An Organic Acid-Tolerant *HAA1*-Overexpression Strain from Industrial Bioethanol Yeast

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***Abstract*—**Organic acids are an effective agent for the prevention of bacterial contamination, but it negatively affects the fermentation ability of *S. cerevisiae*. We showed that overexpression of the *HAA1* gene, which encodes a transcriptional activator, could be a useful molecular breeding method for organic acid -tolerant yeast strains. We constructed a *HAA1*-overexpressing diploid strain (*MAT* **a**/α, named ER HAA1-OP) derived from the industrial bioethanol strain Ethanol Red (ER). ER HAA1-OP showed tolerance to organic acids. It was suggested that the tolerance to organic acids was dependent on the increased expression of *HAA1*.

***Keywords*—** bioethanol; organic acids tolerance; *Saccharomyces cerevisiae*; sugarcane molasses

I. Introduction

The utilization of bioethanol as an alternative to fossil fuels has attracted much attention in the effort to combat global warming and improve energy reserves. Bacterial contamination is known as a major cause of reductions in the yield of ethanol from such feedstock by yeast, *Saccharomyces cerevisiae* [1]. Various agents have been examined for their potential to control bacteria and thereby avoid the reduction of ethanol yields. It has been reported that potassium metabisulfite and antibiotics effectively inhibit bacterial contamination [2]. In designing a bioethanol production process that eliminates bacterial contamination, we focused on organic acids including acetate as a control agent.

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Acetate, which is a typical organic acid, strongly inhibits bacterial growth and viability at elevated levels; however, the addition of high concentrations of acetate may also reduce the fermentation ability of *S. cerevisiae*. At an external pH below the pKa value, the acetate predominantly assumes an undissociated lipophilic form and can permeate the plasma membrane by simple diffusion. At natural cytosolic pH, dissociation of the acids leads to the release of protons and the respective anions, which induces intracellular acidification [3, 4].

To overcome these conflicting effects of addition, we attempted to construct organic acids tolerant strains from the industrial *S. cerevisiae* strain Ethanol Red (ER).

In this study, we constructed tolerant strain to organic acids derived from the bioethanol yeast *S. cerevisiae* ER. Because organic acids show a negative effect on the growth of contaminated bacteria and is a safe and inexpensive reagent for inhibiting bacterial growth, we examined whether the combined use of organic acids and a strain tolerant to organic acids would be suitable for industrial ethanol production.

II. Materials and Methods

A. Yeast strains

As the parent strain, we employed ER (kindly provided by Le Saffre), which has been developed for industrial ethanol production. Two haploid strains derived from ER, ER-6c (*MAT* ***a***) and ER-3a (*MAT* α), and their *ura3Δ0* derivatives were used to construct the *HAA1*-overexpressing strain [5].

B. Construction of the HAA1-overexpressing strain

Construction strategy of the *HAA1*-overexpressing strain was illustrated in Figure 1. In Brief, the promoter region of the *TDH3* gene, which allows constitutive expression at a high level, was fused with the *URA3* marker gene and then inserted upstream of the start codon of the *HAA1* gene [6]. Diploid strains were constructed by mating haploid strains of opposite mating types in YPD medium. Overnight cultures of each haploid parent were mixed and incubated at 30°C for 4 h without shaking. The mixtures were diluted 50-fold with fresh YPD medium. After cultivation overnight at 30°C, the mating

mixture was plated onto YPD agar. Diploid strains were selected on the basis of colony size, yielding strain ER HAA1-OP. Diploid formation was confirmed based on spore formation ability and PCR analysis of the *MAT* loci.

C. Quantitative real-time PCR analysis

To determine the expression level of the *HAA1* gene in each overexpressing strain, quantitative real-time PCR (qRT-PCR) analysis was performed. Total RNAs were extracted from logarithmically growing cells using the hot phenol method. Synthesis of the cDNAs from the total RNAs was performed using a PrimeScript II High Fidelity RT-PCR kit (Takara, Ohtu, Japan) with an oligo dT primer. Real-time PCR was carried out using a FastStart Essential DNA Green Master (Roche, Mannheim, Germany) and LightCycler nano (Roche). Data analysis was performed using the LightCycler Nano software version 1.0 (Roche).

D. Organic acids stress tolerance test

To test the organic acids tolerance of the yeast transformants, each yeast strain was cultured in YPD medium until OD_{600} ~4-5 and then serially diluted, 10-fold at a time, and 4 µl of each diluted solution was applied to YPD agar medium containing 0.5% (w/v) , 0.3% (w/v) formate, or 9% (w/v) lactate.

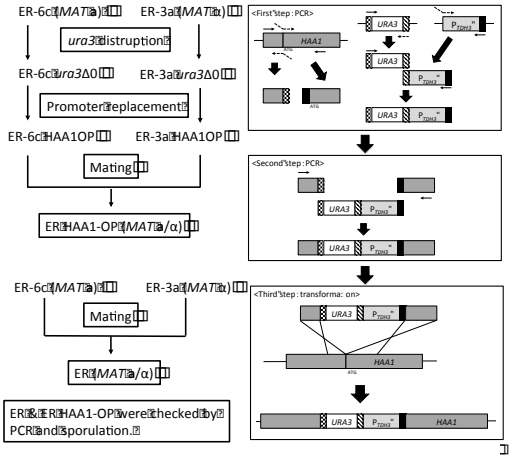

Fig. 1 Strategy for *HAA1*-overexpresing strain

III. RESULTS

A. Construction of a diploid HAA1-overexpressing strain and confirmation of the HAA1 gene expression level.

To construct a diploid strain overexpressing *HAA1* gene derived from *S. cerevisiae* ER, we employed a strategy of mating haploid strains that overexpressed the *HAA1* gene. Several haploid strains were obtained by tetrad dissections from ER and their fermentation patterns were analyzed. Because the fermentation abilities of ER-6c and ER-3a were intermediate between those of the other haploid strains, we used ER-6c and ER-3a as the *MAT* ***a*** haploid strain and *MAT* α haploid strain, respectively. Mating was performed between strains ER-6c HAA1-OP and ER-3a HAA1-OP to obtain the diploid *HAA1*-overexpressing strain ER HAA1-OP (*MAT a/α*). Diploid formation was confirmed by both spore formation ability and PCR analysis of *MAT* loci.

To estimate the expression levels of *HAA1* and Haa1-regulated genes in HAA1-OP, qRT-PCR analysis was carried out. The results indicated that the expression levels of these overexpressing strains were approximately 7-8 times higher than that of parent strain. In Figure 2, the expression levels of the HAA1 gene in ER HAA1-OP and parental cells grown in YPD medium are shown. These results show that the expression level of the *HAA1* gene in ER HAA1-OP is 6 times higher than that in the parent strain.

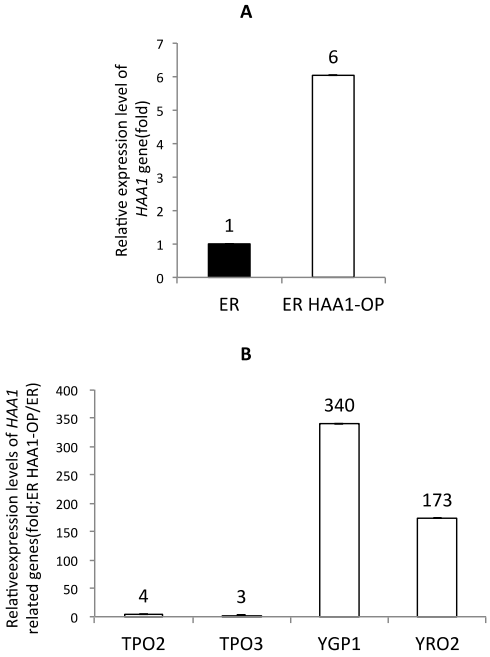

Fig. 2. Expression levels of *HAA1*-overexpressing strains. Expression levels were monitored in RNAs extracted from cells of *S. cerevisiae* ER and ER HAA1-OP.

B. Stress tolerance to organic acids of the HAA1-overexpressing strain

To determine the effects of the overexpression of *HAA1* gene on stress tolerance to organic acids, the stress tolerance to organic aicds of ER HAA1-OP (Fig. 3) was evaluated. As expected, ER HAA1-OP showed drastically higher tolerance to organic acids than each parent strain.

We also found that ER HAA1-OP showed tolerance to formate and lactate (Fig. 3).

IV. DISCUSSION

In this study, we showed the possibility of designing an effective bioethanol production system with the addition of organic acids. Previously, we reported that an

HAA1-overexpressing strain derived from a laboratory strain showed higher tolerance, and that the overexpression of *HAA1* induced the expression of Haa1-regulated genes, including *TPO2* and *TPO3*, the products of which are considered major facilitator superfamily transporters of the *S. cerevisiae* plasma membrane [6]. In the present study, the constitutive *HAA1*-overexpressing diploid strain (ER HAA1-OP) was constructed from the industrial yeast strain *S. cerevisiae* ER. The ER HAA1-OP strain showed not only higher organic acids tolerance by spot assay but also higher fermentation ability (data not shown) in the presence of 0.5% acetate than the wild-type strain. We consider the combined use of strains tolerant to organic acids, including and lactate, to be suitable for industrial ethanol production from molasses, because organic acids are safe and inexpensive reagents for inhibiting bacterial growth [2, 7]. By making antibiotics unnecessary, our proposed ethanol production system offers several advantages. One of the most important is that the waste generated during antibiotic-free bioethanol production can be used safely as forage or fertilizer. In the future, inexpensive methods for preparing organic acids, such as recycling, need to be investigated for industrial application. However, ER HAA1-OP clearly has the ability to produce ethanol in molasses medium containing 0.5% acetate.

In future studies, it may be useful to pursue a bioprocess which eliminates the heat sterilization process of feedstock, which is one of the most energy-consuming steps in bioethanol production. We believe that one of the key technologies in the design of economic and environmentally friendly processes is the improvement of industrial microorganisms, such as yeast and bacteria, by enhancing characteristics such as their tolerance to environmental stress. To enable the breeding of such microorganisms, detailed elucidation of their stress-tolerance mechanisms will be needed.

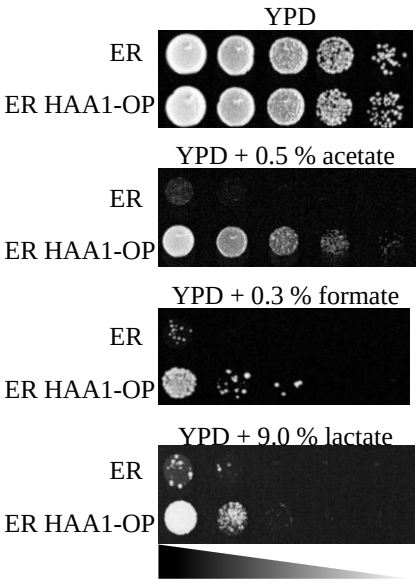

Fig. 3 Organic stress tolerance of *HAA1*-overexpressing strains. ER and ER HAA1-OP were spotted onto YPD medium, YPD medium containing 0.5% (pH 4.5) , YPD medium containing 0.3% formate, and YPD medium containing 9.0% lactate (pH 2.6), and incubated at 30^{o}C

V. Conclusion

We suggested the possibiity of designing an effective bioethanol production system with the addition of organic cids. In this study, the constitutive *HAA1*-overexpressing diploid strain was constructed from the industrial yeast strain *S. cerevisiae* ER. The ER HAA1-OP strain showed not only higher organic acids tolerance by spot assay but also higher fermentation ability.

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