A novel approach for the improvement of ethanol fermentation by *Saccharomyces cerevisiae*

Lihua Hou, Xiaohong Cao, and Chunling Wang

Abstract: Fermentation properties under the control of multiple genes are difficult to alter with traditional methods in *Saccharomyces cerevisiae*. Here, a novel genome engineering approach is developed to improve ethanol production in very high gravity fermentation with 300 g/L glucose as the carbon source. This strategy involved constructing aneuploid strains on the base of tetraploid cells. The tetraploid strain was constructed by using the plasmid YCplac33-GHK, which harbored the *HO* gene encoding the site-specific Ho endonucleases. The aneuploid strain, WT4-M, was selected and screened after the tetraploid cells were treated with methyl benzimidazole-2-yl-carbamate to induce loss of mitotic chromosomes. It was found that aneuploid strain WT4-M not only exhibited an increase in ethanol production and osmotic and thermal tolerance, but also an improvement in the sugar–ethanol conversion rate. Notably, WT4-M provided up to 9.8% improvement in ethanol production compared with the control strain. The results demonstrated that the strategy of aneuploidy was valuable for creating yeast strains with better fermentation characteristics.

Key words: aneuploid, ethanol, *Saccharomyces cerevisiae*, very high gravity.

Résumé : Les propriétés de fermentation contrôlées par de multiples gènes sont difficiles à modifier par les méthodes traditionnelles chez *Saccharomyces cerevisiae*. Une nouvelle approche d'ingénierie du génome a été développée ici afin d'améliorer la production d'éthanol par la fermentation à très haute gravité de glucose 300 g/L utilisé comme source de carbone. Cette stratégie a impliqué la construction de souches aneuploïdes à partir de cellules tétraploïdes. La souche tétraploïde a été construite à l'aide du plasmide YCplac33-GHK, qui comprend le gène *HO* codant les endonucléases de restriction Ho. La souche aneuploïde WT4-M a été sélectionnée et criblée après que les cellules tétraploïdes aient été traitées au méthyl benzimidazole-2-yl-carbamate afin d'induire une perte chromosomique lors de la mitose. La souche aneuploïde WT4-M montrait non seulement une augmentation de production d'éthanol et une tolérance osmotique et thermique, mais elle montrait aussi une amélioration du taux de conversion sucre–éthanol. WT4-M démontrait notamment une amélioration de 9,8 % de production d'éthanol comparativement à la souche contrôle. Ces résultats ont démontré que la stratégie de l'aneuploïdie était appropriée pour créer des souches de levures possédant de meilleures caractéristiques de fermentation.

Mots-clés : aneuploïde, éthanol, *Saccharomyces cerevisiae*, très haute gravité.

[Traduit par la Rédaction]

Introduction

As a result of the imminent decline in the availability of global fossil fuel energy and increasing concern over the issue of environment protection, partial substitution of fossil fuels with bioethanol has become an important renewable energy strategy. For technical reasons, ethanol production is mainly achieved at present through fermentation of starch- or sugar-based feedstock by *Saccharomyces cerevisiae*. The cost of feedstock accounts for an important portion of the total production cost in starch- or sugar-based ethanol fermentation, which, to a large extent, hampers the development of the ethanol industry. Hence, increasing the sugar–ethanol conversion rate and reducing the fermentation cost through implementation of high-gravity fermentation are key subjects of development for the starch- and sugar-based ethanol industry (Lin and Tanaka 2006). Very high gravity (VHG) fermentation is common in the ethanol industry; however, yeast is exposed to greater osmotic stress at the beginning of the process and to greater ethanol stress at the end of a batch than in conventional fermentation (Rautio et al. 2007). Greater ethanol and osmotic stress results in decreased growth rate, lower viability, prolonged fermentation cycle, and more residual sugar under VHG conditions (Devantier et al. 2005*a*).

Ethanol and osmotic stress tolerance, the sugar–ethanol conversion rate, and other fermentation characteristics are complex traits under the control of multiple genes that are broadly distributed throughout the genome in yeast (Steinmetz et al. 2002). These fermentation properties are difficult to alter with classical breeding, metabolic engineering, or other genetic manipulation methods with specific genes or pathways as targets. Therefore, employing a whole-genome engineering approach will be an effective way to manipulate yeast strains. The aim of this work was to find a new strat-

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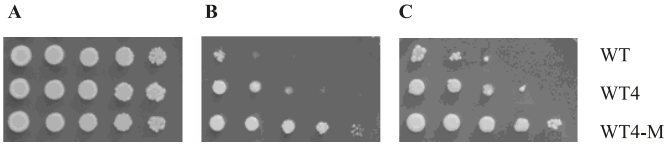
Fig. 1. Serial dilution assay of aneuploid strain WT4-M, tetraploid strain WT4, and diploid strain WT on different plates incubated at 30 °C for 4 days. (A) Nonselective (YPD) plates; (B) YPD plates containing 12% ethanol by volume; (C) YPD plates containing 300 g/L glucose. Experiments were carried out at least in triplicate, and one representative experiment result is shown.



egy by which yeast strains with better fermentation characteristics can be effectively and rapidly constructed.

Polyploidy and aneuploidy have often been observed in industrial yeasts (e.g., baking, brewing, distilling, and wine yeasts) (Bidenne et al. 1992; Vezinhet et al. 1990). However, diploid strains selected artificially are widely used in alcohol fermentation industries. During VHG fermentation, polyploidy and aneuploidy may confer on cells a greater level of protection against the occurrence of spontaneous lethal or detrimental recessive mutations or change gene dosage, thus improving fermentation performance (Salmon 1997).

In this study, a tetraploid strain was constructed from the diploid industrial strain via the plasmid pYCplac33-GHK carrying the Ho endonuclease gene (*HO*), which is under the control of a galactose-inducible promoter, and the *kanMX* gene as selectable marker. The aneuploid cells were produced by treating tetraploid cells with methyl benzimidazole-2-yl-carbamate (MBC), which induced mitotic chromosome loss in polyploid cells at a high frequency (Wood 1982). Moreover, the aneuploid strain, WT4-M, which had the best fermentation characteristics, was selected and screened by cultivation in VHG fermentation media.

Materials and methods

Yeast strains and growth conditions

The aneuploid and tetraploid strains used in this study were derived from the diploid industrial strain TH-AADY (WT) of *S. cerevisiae* (Angel Yeast, Yichang, China).

A serial dilution assay was carried out to evaluate the stress resistance of the yeast. The log-phase cells were added to 1 mL fresh YPD medium (2% peptone, 1% yeast extract, and 2% glucose). The number of log-phase cells in 1 mL of the cell suspension was determined: 10 μL aliquots with the same cell number were taken, diluted 10 000-fold, and 3 μL of the diluted cell suspension was spotted on YPD plates containing 15% (*v*/*v*) ethanol, YPD plates containing 25% (*m*/*v*) glucose, and nonselective (YPD) plates, respectively. The plates were incubated at 30 or 40 °C for 3 days and photographed to record the stress tolerance of the yeast strains.

Plasmid construction

The plasmid YCplac33 (RD Gietz 1988) was used to clone the *HO* gene, which was amplified with primers HO-Sense, 5′-GGGCCCGAGCTCATGCTTTCTGAAAACACGAC-3′ (*Sac*I), and HO-Anti, 5′-GGGCCCCCCGGGGTTAAGACTGCATTCATCAC-3′ (*Sma*I). The fragment was then digested by *Sac*I–*Sma*I and inserted into the corresponding sites of YCplac33, creating pYCplac33-H. Subsequently, the promoter of *GAL2* was amplified, using primers GAL-Sense, 5′-GGGCCCTCTAGACGCCAGATCTGTTTAGCTTG-3′ (*Eco*RI), and GAL-Anti, 5′-GGGCCCGAGCTCAGTATCGAATCGACAGCAGT-3′ (*Sac*I). The resulting PCR product was introduced into the *EcoR*I and *Sac*I sites of pYCplac33-H after digestion, generating pYCplac33-GH. Afterwards, the primers Kan-Sense, 5′-GGGCCCGAATTCGCGAAGGCACATCTATTAC-3′ (*Xba*I), and Kan-Anti, 5′-GGGCCCGCATGCGTTCTCCTCAACTGCCATTA-3′ (*Sph*I), were used to amplify *kanMX* from the plasmid pFA6-*kanMX4*. The resulting PCR product was cloned into the *Xba*I and *Sph*I sites of pYCplac33-GH, forming plasmid pYCplac33-GHK.

Mating-type analysis

To analyse the mating type of yeast cells (Haber 1998), colony PCR was carried out with primer MAT-Sense, 5′-AGTCACATCAAGATCGTTTATGG-3′; MAT-a, 5′-GCACGGAATATGGGACTACTTCG-3′; and MAT-α, 5′-ACTCCACTTCAAGTAAGAGTTTG-3′. If the PCR product is 544 bp, the mating-type of the cells is MATa. On the contrary, they are MAT-α cells when the fragment is 404 bp. MATa/α is determined when there are 2 fragments of 544 and 404 bp.

Flow cytometry analysis

Flow cytometry analysis (FCA) is a simple, fast, and reliable method for the analysis of genetic stability in yeast strains (Carlson et al. 1997; Hegemann et al. 1999). The log-phase cells were fixed with 70% EtOH and stained with propidium iodide, which bound DNA quantitatively and emitted red fluorescence with an intensity corresponding to the cellular DNA content (Ormerod and Kubbies 1992).

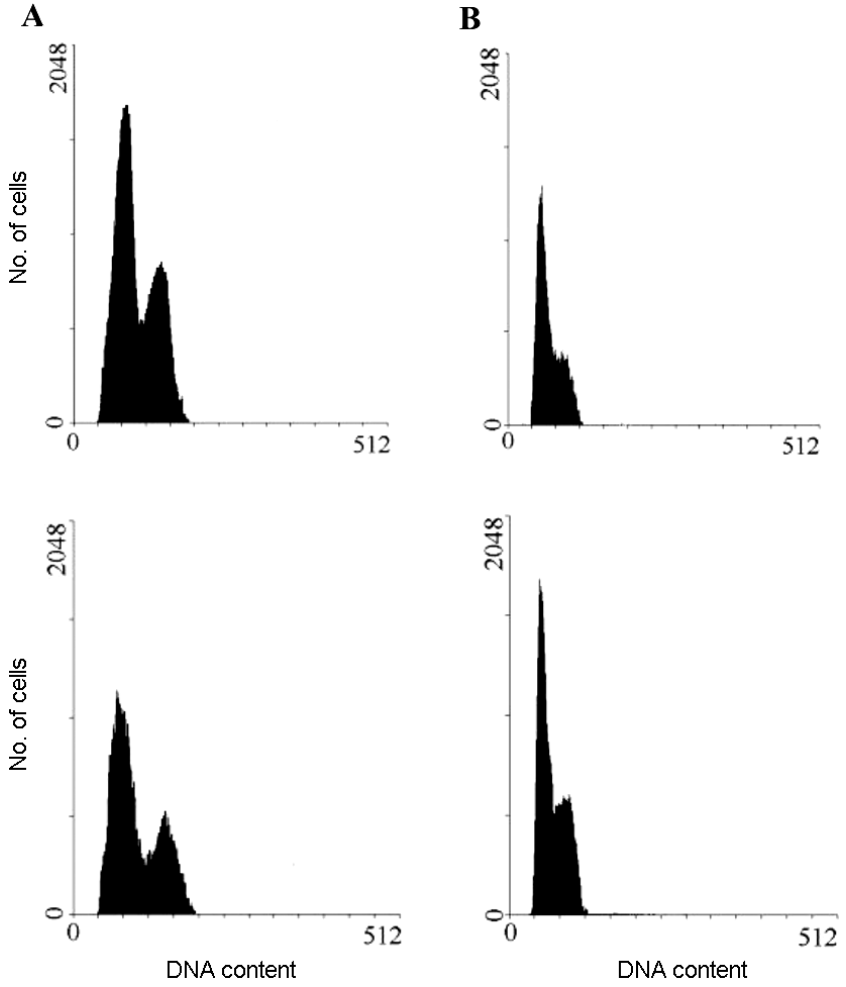
MBC treatment

MBC treatment was carried out as described by Wood (1982), with some modifications. Cells were grown overnight in liquid YPD medium to mid-log phase, and the culture was diluted to 5×10^6 cells/mL. The cells were subsequently treated with MBC (at a final concentration of 0.5 mmol/L) in liquid YPD medium at 23 °C and 200 r/min for 24 h. MBC was then removed from the culture by filtering the cells (0.45 μm pore size, type HA), and the cells were washed with 12 volumes of YPD liquid medium without glucose. Finally, the cells were suspended and cultivated in a fermentation medium containing 300 g/L glucose.

Fermentation conditions

The fermentation medium, which was prepared from corn powder by the double-enzyme hydrolyzed method as previously described (Kong et al. 2007), contained 300 g/L glucose, 0.5 g/L $(NH_4)_2HPO_4$, and 0.5 g/L K_2HPO_4. The inoculum was cultured in YPD medium at 200 r/min and 30 °C until the cell optical density (OD_{600}) reached 1.0. The cells collected by centrifugation at 25 °C and 1000*g* were then transferred to 250 mL cap-covered flasks, with an initial cell number of 2.5×10^7 per mL. The working volume of the flasks was set at 150 mL. The fermentation cultivations were carried out at 30 °C and 150 r/min, and pH 5.0

Fig. 2. Flow cytometry analysis of aneuploid strain WT4-M (A) and diploid strain WT (B). The initial cells (top) and the cells grown for 15 generations (bottom) were stained with propidium iodide. The horizontal axis indicates DNA content (channel), and the vertical axis indicates the number of cells (count). The first and second peak in the histogram represent prereplication and postreplication cells, respectively.



was controlled by adding 2 mol/L NaOH. Fermentations were repeated at least 3 times. Statistical significance ($p < 0.05$) was determined using the SAS statistical analysis program, version 8.01 (SAS Institute Inc. 2003).

Sampling and analysis

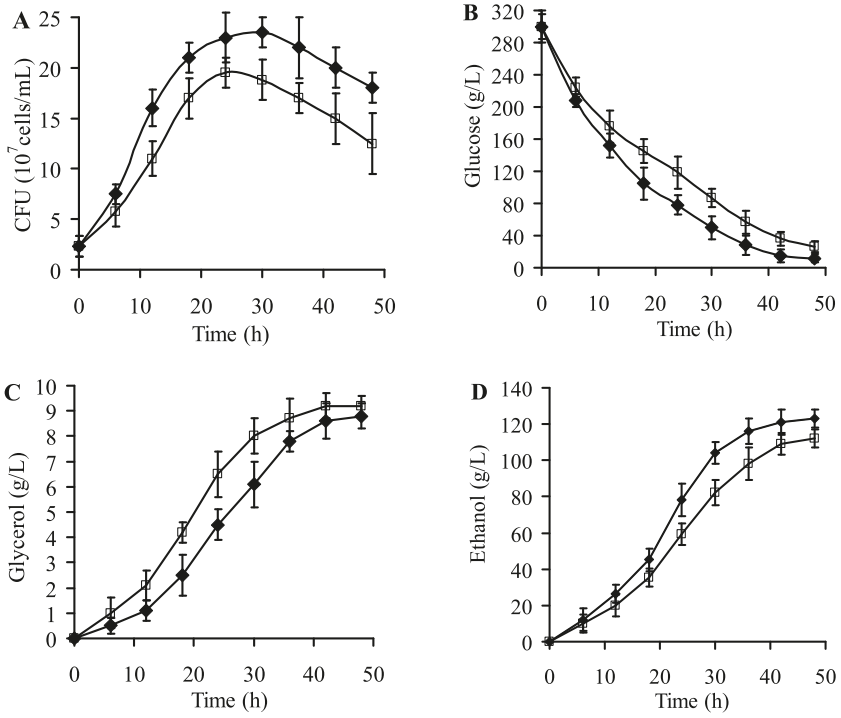
During the VHG fermentation, samples were taken every 6 h to measure colony-forming units per millilitre, determined by counting the number of colonies and multiplying the mean by the dilution factor. At the same time, supernatant analysis was conducted to identify concentrations of glucose, glycerol, and ethanol via high-performance liquid chromatography (HPLC) (HP1100, Hewlett Packard, Tokyo, Japan), using previously described modified methods (Chen and Peterson 1994). An XDB-C8 column (Agilent, Santa Clara, Calif., USA) for determination of ethanol concentration was eluted with 0.25 mmol/L H_2SO_4 (pH 3.1) at 30 °C. Glycerol and glucose were analyzed by a ZORBAX carbohydrate column (Agilent) eluted with 75% acetonitrile at 30 °C. Meanwhile, detection was carried out using a differential refractive index detector (RI-8120/8120P) with a flow rate of 1 mL/min. In addition, the concentration of another byproduct of ethanol fermentation, acetate, was determined by gas chromatography (Shimadzu GC-14B; Shimadzu Corporation, Kyoto, Japan) analysis with a DB-WAX capillary column (Shimadzu Corporation). N_2 was used as a carrier gas and H_2 as a flaming gas, with both at a flow rate of 32 mL/min.

Results

Strain construction and selection by means of MBC treatment

The plasmid pYCplac33-GHK was transformed into the diploid industrial strain by the lithium acetate method (Schiestl and Gietz 1989). After inducement of *HO* expression by galactose, transformants switched mating type, yielding MATa/a and MATα/α diploid cells, as confirmed by switching assays (Houston et al. 2004). The plasmid pYCplac33-GHK in the MAT a/a and MAT α/α cells was then lost by cultivation in liquid YPD medium, respectively (Houston et al. 2004). A polyploid strain, WT4, was constructed by the fusion of the above diploid cells of complementary *MAT* genotype to each other. WT4 was subsequently inoculated onto sporulation plates, and asci were dissected. Sporulation and ascus dissections of WT4, in which all 4 spore clones did not cross (data not shown),

Fig. 3. Changes in measured parameters during microanaerobic batch cultivation of aneuploid strain WT4-M (black diamond, ◆) and diploid strain WT (white square, □), respectively, with an initial cell number of 2.5×10^7 cells and an initial glucose concentration of 300 g/L: (A) colony-forming units (CFU), (B) consumption of glucose, (C) formation of glycerol, and (D) production of ethanol. Results are the mean of at least 3 independent experiments. Error bars in the figure represent SD. Statistical significance was determined using the SAS statistical analysis program, version 8.01. The data were analyzed using a Student's *t* test; $p < 0.05$ was considered significant.



were conducted to verify that WT4 was a tetraploid strain (Mayer and Aguilera 1990).

To obtain the aneuploid strain, tetraploid cells were treated with the mitotic inhibitor MBC. All mutant cells were then cultivated in fermentation medium containing 300 g/L glucose for about 10 d. The majority of cells died because of decreasing carbon source and increasing ethanol concentration, while a few living cells with higher ethanol tolerance remained in the culture medium. The cells from the above medium were harvested and then spread onto YPD plates containing 12% ethanol by volume. After 2–3 days, 36 colonies appeared. As a control, no colonies were observed when diploid strain WT and tetraploid strain WT4 were plated onto the YPD plates with 12% ethanol by volume. Subsequently, the 36 colonies were evaluated in fermentation media (data not shown), and the strain with the best fermentation properties, WT4-M, was selected and screened. WT4-M could not sporulate on sporulation plates, which suggested it was probably an aneuploid strain (Salmon 1997). It was found that DNA content per 10^9 cells of WT4-M, WT4, and WT was 78.13 ± 0.42 μg, 96.42 ± 0.47 μg, and 47.95 ± 0.38 μg, respectively. The results confirmed that WT4-M was an aneuploid strain.

Ethanol tolerance test of the selected strain

To understand the effect of several adverse conditions on the selected strains, serial dilution assay was carried out under stress conditions. Figure 1 shows the ethanol and osmotic tolerances of aneuploid strain WT4-M, tetraploid strain WT4, and diploid strain WT, respectively. Enhancing the ethanol resistance, which is closely related to ethanol productivity, is a useful approach to improve fermentation performance, since ethanol is toxic to cells (Jones 1989). Moreover, osmotic tolerance is also a key trait of yeast strains in ethanol fermentation, especially in VHG fermentation. The results indicated that the stress tolerance of WT4-M was greater than that of WT4 and WT. Because WT4-M provided the most desirable phenotype with respect to elevated alcohol and osmotic tolerance, the aneuploid strain was the focus of the remainder of this work.

Stability analysis of the selected strain

To further investigate the stability of WT4-M during propagation, the initial cells and the cells that had grown for 15 generations were analyzed by FCA. Figure 2 displays the ploidy state of aneuploid strain WT4-M and diploid strain WT, as determined by FCA. The data indicated that WT4-M kept its genetic stability during propagation. The data (Fig. 2) suggested that WT4-M was still aneuploid during propagation, although Mayer and Aguilera (1990) pointed out that aneuploidy was easy to resolve into euploidy. The reason may be that aneuploid yeast cells can be stabilized after structural chromosome changes, including partial duplication and circular minichromosomes (Niwa et al. 2006).

Fermentation capability of the selected strain

Selected strain WT4-M and control strain WT were evaluated using fermentation media with an initial concentration of 300 g/L glucose. The fermentation characteristics (e.g.,

parameters of growth, consumption of glucose, and production of ethanol) are presented in Fig. 3. The fermentation results of WT4-M for 15 generations (data not shown) were consistent with the data shown in Fig. 3.

As can be seen from Fig. 3A, the growth of WT4-M, with a prolonged exponential phase, was superior to that of the control strain. In this study, the total glucose concentration was initially 300 g/L and around 25 g/L at the end, representing 1 batch fermentation cycle. The glucose utilization rate of WT4-M was distinctly improved, resulting in a residual sugar decrease of 53.8% relative to that of WT at the end of fermentation (Fig. 3B). Moreover, Fig. 3C shows that the glycerol yield of WT4-M was 4.4% less than that of WT. Notably, ethanol production by WT4-M was up to 9.8% greater than that of the control. Fermentation results illustrated the capacity of WT4-M to produce a higher sugar–ethanol conversion rate (42.7%), surpassing the function of WT (40.1%).

Discussion

The demand for ethanol will require the engineering of new yeast strains that can produce ethanol more efficiently. Although polyploid yeast strains have been studied to improve ethanol production (Dilorio et al. 1987; Takagi et al. 1983), aneuploid strains are not used in alcoholic fermentation. Aneuploid strains are often investigated for the genetic and molecular profiles of their unusual cells (Waghmare and Bruschi 2005). Here, the new strategy of construction of aneuploid strains was employed to improve the fermentation characteristics in VHG fermentation. To the best of our knowledge, this is the first description of the fermentation behavior of an artificially constructed aneuploid yeast.

Industrial yeast is known to exhibit naturally high ethanol production. There is limited opportunity for marginal modification to improve ethanol yield in VHG fermentation. As such, it can be said that the amount of ethanol produced by WT4-M was obviously increased in the presented work. The distinct improvement in the amount of ethanol produced by WT4-M (Fig. 3D) was due to the extension of the growth phase (Fig. 3A), and the accumulation of glycerol was optimal (Fig. 3C). Glycerol production by yeast strains has been related to ethanol and osmotic stress tolerance (Hohmann 2002). The high stress tolerance of yeast strains is crucial for the outcome of the fermentation process, with regard to both residual sugar and final ethanol concentration (Devantier et al. 2005*b*). Low residual sugar at the end of fermentation (Fig. 3B) was not only a saving of substrate in economic terms, but it was also advantageous in terms of downstream processing; that is, in distillation and drying of the distilled grains (Devantier et al. 2005*a*). On the other hand, extended growth (Fig. 3A) enhanced the sugar–ethanol conversion rate, resulting in the faster consumption of substrate during VHG fermentation. Under VHG conditions, aneuploid strain WT4-M exhibited good fermentation characteristics, which may be attributed to a favorable adaptation to specific industrial conditions. The reason for these results may be that MBC caused chromosomes loss or mitotic recombination in polyploid cells (Salmon 1997), which led to a change in copy number of genes related to ethanol fermentation. Further research will be focused on the mechanism of the improvement of fermentation properties in aneuploid strain WT4-M.

In conclusion, a novel genome engineering approach has been developed for the improvement of ethanol production by *S. cerevisiae*. The aneuploid strain constructed in this study possessed favorable metabolic traits in VHG fermentation. The approach described here was thus valuable for creating yeast strains with better fermentation characteristics. It should be noted that this strategy has the potential for improving other desired multiplex traits under the control of multiple genes in *S. cerevisiae*.

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