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ORIGINAL RESEARCH PAPER

# Increase of ethanol tolerance of *Saccharomyces cerevisiae* by error-prone whole genome amplification

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**Abstract** *Saccharomyces cerevisiae* was transformed for higher ethanol tolerance by error-prone whole genome amplification. The resulting PCR products were transformed back to the parental strain for homologous recombination to create a library of mutants with the perturbed genomic networks. A few rounds of transformation led to the isolation of mutants that grew in 9% (v/v) ethanol and 100 g glucose $l^{-1}$ compared to untransformed yeast which grew only at 6% (v/v) ethanol and 100 g glucose $l^{-1}$.

**Keywords** Ethanol tolerance · *Saccharomyces cerevisiae* · Whole genome amplification

## Introduction

The evolution of the complex traits of a whole cell biocatalyst always requires the simultaneous alteration of the expression levels of many genes, which may not be easily achieved by sequential multi-gene modification (Alper et al. 2006). Taking ethanol tolerance of a microorganism as an example, it potentially involves the interaction of 40–60 genes (Patnaik 2008). Moreover, the identification of genes for perturbation may be largely unanticipated by conventional pathway analysis. Consequently, novel metabolic strategies towards whole cell metabolic network manipulation, such as genome shuffling and global transcription machinery engineering, have been developed (Crameri et al. 1998; Alper et al. 2006).

Since the global transcription factors of most microorganisms have not yet been identified, this limits the wide utilization of the technique. As for the conventional genome shuffling method, it relies on protoplast fusion which involves protoplast preparation and cell wall regeneration that are relatively not easy to handle. In addition, UV or chemical mutagens are generally employed to create the starting strains. This prompted us to develop new tools to engineer the complex phenotypes of microorganisms.

We present here a method for improving the complex phenotypes of *Saccharomyces cerevisiae* by error-prone whole genome amplification (ep-WGA). The resulting PCR products of ep-WGA were transformed back into the parental yeast strains for homologous recombination, followed by selection or screening for the improved phenotypes. We applied this method to evolve *Saccharomyces cerevisiae* for higher ethanol tolerance. This was efficient and represents an alternative to the conventional genome shuffling approach for generation of complex phenotypes.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10529-011-0518-7) contains supplementary material, which is available to authorized users.

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## Materials and methods

### Microorganisms, oligos and culture medium

*Saccharomyces cerevisiae* INVSc1 strain (MATα *his3Δ1 leu2 ade2-1 trp1-289 ura3-52*) from Invitrogen was employed as the host for yeast transformation. Random 15-mer oligos (173.5 μg) were synthesized by 1st BASE. Yeast complex medium, YPAD, containing 2% (w/v) yeast extract, 2% (w/v) peptone, 0.05% (w/v) adenine hemisulfate, and 2% (w/v) dextrose was used to cultivate yeast cells. Ethanol was supplemented to the culture medium for selection of transformants which indicated higher ethanol tolerance.

### DNA manipulation

The genomic DNA (gDNA) of *S. cerevisiae* INVSc1 was isolated from 2 ml overnight cell culture. Ep-WGA was performed with 1–100 pg gDNA, error-prone dNTPs containing 1 mM each dCTP and dTTP, 0.2 mM each dATP and dGTP, 33.3 uM 15-mer random primers, 0.15 mM $MnCl_2$, mutation buffer composed of 7 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatine in 10 mM Tris/HCl buffer (pH 8.3), and 0.1 U of *Taq* DNA polymerase. Basically, we modified the WGA protocol developed by Zhang et al. (1992) by including $MnCl_2$ for introduction of random mutations during PCR amplification. The amplified PCR products were ethanol-precipitated and transformed into electro competent *S. cerevisiae* for homologous recombination.

The transformants were selected through multiple rounds in YPAD medium supplemented with an initial 7% (v/v) ethanol and 100 g glucose $l^{-1}$ since non-transformed *S. cerevisiae* INVSc1 can only tolerate an initial 6% (v/v) ethanol and 100 g glucose $l^{-1}$ in the culture medium. After electroporation, the transformants were inoculated into 20 ml selective medium in a 50 ml conical centrifuge tube which was securely capped to prevent ethanol evaporation and incubated at 30°C with shaking at 220 rpm. Once cells grew, 50 μl of the culture was transferred into another 20 ml freshly-prepared selective medium for the second round of selection. Altogether, three rounds of selection were performed to confirm the growth of cells in the selective medium. The liquid culture was streaked on YPAD agar to isolate a few single colonies which were re-inoculated into 5 ml selective medium to verify their growth under those conditions. Yeast cells that grew in the selective medium and had the highest ($OD_{600}$) were selected for the next round of evolution.

### Further molecular crossing

DNA was isolated from one of the final mutants, digestion with *Sau*I, and the resulting fragments were precipitated with ethanol and transformed into another mutant. The transformants were selected in culture medium supplemented with an initial 9% (v/v) ethanol and 100 g glucose $l^{-1}$.

## Results and discussion

### Methodology development

As indicated in Fig. 1a, random point mutations were introduced by ep-WGA to the parental strain gDNA, creating a pool of small sized PCR products (Zhang et al. 1992). The PCR products were transformed back into the parental strain for homologous recombination in vivo. The recombinant yeasts were then selected or screened for the improved phenotypes. Additional rounds of evolution were carried out on the improved mutants for further phenotype improvement by recursive ep-WGA. After a few rounds of evolution, the mutants with the improved traits might have different point mutations in their genomes. Further improvement of the phenotypes could be created by combining the beneficial point mutations in various mutants via homologous recombination as proposed in Fig. 1b. Thus, gDNA from one of the mutants created by previous iterative cycles of ep-WGA was isolated, digested with *Sau*I, and transformed into the selected mutant, followed by selection or screening for the best candidates with the further improved phenotypes.

### Creation of recombinant *S. cerevisiae* with higher ethanol tolerance

We applied the method shown in Fig. 1 to evolve *S. cerevisiae* for higher tolerance to ethanol. As expected, electrophoresis of the PCR products of ep-WGA yielded a smear on the agarose gel. After transformation of the resulting PCR products back

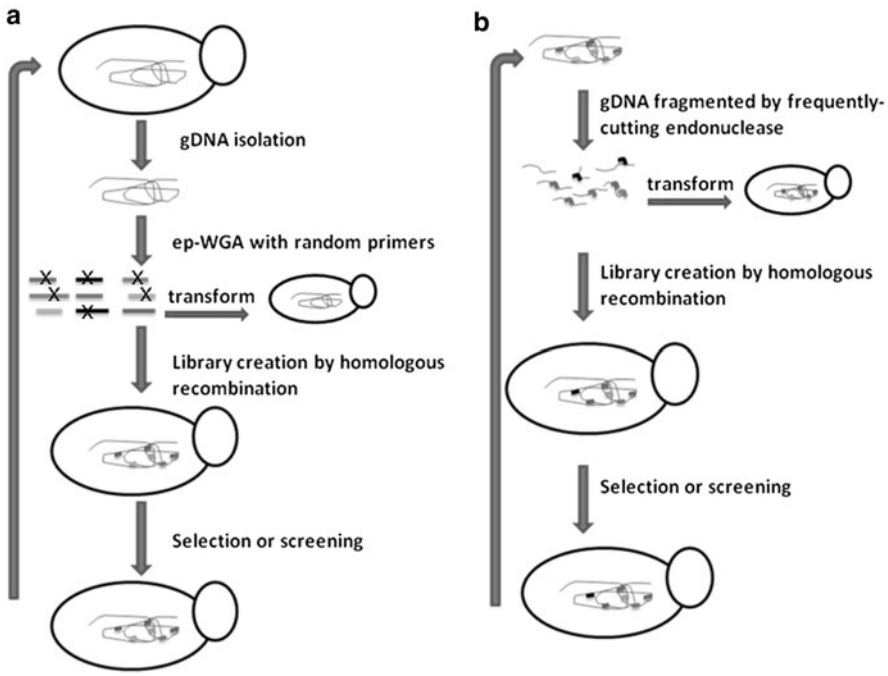


**Fig. 1** The scheme for evolving *S. cerevisiae* by recursive ep-WGA, transformation and selection or screening. **a** Evolving *S. cerevisiae* by ep-WGA for complex phenotype improvement. '×' stands for a random point mutation. **b** Procedure for further phenotype improvement of *S. cerevisiae*

into parental strain, mutants with the perturbed genome were selected in medium supplemented with ethanol. As indicated in Fig. 2 and Supplementary Fig. 1, the first round evolution and selection led to the isolation of a few recombinant *S. cerevisiae* mutants that grew in YPAD medium with 7% (v/v) ethanol and 100 g glucose $l^{-1}$. Using the resulting mutant GS-116 with the highest cell density as a parental strain, another two rounds of evolution were performed and this led to the isolation of mutants which grew in medium with 8.5% (v/v) ethanol and 100 g glucose $l^{-1}$.

Using these mutants as the parental strains, we further evolved those strains for higher ethanol tolerance by following the procedure in Fig. 1b. Three rounds of selection resulted in the isolation of

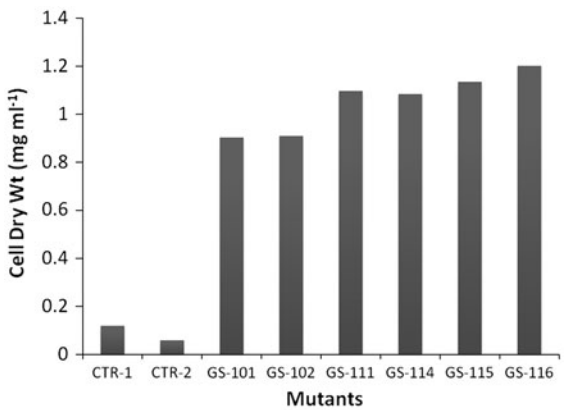


**Fig. 2** The growth of the mutants created by ep-WGA in YPAD medium supplemented with an initial 7% (v/v) ethanol and 100 g glucose $l^{-1}$. Cell biomass was analyzed after 6 days. CTR stands for the control parental yeast strain without evolution. GS represents mutant evolved via ep-WGA

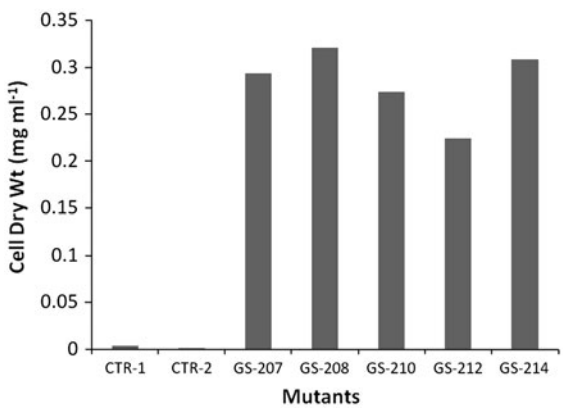


**Fig. 3** The growth of the mutants in YPAD medium supplemented with an initial 9% (v/v) ethanol and 100 g glucose $l^{-1}$. Cell biomass was analyzed after 6 days. CTR stands for the control mutant selected from the previous evolution cycles which was able to grow in medium supplemented with an initial 8.5% (v/v) ethanol and 100 g glucose $l^{-1}$. GS represents mutant

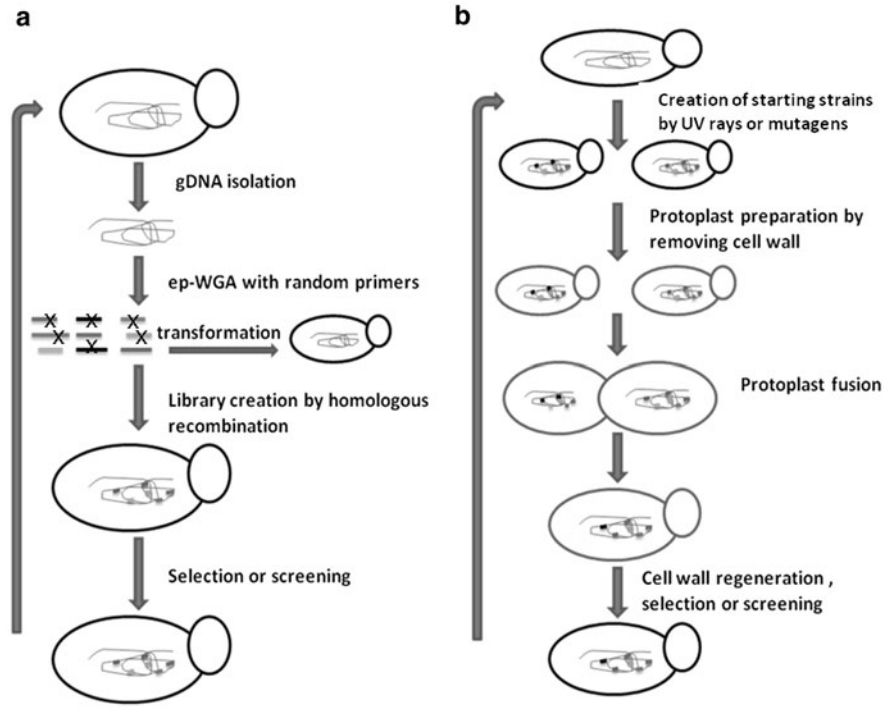


**Fig. 4** A comparison of the new method developed in this study with the conventional genome shuffling for evolving *S. cerevisiae* for complex phenotype engineering. The procedure of the new method developed in this study (**a**) and the conventional genome shuffling (**b**)

cells that grew in medium supplemented with an 9% (v/v) ethanol and 100 g glucose $l^{-1}$ (Fig. 3; Supplementary Fig. 2).

A comparison of the evolving method developed in this study with the conventional genome shuffling is illustrated in Fig. 4. Similarly, both methods rely on homologous recombination in the target microorganisms. However, the distinctive feature of our method is that gDNA is directly manipulated by in vitro ep-WGA rather than protoplast fusion in vivo. In addition, parental strains in the conventional genome shuffling are created by a complicated and time-consuming process of mutagenesis followed by protoplast fusion (Crameri et al. 1998; Xu et al. 2008). Instead, our new method expedites the procedure by simple ep-WGA and transformation.

The maintenance energy requirement of cells can be influenced by various parameters, such as a particular nutrient limiting growth, the presence of growth inhibitory substances, and an increase in osmotic pressure (Stouthamer and Bettenhaussen 1973; Lee et al. 1979). Similarly, we observed higher ethanol concentration accompanied by slower rate of cell growth. The evolved mutants grew slower in YPAD medium with 9% (v/v) ethanol than 7% (v/v) ethanol. A possible reason for this change is the higher maintenance energy requirement in culture medium with an initial higher concentration of ethanol.

Our method presents an alternative to the conventional genome shuffling method. In this study, we took advantage of the known high frequency of homologous recombination in *S. cerevisiae* and evolved *S. cerevisiae* for higher ethanol tolerance by multiple-round ep-WGAs. This method may also be extended to other microorganisms, depending on the homologous recombination frequency and the availability of genetic tools for transformation in those microorganisms. Characterization of the mutants by proteomic and whole genomic studies is currently under way.

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## References

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568

Crameri A, Raillard SA, Bermudez E, Stemmer WP (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. Nature 39:288–291

Lee KJ, Tribe DE, Rogers PL (1979) Ethanol production by *Zymomonas mobilis* in continuous culture at high glucose concentration. Biotechnol Lett 1:421–426

Patnaik R (2008) Engineering complex phenotypes in industrial strains. Biotechnol Prog 24:38–47

Stouthamer AH, Bettenhaussen C (1973) Utilization of energy for growth and maintenance in continuous and batch cultures of microorganisms. A reevaluation of the method for the determination of ATP production by measuring molar growth yields. Biochim Biophys Acta 301:53–70

Xu B, Jin Z, Wang H, Jin Q, Jin X, Cen P (2008) Evolution of *Streptomyces pristinaespiralis* for resistance and production of pristinamycin by genome shuffling. Appl Microbiol Biotechnol 80:261–267

Zhang L, Cui X, Schmitt K, Hubert R, Navidi W (1992) Whole genome amplification from a single cell: implications for genetic analysis. Proc Natl Acad Sci USA 89:5847–5851