafilm and incubated at 30°C for several days. Colonies growing on this medium were transferred to fresh solid YEPG medium for subculture and maintenance. All media and chemicals were purchased from Merck (Germany), unless otherwise stated.

EMS mutagenesis EMS was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Mutagenesis was induced using EMS according to Hahn's method (Hahn, S. Laboratories, www.bio.com/protocolstools/protocol.jhtml, 1995). For mutagenesis, wild-type yeast was grown in 50 ml of YEPG broth at 30°C with shaking at 120 rpm overnight. Then, one aliquot of 1×10^8 yeast cells measured on the basis of the optical density (O.D.) at 600 nm was transferred to a 15-ml tube. After centrifugation and washing of cells in 5-ml sterile distilled water (S.D.W.), the pellets were resuspended in 1.7 ml sodium phosphate buffer (0.1 M and pH 7.0). Yeast suspensions were transferred to sterile glass test tubes and 50 µl of EMS was added to each tube in a biological safety cabinet. The final volume of EMS was nearly 3%. All tubes were incubated at 30°C on a roller shaker for 60 min.

Recovery of EMS-mutagenized cells After each 15-min interval, mutagenesis was stopped by adding 8 ml of 5% sterile sodium thiosulphate solution. Samples of these suspensions that contain EMS-mutagenized cells, were diluted with S.D.W. and a specific volume of these suspensions was inoculated to solid YEPG for estimation of cell viability at each time point (24, 26).

Isolation of resistant mutants One hundred microliters of mutagenized yeast suspension were transferred to 10 ml of YEPG broth in an Erlenmeyer flask and incubated at 30°C for 18 h without shaking. Then, several 50-µl aliquots of yeast suspension, containing nearly 1×10^5 yeast cells, were added to ALP solid medium containing 2–12% (v/v) ethanol. The plates were sealed with parafilm and incubated at 30°C for colony formation (2). Inoculation of mutagenized yeast culture to ALP solid media that contained 8% to 12% (v/v) ethanol was repeated many times for isolation of mutant strains resistant to high concentrations of ethanol.

Bioethanol production and detection Different yeast mutant strains were grown in YEPG broth after overnight incubation at 30°C with shaking at 120 rpm until they reached an $O.D._{600}$ of 3. Then, 100 µl of each yeast suspension was added to 900 µl of fermentation medium (9.4% glucose, 0.4% urea, and 0.65% peptone) in a microfuge tube (1). After inserting of a 5-ml sterile syringe into the cap of a microfuge tube, all tubes were incubated at 30°C with shaking at 120 rpm for 5 d using a specific rack with a 45° angle. The produced gas was recorded daily during fermentation for all samples. Finally, bioethanol production by different mutants was estimated by the distillation-colorimetric method. In this method, after assembling the distillation system, 0.5 ml of microfuge tube content was injected to a specific container and exposed to water vapor for 10 min. Water vapor can translocate the produced bioethanol to the collector of the system that contained 20 ml of dichromate-sulfuric acid (12 g of potassium dichromate in 500 ml of 18 N sulfuric acid) solution. The volume of this solution was adjusted to 100 ml with S.D.W. and the absorption of the solution was measured at 574 nm using a spectrophotometer (Specord S; Zeiss, Jena, Germany). Bioethanol concentration in each sample was determined using a standard curve of ethanol (27).

Trehalose detection Trehalose was detected according to Sharma and Trevelyan's method (3, 28). From each yeast suspension in YEPG broth, 5 ml was added to a test tube. Yeast cells were washed twice with cold S.D.W. and all test tubes were placed on ice. To each test tube, 4 ml of cold *tri*-chloro acetic acid (TCA, 0.5 M) was added, and the contents in tubes were mixed at 10-min intervals up to 30 min. Centrifugation ($4000 \times g$) was performed and supernatant was collected in a clean 50-ml volumetric container. After adding TCA solution, centrifugation and supernatant collection were repeated. The final volume of collected TCA solution was adjusted to 50 ml with cold S.D.W. and 1 ml of it was transferred to another clean test tube containing 5 ml of Anthron reagent solution (0.8 g of anthron in 500 ml of 27.4 N sulfuric acid). All tubes were placed on boiling water bath for 10 min and then, the $O.D._{620}$ of each solution was determined. The trehalose content of each sample was compared with the standard curve and recorded.

Invertase assay Invertase activity in yeast cells was estimated according to Osho's method (15). Sucrose solution (4% w/v), as an appropriate substrate, was prepared in acetate buffer (0.1 M, pH 5.0). Yeast cells of specific mass (0.01 g) were exposed to 2 ml

sucrose solution. After incubation at 30°C for 5 min and centrifugation (4000×*g*), 0.5 ml of supernatant was mixed with 1.5 ml of dinitro salicylic acid (DNS) (1% DNS, 2% NaOH, and 20% sodium-potassium tarterate) reagent. This mixture was placed on boiling water bath for 10 min. Then, the absorbance of colored mixture was measured at 540 nm using a spectrophotometer. According to standard curve of glucose, enzyme activity was calculated on the basis of free hexoses content, which was produced after adding the substrate. The amount of enzyme that released 1 μmole of the reduced sugar per minute was defined as one unit of invertase activity.

Resistance to exogenous methanol, ethanol, isopropanol, and 1-butanol For evaluation of alcohol resistance in broth medium, 50 μl of each yeast suspension with an $O.D._{600}$ equal to 1.0 was inoculated to 10 ml of YEPG broth containing 1–13% (v/v) methanol, 2–14% (v/v) ethanol, 1–9% (v/v) isopropanol, or 0.5–1.5% (v/v) 1-butanol in capped test tubes. After incubation at 30°C on a rotary shaker, growth rate was estimated using a spectrophotometer for up to 48 h (15). This experiment was repeated to determine resistance to ethanol using a bio-photorecorder (TVS062CA; Advantec, Tokyo) for continuous growth recording at 30°C for 48 h. These experiments were repeated three times.

Statistical methods Student t-test and ANOVA were carried out for statistical analysis.

RESULTS

In mutagenesis induced using EMS, the viability of yeast cells was determined by the standard plate counting method. Cell viability decreased with time. The viabilities at the start of EMS treatment and 30 min after the treatment were 100% and 67%, respectively. This decreased to 43% after 45-min exposure (Fig. 1). On the basis of viability variation at different time points, we assumed that EMS affected yeast cells actively, and nearly 40% of yeast cells survived and EMS mutagenized the cells after 45 min. Then, we used this yeast suspension to isolate resistant mutagenized cells because their cells viability was suitable for resistant mutant screening. These differences in viability were significant at 1% level of statistical significance (p value < 0.01).

After inoculation of recovered mutagenized cells to ALP medium supplemented with different ethanol concentrations (2–12% v/v) and after incubation, resistant colonies were isolated. To isolate mutagenized cells resistant to high concentrations of ethanol, we transferred many 50-μl samples of the above-mentioned yeast suspension to ALP medium that they had different ethanol concentrations between 2–12% (v/v). About 1×10^6 mutagenized yeast cells were inoculated to ALP medium. In each ALP-medium-containing plate with ethanol concentrations of 2%, 4% and 6% (v/v), many colonies were formed after incubation. However, in ALP-medium-containing plate with 8% and 10% (v/v) ethanol, yeast colonies did not form in several plates. Therefore, we repeated this experiment for isolation of mutant cells resistant to 8% and 10% (v/v) ethanol. Finally, we were able to isolate six independent colonies, which were named mut1 to mut6.

Bioethanol production in all mutants and wild-type yeast strains was carried out by a small-scale fermentation method. The mut1 strain showed the highest production of bioethanol, *i.e.*, 6.2% (w/v), which was 17.3% higher than that of the wild type (Fig. 2). Bioethanol production by the mut2 strain was slightly higher than that by the wild type, but the production by other mutant strains was lower than that by the wild type. Differences in bioethanol production in all strains were statistically significant at 1% level (p value < 0.01).

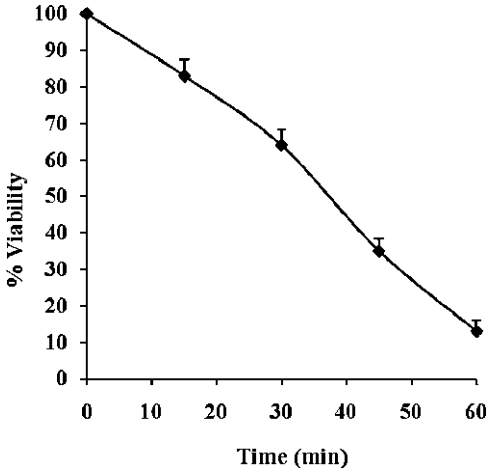

FIG. 1. Yeast viability changes during mutagenesis induced by EMS in rotary shaker at 30°C and 120 rpm. Standard deviations are indicated in the graph by bars.

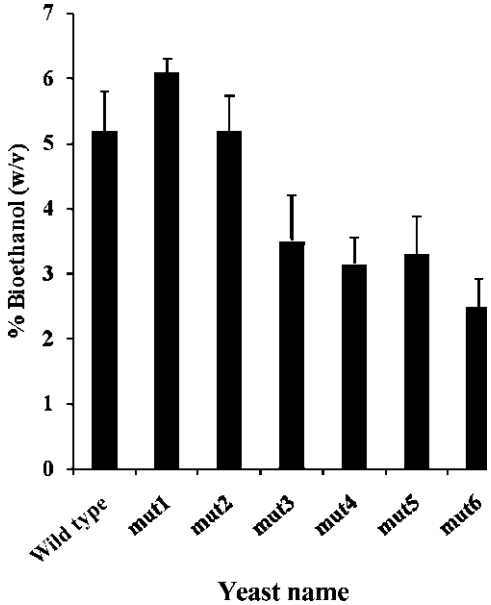

FIG. 2. Bioethanol productions in wild-type and mutant yeast strains by microfermentation method using microtubes containing fermentation medium after 5 d at 30°C with shaking at 120 rpm. Standard deviations are indicated on all columns by bars.

Trehalose content (Fig. 3) and the activity of invertase enzyme (Fig. 4) were also determined in all yeast strains. Although trehalose contents in mut1, mut2, and mut3 strains were higher than that in the wild type, those in other strains were lower than that in the wild type. Differences in trehalose content in all examined strains were significant at 1% level (p value < 0.01). Interestingly, all mutant strains showed higher invertase activities than the wild type. Mut2 showed the highest invertase activity, *i.e.*, 10.5 U. In mut4, mut5,

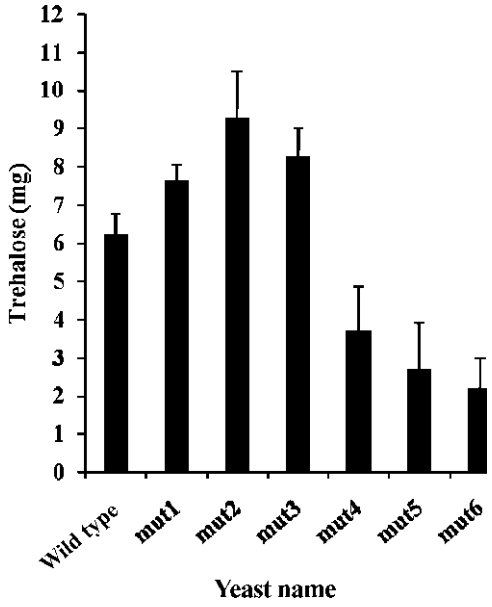

FIG. 3. Trehalose contents in wild-type and mutant strains. Standard deviations are indicated on all columns by bars.

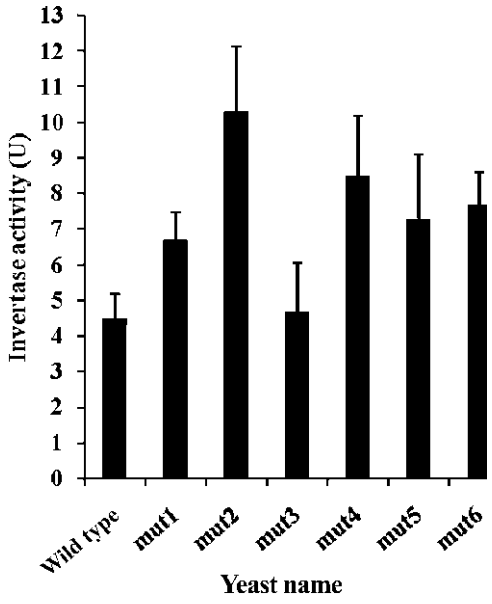

FIG. 4. Invertase activities in wild-type and mutant strains. Standard deviations are indicated on all columns by bars.

and mut6, trehalose contents were lower than that in wild-type cells, whereas invertase activity was higher than the normal activity. Differences in content in relation to invertase activity were significant at 1% level (p value < 0.01).

As we expected, mut1 as a high-bioethanol producer tolerated 10% and 11% (v/v) exogenous ethanol better than the wild type. In addition, mut1 was able to grow in YEPG broth supplemented with 12% (v/v) ethanol and showed measurable growth under this condition (Fig. 5). In spite of mut1 resistance to ethanol, there was a difference in growth rate in the presence of other organic solvents such as methanol, isopropanol, and 1-butanol. The mut1 strain did not tolerate methanol, isopropanol, and 1-butanol similarly to the wild type (Fig. 6). The results for 1-butanol are not shown in Fig. 6.

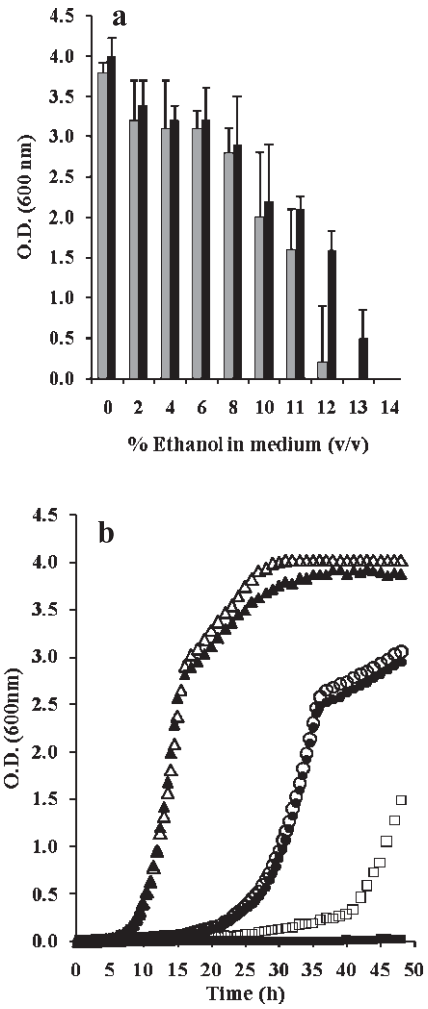

FIG. 5. Growth rates of wild-type and mut1 strain in the presence of different concentrations of ethanol in roller shaker (a) and bio-photorecorder (b) at 30°C and 70 rpm for 48 h. (a) Symbols: open bars, wild-type; closed bars, mut1. (b) Filled and open triangles indicate wild and mut1 strains in YEPG without ethanol respectively, filled and open circles indicate wild and mut1 strains in YEPG plus 10% (v/v) ethanol respectively, and filled and open squares indicate wild and mut1 strains in YEPG plus 12% (v/v) ethanol respectively.

DISCUSSION

EMS is a mutagenic agent that induces point mutations in a DNA molecule by A-T transition to G-C. In the presence of EMS, native sequences of affected genes are changed and their related products are modified structurally, causing inactivation of functional proteins (29). Wahlbom *et al.* (21) in 2003 and Khattab *et al.* (23) in 2005 reported that EMS is a suitable mutagen for related purposes. In addition, French *et al.* in 2006 reported that EMS is a powerful chemical mutagen and its effect on cells is related to its concentration in a medium (22). Our findings about mutagenesis induced by EMS were in good agreement with the above reports and

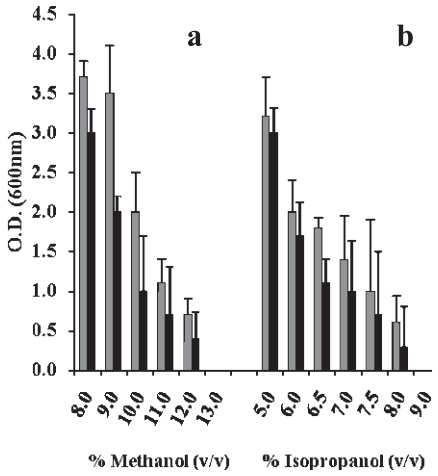

FIG. 6. Growth rates of wild-type (open bars) and mut1 strain (closed bars) in the presence of different concentrations of methanol (a) and isopropanol (b) at 30°C with shaking at 70 rpm for 48 h.

confirmed the usefulness of this potent mutagenic agent for inducing mutagenesis in *S. cerevisiae*.

Among many mutagenized cells of *S. cerevisiae*, only six mutants showed the ability to grow on solid ALP medium supplemented with 10% (v/v) ethanol. Although the growth of wild-type cells was inhibited in the presence of ethanol at concentrations higher than 10% (v/v) (27), mutant cells did not grow in solid medium with 12% (v/v) or higher concentrations of ethanol. The reason for the ability of afew yeast mutant cells to grow in the presence of 10% (v/v) ethanol may be the presence of an additional tolerance mechanism(s) or mutations in their nonvital genes (2). Therefore, the majority of EMS-mutagenized cells were dead under this condition.

Bioethanol production by all mutant strains was performed using the microfermentation method. The advantage of this technique is rapid screening of many yeast strains for bioethanol production in a short time. Our data are in a good agreement with the findings of Sreenath *et al.* in 1996: they reported that small-scale fermentation is suitable for yeast screening for bioethanol production (1). Bioethanol was successfully produced in microtubes for all strains. Among the six resistant mutant strains that we obtained, there was one strain named mut1 that showed bioethanol production higher than that of the wild type. Because of the complexity of metabolic pathways in *S. cerevisiae*, this strain is assumed to harbor mutations in specific genes that are related to bioethanol synthesis. Jimenez *et al.* (27) already reported a phenotypically similar wild-type strain originating from wine. In addition, Bailey *et al.* isolated mutant strains of *S. cerevisiae* that were resistant to catabolite repression (5). Walhbom *et al.* mutagenized a recombinant strain of *S. cerevisiae* to improve xylose utilization and bioethanol production (21). Osho isolated several wild-type strains of *Saccharomyces* yeast from fermenting cashew apple (a tropical fruit) juice that were able to tolerate high concentrations of ethanol and glucose (15). To our knowledge, this is the first report about EMS application to isolation of mutant strains of *S. cerevisiae* that were able to tolerate high concentrations of ethanol and showed higher bioethanol production than the wild type.

The trehalose contents and growth rates of mut4, mut5, and mut6 were lower than those of other mutants. As trehalose has an important role in preservation of proteins and cell membranes, strains with higher trehalose contents such as mut1, mut2, and mut3 were more resistant to ethanol and showed higher growth rates in the presence of 10% (v/v) ethanol. This observation is in agreement with Sharma's finding that trehalose has a role in ethanol tolerance (3).

Periplasmic invertase is encoded by *Suc2* gene in *S. cerevisiae* (17) and its expression controlled tightly by monosaccharides such as glucose and fructose (30). The complexity of expression regulation of the *Suc* gene family causes a higher susceptibility to mutagenic substances such as EMS. Our data on invertase activity in mutant strains were in agreement with the findings of Osho (15) that strains resistant to exogenous ethanol have a higher invertase activity than normal cells. All mutant strains isolated in this study, were able to grow in the presence of 10% (v/v) ethanol on ALP medium and showed a higher invertase activity than the wild type. Therefore, the expression of the invertase enzyme gene was enhanced in all mutant strains but the expression level was not the same in all mutants. In spite of the high invertase activity in all mutant strains, only the mut1 strain showed higher bioethanol production than the wild type. Thus, ethanol production capacity in these mutant strains was not directly related to intracellular trehalose content and invertase enzyme activity. In the mut2 strain, for example, trehalose content and invertase activity were higher than those in the mut1 strain but this strain did not show a higher bioethanol production than mut1. Therefore, the high bioethanol production in the mut1 strain may be due to different mutation(s) in various metabolic pathways and may be multi-factorial.

In summary mut1 can tolerate high concentrations of ethanol. This mutant strain show a high measurable growth rate in the presence of 10% to 12% (v/v) ethanol. The results of this experiment are in agreement with trehalose content and invertase activity data. This mutant strain showed good selectable resistance to exogenous ethanol but was not able to tolerate any of the alcohols examined. Fujita *et al.* reported that there are a number of common genes required for tolerance to ethanol and other alcohols and there are specific genes responsible for resistance to specific alcohols such as ethanol or 1-pentanol, as well (31). It seems that mut1 harbors mutation(s) in specific genes related to tolerance ethanol but not to other alcohols, such as methanol and isopropanol. However, molecular characterization of this mutant strain should be elucidated in the near future particularly in relation to ethanol production capacity.

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