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ORIGINAL RESEARCH PAPER

A comparison of whole cell directed evolution approaches in breeding of industrial strain of *Saccharomyces cerevisiae*

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Abstract
Objective To reduce the fermentation cost in very high gravity fermentations of ethanol using *Saccharomyces cerevisiae*, whole cell directed evolution approaches were carried out.
Results The methods used included cell ploidy manipulation, global transcription machinery engineering and genome shuffling. Ethanol production by the four methods was improved compared with the control. Notably, the ethanol yield of a strain constructed by genome shuffling was enhanced by up to 11 % more than the control reaching 120 g ethanol/l in 35 h using a very high gravity fermentation with 300 g glucose/l.
Conclusion Genome shuffling can create strains with improved fermentation characteristics in very high gravity fermentations.

Keywords Bioethanol · Ethanol production · Genome shuffling · *Saccharomyces cerevisiae* · Very high gravity fermentation · Whole cell directed evolution

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Introduction

The demand for ethanol as a biofuel is expected to increase because of concerns related to national security, economic stability, environmental impact and climate changes. For technical reasons, however, ethanol production is mainly achieved at present through fermentation of starch- or sugar-based feedstock by *Saccharomyces cerevisiae*. The cost of the feedstock accounts for a major portion of the total production costs. Hence, reducing the fermentation cost by implement in very high gravity fermentations (VHG) which are common in ethanol industry, are key subjects of development for the starch- and sugar-based ethanol industry (Lin and Tanaka 2006). During VHG fermentations, the yeast is exposed to an osmotic stress at the beginning of the process and to an ethanol stress at the end of a batch (Rautio et al. 2007). Ethanol and osmotic stresses result in decreased growth rate, lower viability, higher energy consumption, prolonged fermentation cycle and more residual sugar (Devantier et al. 2005).

Glycerol is an important byproduct, utilizing 4 % of the carbon source during anaerobic fermentation of *S. cerevisiae* (Nissen et al. 2000). Therefore, ethanol production of *S. cerevisiae* can be improved by minimizing glycerol formation by using mutants in which *GPD1* encoding glycerol-3-phosphate dehydrogenase, and *FPS1* encoding a channel protein which mediates glycerol export were knocked-out (Tamás et al. 2003), and by overexpressing *GLT1*

encoding glutamate synthase (the GS-GOGAT system) to solve the redox problem (Pronk 2001). The approach of metabolic pathway modification was used to achieve a higher ethanol yield and a lower glycerol production in VHG fermentations (Ostergaard et al. 2000). However, metabolic pathway modification is laborious because several genes need to be modified one by one. This one-by-one strategy is, though, ineffective in a most simple cells such as *Escherichia coli* (Patnaik 2008).

Engineering yeast transcription machinery reprograms gene transcription to generate cellular phenotypes crucial for technological applications through regulating a TATA-binding protein (TBP) encoded by *SPT15* (Alper 2006). This approach has already been employed in yeast to improve ethanol tolerance (Alper et al. 2006); however, only a standard laboratory yeast strain was used in this work.

Aneuploidy has often been applied in industrial yeasts (e.g. baking, brewing, distilling and wine yeasts) (Bidenne et al. 1992). Under VHG conditions, aneuploidy may give rise to cells with a greater level of protection against the occurrence of spontaneous lethal or detrimental recessive mutations or change gene dosage (Salmon 1997). In addition, genome shuffling can achieve the preferred multiple traits using the recursive multiparental protoplast fusion (Zhang et al. 2002) or yeast sexual and asexual reproduction by myself (Hou 2009). The strategy has been successfully applied in prokaryotic and eukaryotic cells including *S. cerevisiae* (Gong et al. 2009).

Materials and methods

Strains construction

The engineered strains of *S. cerevisiae* were derived from a wild-type diploid industrial strain TH-AADY (Angel Yeast Co., Ltd., China), which were used as the initial strain and the control strain (WT). Table 1 shows the data of four engineered strains. The engineered strain, H315, (Hou et al. 2009) was constructed, based on global transcription machinery engineering (gTME). The engineered strain WT4-M was obtained by yeast cell ploidy manipulation (Hou 2010). The engineered strain S3-10 was achieved using genome shuffling (Hou et al. 2010). The engineered strain, FTG2, is kept in our laboratory (Cao et al. 2007; Kong et al. 2007; Zhang et al. 2007).

Cultivation conditions

Serial dilution assay was carried out to evaluate the ethanol tolerance. The cells were added to 1 ml fresh YPD medium with 2 % (w/v) peptone, 1 % (w/v) yeast extract and 2 % (w/v) glucose. After cultivation at 30 °C for 2 h, the cells were serially diluted. 3 µl of the indicated dilutions were then spotted on the YPD plates containing 15 % (v/v) ethanol, 30 % (w/v) glucose and non-selective (YPD) plates, respectively. The plates were then incubated at 30 °C for 3 days.

Fermentation conditions

The yeast was cultured in YPD media at 30 °C until OD_{600} reached 1 and then transferred to the following flasks at 10 % (v/v). Anaerobic batch cultivations were performed in the cap-covered flasks (500 ml) with a working volume of 150 ml at 200 rpm and 30 °C. The fermentation media prepared from corn powder by the double-enzyme hydrolyzed method, contained 30 % (w/v) glucose, 0.5 g $(NH_4)_2HPO_4$ l^{-1} and 0.5 g K_2HPO_4 l^{-1}. During fermentations, pH 5 was kept by adding 2 M NaOH at every sampling. Fermentation experiments were carried out in triplicate and one representative experiment was shown.

Measurement of CFU, glucose, glycerol and ethanol

Colony-forming units (CFU) were enumerated by counting a sufficient number of colonies and multiplying the average by the appropriate dilution factor. The content of glycerol and glucose in the fermentation broth was determined by HPLC using differential refractive index detector and Agilent ZORBAX carbohydrate column (Agilent, Beijing, China) eluted by 75 % (v/v) acetonitrile. Ethanol was determined by GC.

Results and discussion

Methods for genetic manipulation in *S. cerevisia*

Genes *SPT15* and *SPT3*, encoding two general transcription factors, were cloned into the YEplac195

Table 1 The data of four engineered strains

Srains	Characteristic
H315	Overexpression of *SPT3* and *SPT15*
WT4-M	Aneuploidy
S3-10	Obtained by genome shuffling
FTG2	Deletion of *FPS1*and *GPD*, and overexpression of *GLT1*

SPT15 and *SPT3* are two general transcription factors. *GPD1* encodes glycerol-3-phosphate dehydrogenase, *FPS1* encodes a channel protein which mediated glycerol export and *GLT1* encodes glutamate synthase to solve the redox problem

vector, generating plasmid YEplac195-*SPT15–SPT3*. *SPT15* gene was always under the control of the strong *TEF1* promoter, while *SPT3* gene was under the control of the strong *PGK1* promoter. The resulting plasmid YEplac195-*SPT15–SPT3* was transformed into strain WT by the lithium acetate method (Schiestl and Gietz 1989). Thereby, the engineered strain H315 was achieved on the base of gTME.

The tetraploid strain was obtained by using the plasmid YCplac33-GHK, which harbored the *HO* gene under the control of a galactose inducible promoter *GAL2* and *KanMX4* gene as the selective gene marker. The aneuploid strain, HLH24-M, was selected and screened after tetraploid cells were treated with methyl benzimidazole-2-ylcarbamate to induce loss of mitotic chromosome. The resulting aneuploid strain was investigated the stability of WT4-M during propagation (Hou 2010). Therefore, the engineered strain HLH24-M was obtained by yeast cell ploidy manipulation.

Using yeast sexual and asexual reproduction by itself instead of PEG-mediated protoplast fusion, mutant diploid cells were shuffled through highly efficient sporulation and adequate cross among the haploid cells. After three rounds of genome shuffling the best performing strain, S3-10, was obtained on a plate containing ethanol at 15 % (v/v). Hence, the engineered strain S3-10 was got using the novel genome shuffling (Hou et al. 2010).

Analysis of stress resistance

Serial dilution assay was carried out under high concentrations of ethanol (15 % v/v) and glucose (30 % w/v) to examine the effect of the stress conditions on the engineer strains and the control strain. Figure 1 shows the results of the engineered strains, HLH315, FTG2, WT4-Mand S3-10 along with the control strain, WT, under different stress conditions. Osmotic stress and ethanol tolerance of HLH315 were greater than the control. This can be explained by the fact that Spt15p and Spt3p, as an ensemble, can provide stronger resistance to ethanol and glucose. Spt3p interacts with TBP encoded by *SPT15*, which is required for the efficient recruitment of TBP to the SAGA-dependent promoters of many genes (Bhaumik and Green 2002), e.g. *ADH1* encoding alcohol dehydrogenase isoenzymes involved in ethanol metabolism.

As shown in Fig. 1, ethanol and osmotic stress-resistance of FTG2 was not enhanced compared with the control. This was because glycerol was formed and accumulated inside the cell where it worked as an efficient osmolyte protecting the cell against lyses under osmotic stress conditions (Nevoigt and Stahl 1997). The stress tolerance of FTG2 decreased because of the deletion in the glycerol synthesis pathway. In addition, the results also indicated that WT4-M and S3-10 possessed greater stress tolerance than WT. The main reason was that the engineered strains WT4-M and S3-10 were selected and screened by the improvement of stress resistance.

Very high gravity (VHG) fermentations

Here, we designed a breeding programme to evaluate the efficiencies of the above methods. As mentioned above, several phenotypes related to the ethanol yield including characteristics of growth, glycerol yield and residual sugar were chosen. The engineered strains, FTG2, HLH315, WT4-M and S3-10, along with the control strain WT were evaluated in VHG fermentation with an initial concentration of 300 g glucose l^{-1}. The fermentation characteristics (e.g. parameters of

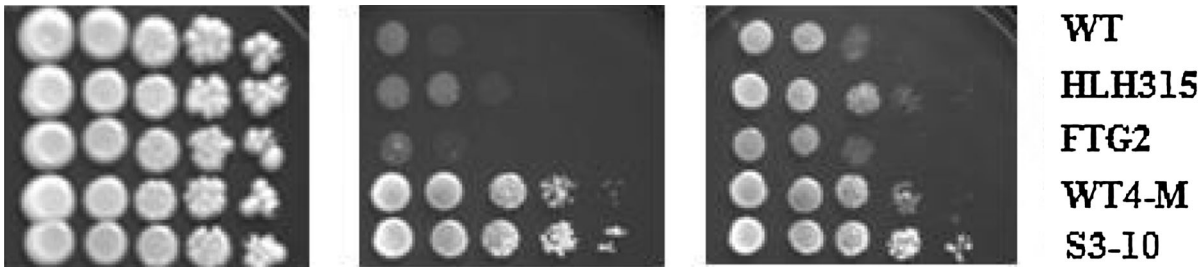

Fig. 1 Serial dilution assay of the engineer strains and the control strain WT on different plates and incubation at 30 °C. (*Left*) non-selective YPD plates; (*middle*) YPD plates containing 30 % (w/v) glucose; (*right*) YPD plates containing 14 % (v/v) ethanol. Experiments were carried out at least in triplicate. One representative experiment is shown

CFU, consumption of glucose, production of glycerol and ethanol) are presented in Fig. 2.

As can be seen from Fig. 2a, the CFU of the engineered strains, WT, FTG2, HLH315, WT4-M and S3-10, were increased which agreed with the stress tolerance of these strains. At the same time, the growth characteristics of the engineered strains with a prolonged growth phase, was superior to those of the control strain. The higher viable cell count and longer exponential growth phase were key reasons of the increase in ethanol production during VHG fermentations. The data showed the growth activity of S3-10 was strongest among these strains, which was the most important reason of the low residual sugar and elevated ethanol production.

Glucose started at 300 g l^{-1} and ended at ~15 g l^{-1} after the VHG fermentation of about 48 h. Glucose utilization rates of FTG2 and HLH315 were slightly better than that of WT, though no obvious differences between the three strains were observed (Fig. 2b). Improvement in glucose utilization of WT4-M and S3-10 was achieved, resulting in the faster overall consumption of substrate and the faster overall ethanol production. In particular, residual sugar of S3-10 decreased by 71 % more than that of WT at the end point of fermentation. Low

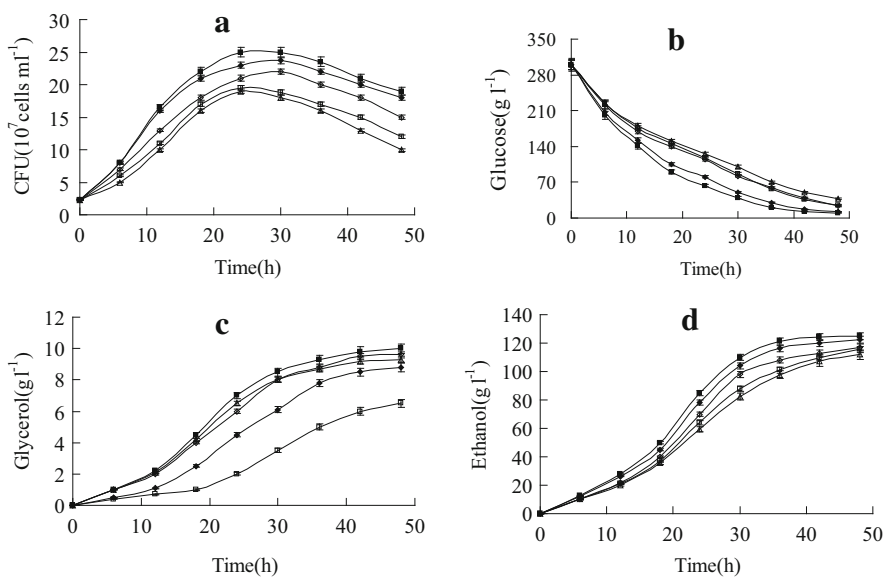

Fig. 2 Changes in measured parameters during microanaerobic batch cultivation of The engineered strains FTG2 (*white square*), HLH315 (*white diamond*), WT4-M (*black diamond*), S3-10 (*black square*) and the control strain WT (*white triangle*), with initial cell number of 2.5×10^7 and glucose at 300 g l^{-1}: **a** CFU, **b** consumption of glucose, **c** formation of glycerol and **d** production of ethanol. *Figure* indicates the average corresponding to at least three independent experiments. Statistical significance is determined using the SAS statistical analysis program, ver. 8.01 (North Carolina, USA). *Error bars* in the figure shows standard deviations and differences are considered significant at $P < 0.05$

residual sugar was not only a saving of substrate in economic terms, but also, it is advantageous in downstream processing, e.g. in distillation and drying of the distillers dried grains (Devantier et al. 2005). The results suggested the faster consumption of substrate was resulted from the extended growth, thus enhancing the sugar-ethanol conversion rate during VHG fermentations.

From Fig. 2c, there was no difference in glycerol yield between WT4-M and the WT. Compared with the control, the glycerol yields of FTG2 and HLH315 were decreased. The decrease in glycerol production of FTG2 is due to the deletion of *GPD1* in the glycerol synthesis pathway. The reason for the decrease in glycerol yield of HLH315 may be that *SPT3* was in concerned with both the activation and repression of transcription (Lee et al. 2000). On the other hand, the glycerol content of S3-10 was higher than that of WT, resulting in the stronger stress tolerance. High stress tolerance of the yeast strain was crucial for the outcome of the fermentation, with regard to both residual sugar and final ethanol content. The data also indicated that the low level concentration of glycerol was not advantage for VHG fermentation.

In the case of ethanol production, improvements in ethanol yields of HLH315, FTG2, WT4-M and S3-10 were achieved, surpassing that of WT (Fig. 2d). Ethanol production by HLH315, FTG2 and WT4-M was increased by 4.5, 3.1 and 9 %, respectively. Notably, S3-10 provided up to 10.96 % improvement in ethanol yield, compared with the WT. The distinct improvements in ethanol could be explained by the elevated stress tolerance, the extended growth phase and the decreased residual sugar. The results illustrated that the engineered strains S3-10 constructed using genome shuffling, exhibited the best fermentation characteristics under VHG conditions.

Comparison of four methods

Ethanol production of FTG2 was slightly better than those of WT in VHG fermentations. Thus, the method of metabolic pathway modification was not good for the improvement of fermentation characteristics in VHG fermentations. Therefore, the whole cell directed evolution approaches including cell ploidy manipulation, gTME and novel genome shuffling, were developed to improve the fermentation performance during VHG fermentations. The data indicated that, under our experimental conditions, modulation of the expression level of the *SPT15* and *SPT3* gene could, to some extent, improve HLH315′s ethanol production. However, the improvement of fermentation performance was far inferior to those of WT4-M and S3-10. The results suggested that the technique of gTME was not a better selection for VHG fermentation.

As for yeast cell ploidy manipulation, WT4-M exhibited enhanced ethanol and osmotic stress tolerance and increased ethanol yield, suggesting this method could improve its fermentation performance. Compared with the above these methods, the strategy of genome shuffling could greatly accelerate the improvement of more phenotypes of yeast. The results demonstrated that genome shuffling was valuable for creating yeast strains with desired multiplex traits during VHG fermentations.

During VHG fermentation the properties under the control of multiple genes is difficult to change by classical breeding, metabolic engineering and other genetic manipulation methods with specific genes or pathways as targets (Teixeira et al. 2012). However, the whole genome engineering approaches involving yeast cell ploidy manipulation and genome shuffling can be employed to improve the fermentation performances under VHG conditions. The results suggested genome shuffling was the most effective way to significantly manipulate yeast strains.

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