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Characterization of an interesting novel mutant strain of commercial *Saccharomyces cerevisiae*

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Abstract
The yeast strains that are resistant to high concentration of ethanol have biotechnological benefits and are suitable models for physiology and molecular genetics research fields. A novel ethanol-tolerant mutant strain, mut1, derived from the commercial *Saccharomyces cerevisiae* showed higher ethanol production, and also demonstrated resistance to ethanol but not to other alcohols, such as methanol, 2-propanol, and 1-butanol. To characterize mut1, the strain's resistance to other organic compounds and osmotic and cell wall stresses were examined. The growth of the mut1 strain in the presence of ethyl n-caproate and 3-methyl butyl acetate, which were metabolic derivatives of ethanol, was found to be less than the wild type. On the other hand, the growth of the mut1 strain in the presence of 50% (w/v) sucrose and 1M NaCl was similar to that of the wild type. The sensitivity to cell wall digestive enzyme, zymolyase, was also similar in both wild and mut1 strains. Finally, the mut1 strain showed resistance to homocysteine and serine but was sensitive to methionine. These results suggest that the ethanol resistance of the mut1 strain may be more related to the ethanol metabolic and signalling pathways rather than the enhanced stress resistances relating to the membrane or cell wall compositions.

Keywords**:** Ethanol; Tolerance; Metabolic and signalling pathways; Fermentation

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INTRODUCTION

Saccharomyces cerevisiae is a model microorganism for different fields of biology and biotechnology. In addition, mutant strains of the yeast *S. cerevisiae* can be used either directly in applied aspects of fermentation process or in reverse metabolic engineering (Hashimoto *et al*., 2005). Among a great variety of stresses during the fermentation process, ethanol is the main stress factor (Martini *et al*., 2004). The toxic effects of ethanol involve the synthesis of stress proteins, disruption of cell membranes, modulation of ion exchange, permeability of the cytoplasmic membrane, accumulation of high levels of trehalose in the cytoplasm, inactivation of some enzymes such as crucial glycolytic enzymes and ATPase, damage to the mitochondrial DNA, reduction of metabolic activities, and decreasing of growth rate and fermentation ability (Wang *et al*., 2007; Aguilera *et al*., 2006; Birch and Walker, 2000). In fact, ethanol, acetic acid, and hydrogen peroxide are currently known as inducible agents of apoptosis in yeast cells (Kitagaki *et al*., 2007).

With regard to growth rate of yeast in the presence of a stress factor such as ethanol, it is obvious that strains resistant to environmental stresses have distinct mechanisms to overcome unfavourable conditions. All stress factors can induce vast changes in the expression of yeast genes, because cells must rapidly adapt themselves to a new situation (Cheraiti *et al*., 2008; Katasushiro *et al*., 2000). More than 200 genes are involved in response to different stress conditions

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(Rokhlenko *et al*., 2007; Alexandre *et al*., 2001). Many factors have been reported to play effective roles in yeast's ethanol resistance. The most important factors include intracellular accumulation of trehalose, the degree of unsaturation of fatty acids in the cytoplasmic membrane, induction of heat-shock proteins, activation of a plasma membrane ATPase, induction of heat-shock proteins, selective mRNA export, and ergosterol biosynthesis (Watanabe *et al*., 2007). Therefore, improvement of a stress resistance mechanism could be effective in the construction of yeast strains that produce high levels of ethanol.

Recently, a mutant *S. cerevisiae* strain, mut1, which showed specific ethanol tolerance and higher ethanol production was isolated (Mobini-Dehkordi *et al*., 2008). In this study, in order to know the mechanism of ethanol tolerance, the mut1 strain was characterized physiologically. The results suggest that mut1 is not a stress resistant mutant relating to membrane and cell wall composition; rather it may be a mutant of the ethanol metabolic and signalling processes that uses in microbial biotechnology as a suitable host and in microbial genetics as a powerful research tool.

MATERIALS AND METHODS

Microorganism, storage and cultivation: Commercial *Saccharomyces cerevisiae* was obtained from S.I. Lesaffre Company in France. The strain was stored on a slant of yeast-extract chloramphenicol agar (0.5% yeast extract, 2% glucose, 1.49% agar-agar and 0.01% chloramphenicol, w/v) at 4°C. After mutagenesis approach based on Hahn's method (www.bio.com/protocolstools/protocol.jhtml) using ethyl-methane sulfonate (EMS), yeast cells were grown on yeast-extract peptone dextrose (YPD) agar (1% yeast-extract, 1% peptone, 2% glucose and 1.5% agar-agar). Cell viability was estimated by the standard plate counting method. In order to grow strains resistant to exogenous ethanol, a petridish containing aerobic low peptone (ALP) agar (1.5% agar, 0.1% $(NH_4)_2SO_4$, 0.05% pancreatic digest of casein, 0.05% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.02% KCl, 0.02% phenol red, (w/v) and 8-12% (v/v) ethanol) was inoculated with 100 µl mutagenized cell suspension, and then sealed tightly by parafilm and incubated at 30°C for several days. The resulting colonies were then subcultured by being transferred to fresh solid YPD medium (Nozawa *et al*., 2002). All media and chemicals were purchased from the Sigma Company (St. Luis, USA) except of few items that purchased from the Japan Companies.

Evaluation of resistance to exogenous chemicals: For evaluation of resistance to different chemicals in broth medium, once an optical density of 1.0 was recorded at 600 nm (OD_{600}), 100 µl of each of the yeast cultures with was used to inoculate 3.9 ml of YPD broth supplemented with 0.1%-0.4% (v/v) ethyl n-caproate (Wako, Japan), 0.5%-1.5% (v/v) 3-methyl butyl acetate (TCI, Tokyo, Japan), 0.5%-4% (v/v) ethyl acetate (Katayama, Japan), 0.5%-2% (w/v) homocysteine (Sigma), in caped test tubes of a biophotorecorder (TVS062CA, Advantec, Tokyo, Japan). This machine is capable to determine optical density of test tubes in each 30 min interval continually and

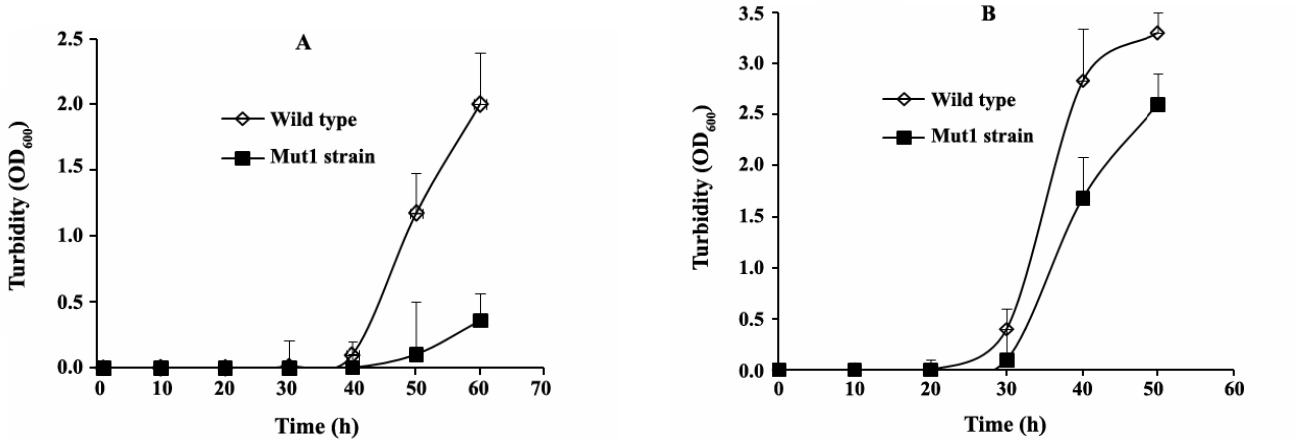

Figure 1. A: The growth rate of the wild and mut1 strains in the presence of 0.4% (v/v) ethyl n-caproate and B: 1.5% (v/v) 3-methyl butyl acetate in YPD broth. This experiment was performed in three repeats at 30°C and 70 rpm for 60 h.

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record all data. In addition, the growth rate of each of the yeast strains was also evaluated in 4 ml of O medium (5% glucose, 1% peptone, 0.5% yeast extract, 0.4% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4$ and 0.15% methionine, w/v, pH 6) in caped test tubes. Based on these experiments, the growth rate of yeast strains in the presence of different chemicals was recorded at 30ºC for 72 h continuously (Cheraiti *et al*., 2008).

Evaluation of osmotic pressure resistance: For evaluation of the effects of osmotic pressure on the growth rate of the two yeast strains, 100 µl of each yeast suspension (as mentioned above) was used to inoculate 3.9 ml of YPD media, each containing 1M NaCl and 50% (w/v) sucrose, in caped test tubes of a biophotorecorder. The growth rates were recorded at 30ºC for 72 h, continuously (Birch and Walker, 2000).

Sensitivity to cell wall lysis enzyme: For evaluation of sensitivity of yeast cell walls to the zymolyase-100T (Seikagaku America, Taiwan) enzyme, log phase cells grown in YPD at 30ºC were washed once with water, and resuspended in Na-phosphate buffer (0.1 M and pH 7.5) to an OD_{600} of 1.50. At time zero, zymolyase solution was added to a final concentration of 10 µg/ml and the decrease in turbidity was measured at 30ºC with an automatic detector such as biophotorecorder (Birch and Walker, 2000).

Statistical analysis: The t-Test and analysis of variance (ANOVA) were used for the analysis of all experimental data that were obtained from experiments carried out in triplicate. SPSS 14.0.0 (2005) software was used for the purpose of these analyses, and standard deviations were calculated for each experiment.

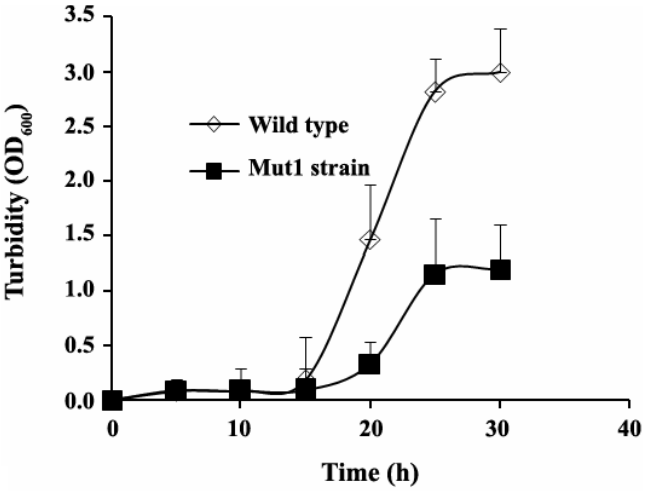

Figure 2. The growth rate of the wild and mut1 strains in the presence of 5% of ethyl acetate. This experiment was performed in three repeats at 30°C and 70 rpm for 30 h.

RESULTS

We isolated six mutant strains of *S. cerevisiae* that were resistant to 10% (v/v) ethanol. One of them was named mut1 that grew in ALP containing 10% (v/v) ethanol better than others. The growth rate of the mut1 strain was very low in the presence of 0.4% (v/v) ethyl n-caproate and 1.5% (v/v) 3-methyl butyl acetate (Figs. 1A, B) when compared to the rapid growth of the wild type. Based on the ANOVA test, these differences were significant at 1% level ($p<0.01$). On the other hand, the mut1 strain was more sensitive to ethyl acetate than the wild type and showed weak growth under this condition (Fig. 2). The difference between the two strains in the presence of ethyl acetate was also found to be significant at 1% level ($p<0.01$). In addition, both wild and mut1 strains showed proper growth

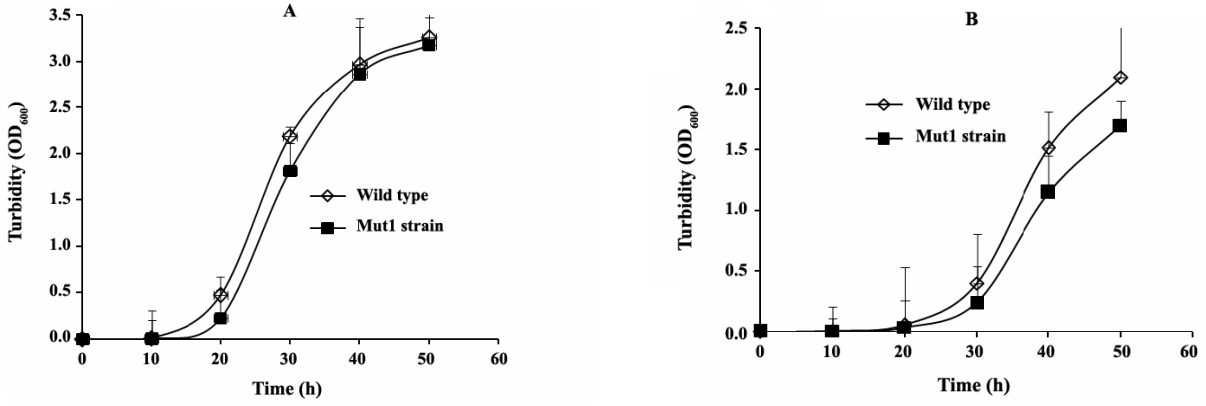

Figure 3. A: The growth rate of the wild and mut1 strains in the presence of 50% (w/v) sucrose and B: 1M NaCl in YPD broth. This experiment was performed in three repeats at 30°C and 70 rpm for 50 h.

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rates in the presence of 50% (w/v) sucrose and 1M NaCl, but the wild type was more resistant to osmotic pressure than the mut1 strain (Figs. 3A, B). The difference between the strains with regard to osmotic stress conditions was significant at 1% level ($p<0.01$). Although sensitivities of the wild and mut1 strains to the zymolyase-100T enzyme were similar and the cell densities of the two yeast cultures decreased after 10 h, but the mut1 strain was nearly more sensitive than the wild type (Fig. 4). This difference was significant at 1% level ($p<0.01$). Finally, the mut1 strain showed more resistance to homocysteine and serine (data not shown) than the wild type. Conversely, the mut1 strain was more sensitive to methionine than the wild type (Figs. 5A, B). These mentioned differences were significant at 1% level ($p<0.01$).

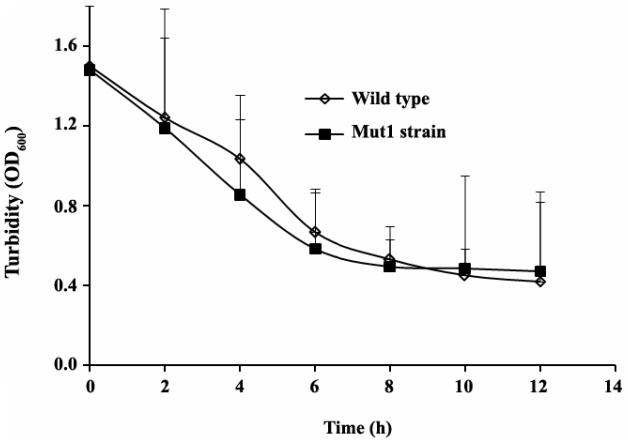

Figure 4. Decrease of turbidity of the wild and mut1 strains in the presence of zymolyase T-100 enzyme in phosphate buffer. This experiment was performed in three repeats at 30°C and 70 rpm for 12 h.

DISCUSSION

Different experiments were performed for the characterization of the mut1 strain. A previous report had shown that the mut1 strain could tolerate only high concentrations of ethanol among the different alcohols, which included methanol, 2-propanol and 1-butanol. Although there are genes in the yeast cell that play a role in resistance to different alcohols (Fujita *et al*., 2006), but the mut1 strain does not show this phenotype. Infact, there are some genes in the yeast cell that are specific for resistance to each alcoholic compound or its derivatives. There is a specific ring finger protein in *S. cerevisiae* for signalling ethanol, 2-propanol, and 1-butanol stresses to the nucleus. Deletions of the *ASR1* gene, can sensitize yeast cells to butanol but not to ethanol (Betz *et al*., 2004). As the mut1 strain is susceptible to 1-butanol, 3-methyl butyl acetate, and ethyl-n-caproate, it is possible that the *ASR1* gene or its related genes are inactivated in the mut1 strain by our mutagenesis approach using EMS. In addition, based on this finding, we have predicted that there are specific genes related to the signalling pathways of methanol, 2-propanol, and other organic chemicals in yeast cells. Since, the mut1 strain showed only selective resistance to ethanol and could not tolerate other chemicals such as ethyl-acetate; it can be concluded that the mutagenesis process has modified

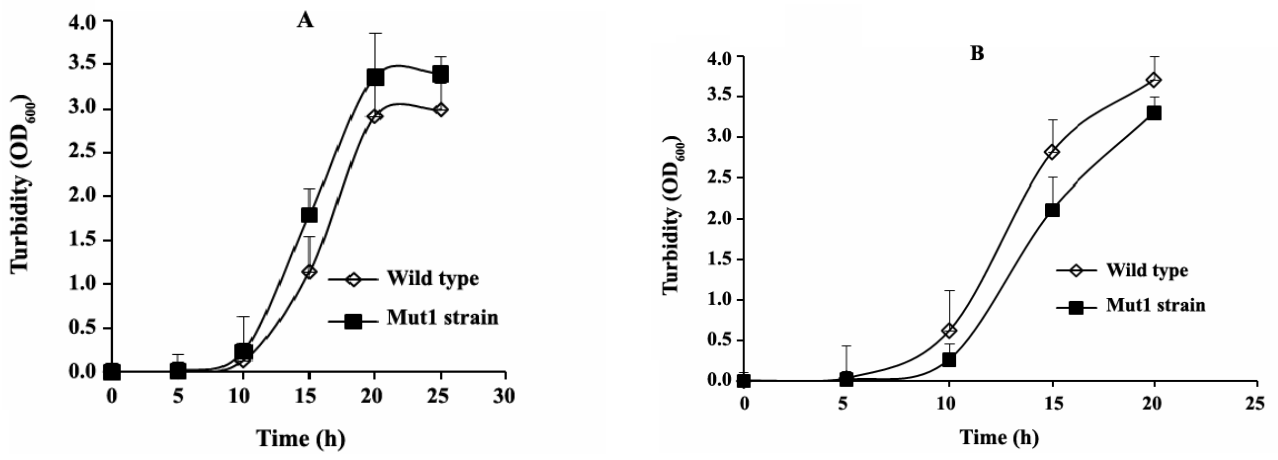

Figure 5. A: The growth rate of the wild and mut1 strains in the presence of 1.5% (w/v) homocysteine and B: in O medium. This experiment was performed in three repeats at 30°C and 70 rpm for 25 h and 20 h respectively.

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the expression levels of the genes resistant to such molecules (Mobini-Dehkordi *et al*., 2008). On the other hand, this might be related to the presence of specific biochemical compounds in the mut1 cells that were active in the presence of ethanol but not functional in the presence of other chemicals. This study shows that the mut1 strain may be considered as highly valuable in molecular biotechnology for the evaluation of specific signalling pathways related to different alcohols and their derivatives.

The mut1 strain could tolerate both high osmotic pressure and ethanol. This finding emphasizes the physiological role of the yeast cytoplasmic membrane, which is in agreement with findings of Aguilera (2006). They reported that ethanol resistance in the wine strains of *S. cerevisiae* was related to ergosterol, oleic acid, and palmitoleic acid contents of the cytoplasmic membrane. In addition, the survival of palmitic acid-enriched cells is better than that of the linoleic acid-enriched cells, in the presence of high concentrations of ethanol (Nozawa *et al*., 2002). Since the cytoplasmic membrane and its type of lipid components can determine yeast cell resistance to ethanol stress, the mut1 strain may harbour biochemical changes in its cytoplasmic membrane composition. In this mutant strain, permeability of the cytoplasmic membrane had changed because hydrophobic compounds, such as ethyl caproate and methyl butyl acetate could penetrate the yeast cells thus affecting their growth cycle. In addition, some amino acids, such as methionine and serine could be transported into the mut1 cells at a faster or slower rate, respectively, when compared to the wild type cells (Rokhlenko *et al*., 2006).

The mut1 strain did not show resistance to compounds that contained the ethyl group, such as ethyl n-caproate and ethyl acetate. Therefore, mut1 harbours specific strategies with regard to its gene expression that can tolerate only exogenous ethanol but not its derivatives. This strain can be used in biotechnology as an interesting host for controlling heterologous gene expression in the presence of specific chemicals. Since both wild type and mut1 strains were sensitive to the cell wall degrading enzyme (zymolyase) and could tolerate osmotic pressure caused by 50% (w/v) sucrose and 1M NaCl, changes in cell wall composition of the mut1 strain was not severe. Therefore, the resistance to ethanol cannot be related to cell wall structure/composition in this strain.

Based on a recent report, deletions of the *URA7* and *GAL6* genes improve ethanol tolerance and fermentation capacity in *S. cerevisiae* (Yazawa *et al*., 2007). The URA7p is the major CTP synthase isozyme in yeast and the GAL6p is cysteine aminopeptidase with homocysteine-thiolactonase activity that protects yeast cells against homocysteine toxicity. Therefore, in a very complex phenomenon in yeast cells, such as ethanol resistance, inactivations of distinct genes in the yeast metabolic pathways cause improvement of ethanol tolerance. As mention above, the GAL6p has an important role in the homocysteine detoxification of yeast cells. Despite the sensitivity of the mut1 strain to many chemicals such as ethyl n-caproate, ethyl acetate, 3-methyl butyl acetate, methionine in the O medium, this strain showed more resistance to homocysteine and serine than the wild type. Therefore, there is a direct relationship between ethanol tolerance and other metabolic pathways in the yeast cell, such as amino acid and pyrimidine synthesis. In addition, since the mut1 strain shows resistance to both ethanol and homocysteine, it must use other genes instead of *GAL6* for homocysteine detoxification. Evaluation regarding the inactivation of the putative genes in the mut1 strain related to the high tolerance of ethanol and specific signaling pathways for different organic chemicals are suggested as future research.

As a result, the mut1 strain to meet changes in the genome is the only feature that can show resistance to ethanol. This property is mainly linked with the yeast metabolic pathways and different genes, regulatory elements, and signaling proteins associate with this interesting phenotype.

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