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Evolutionary engineering of *Saccharomyces cerevisiae* for efficient conversion of red algal biosugars to bioethanol

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HIGHLIGHTS

- Red algae mainly consist of two mono-sugars of galactose and glucose.
- Conversion of both galactose and glucose is required for efficient biofuel production.
- *S. cerevisiae* was evolutionary-engineered to have high galactose-consuming activity.
- Moderate relief of the glucose repression is crucial for fast galactose utilization.
- Evolved *S. cerevisiae* HJ7–14 efficiently fermented *G. amansii* hydrolysates to ethanol.

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ABSTRACT

The aim of this work was to apply the evolutionary engineering to construct a mutant *Saccharomyces cerevisiae* HJ7-14 resistant on 2-deoxy-D-glucose and with an enhanced ability of bioethanol production from galactose, a mono-sugar in red algae. In batch and repeated-batch fermentations, HJ7-14 metabolized 5-fold more galactose and produced ethanol 2.1-fold faster than the parental D452-2 strain. Transcriptional analysis of genes involved in the galactose metabolism revealed that moderate relief from the glucose-mediated repression of the transcription of the GAL genes might enable HJ7-14 to metabolize galactose rapidly. HJ7-14 produced 7.4 g/L ethanol from hydrolysates of the red alga *Gelidium amansii* within 12 h, which was 1.5-times faster than that observed with D452-2. We demonstrate conclusively that evolutionary engineering is a promising tool to manipulate the complex galactose metabolism in *S. cerevisiae* to produce bioethanol from red alga.

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1. Introduction

As a renewable resource for the sustainable production of biofuels, marine biomass of micro- and macro-algae has been receiving increasing attention. Among the various potential sources of marine biomass, red algae (*e.g. Gelidium amansii*) are mainly composed of agar and cellulose, which and deficient in phenolic compounds of lignin and can be readily hydrolyzed to the fermentable sugars of galactose and glucose (Wei et al., 2013). Because of these structural advantages, red algae have been recognized as a promising source of biomass (Kim et al., 2013a), and both galactose and glucose should be metabolized fast and efficiently by microorganisms for biofuels production from red algae. Bioethanol is an alternative biofuel or additive to gasoline and is also used as a chemical feedstock for bio-ethylene production. Commercial production of bioethanol has been performed using corn and sugarcane as a source of biomass, and it was recently reported that construction of demonstration facilities for lignocellulosic bioethanol was under way worldwide (Kolfschoten et al., 2014; Richard, 2010). Meanwhile, the yeast *Saccharomyces cerevisiae* is a GRAS microorganism and a robust bioethanol producer used for commercial processes (Kim et al., 2013b). In *S. cerevisiae*, glucose can be easily converted to bioethanol via the glycolysis whereas galactose is metabolized by the more complex Leloir pathway (Supplementary Fig. 1). In this pathway, galactose is taken up by galactose permease (Gal2p) and converted into glucose-6-phosphate by the actions of galactokinase

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(Gal1p), galactose-1-phosphate uridyltransferase (Gal7p), UDP-galactose-4-epimerase (Gal10p) and phosphoglucomutase (two isozymes: Pgm1p and Pgm2p). Because of the complexity of galactose metabolism, galactose is consumed more slowly than is glucose by *S. cerevisiae* (Ostergaard et al., 2000). In addition, the expression of the enzymes involved in galactose metabolism is strictly down-regulated in the presence of glucose, called "glucose repression" (Johnston et al., 1994). The preferential utilization of glucose and slow assimilation of galactose after depletion of glucose by *S. cerevisiae* results in a reduced productivity of ethanol from a mixture of galactose and glucose, compared to that observed with glucose alone. In order to overcome this problem of the step-wise consumption, metabolic engineering approaches of the modulation of target genes have been attempted. Overexpression of a positive regulator of Gal4p or elimination of the negative regulators of Gal6p, Gal80p, and Mig1p improved specific galactose uptake rate and ethanol production rate by 20% and 153%, respectively (Ostergaard et al., 2000; Rønnow et al., 1999). Overexpression of the *PGM2* gene encoding phosphoglucomutase (Bro et al., 2005) and a truncated *TUP1* gene encoding a transcription repressor (Lee et al., 2011) led to an increase in galactose consumption rate, compared to that observed with the corresponding parent strains. Deletion of the *HXK2* gene coding for a hexokinase isozyme relieved the glucose-mediated catabolite repression and enhanced galactose consumption rate in a culture using galactose and glucose (Bae et al., 2014; Kummel et al., 2010). In addition, wild type strains of *S. cerevisiae* were screened newly with high ethanol producing performance from galactose under aerobic conditions (Kim et al., 2014).

Among several available technologies for engineering microorganisms, evolutionary engineering is an attractive approach, as it allows the simultaneous modification of multiple genes (Portnoy et al., 2011; Santos and Stephanopoulos, 2008). Evolutionary engineering accompanied by chemical mutagenesis has been successfully applied to *S. cerevisiae* and *Klebsiella oxytoca* towards improving ethanol production (Uma and Polasa, 1990), acquiring high stress tolerance (Kumari and Pramanik, 2012), and facilitating a decrease in byproduct formation (Han et al., 2013). In this study, we performed evolutionary engineering of a wild type *S. cerevisiae* D452-2 strain in order to construct an evolved strain that is able to grow in a mixture of galactose and 2-deoxy-D-glucose (2-DG; a glucose analog). The evolved strain HJ7-14 was characterized to consume galactose rapidly in both batch and repeated-batch cultures using galactose alone or a mixture of galactose and glucose. Transcription levels of genes involved in galactose metabolism were analyzed to determine the mechanism of rapid utilization of galactose by HJ7-14. Subsequently, to evaluate the industrial application of HJ7-14, we tested its ability to produce bioethanol by using the hydrolysates of the red alga *G. amansii*.

2. Methods

2.1. Laboratory evolution

S. cerevisiae D452-2 (*MATα*, *leu2*, *his3*, *ura3*, *can1*) (Lee et al., 2012) was used as a parent strain for evolutionary engineering. D452-2 was cultured in YP medium (10 g/L yeast extract, 20 g/L bacto peptone) with 20 g/L glucose. After harvesting the cells by centrifugation, cell pellets were suspended in fresh YP medium with 10 g/L glucose and their suspension was mixed with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) at a final concentration of 20 mg/L. After incubation at 30 °C for 1.5 or 3 h, the cell suspension was spread onto YP medium with 20 g/L agar and 20 g/L of galactose. Rapidly growing colonies were selected and cultured in 10 mL YP medium with 10 g/L galactose and 10 g/L glucose at 30 °C, with shaking at 200 rpm. The strains exhibiting a high galactose consumption rate were selected and cultured in YP medium with10 g/L galactose and 10 g/L 2-deoxy-D-glucose (2-DG). By consecutive subcultures, mutants that grew rapidly on galactose were enriched. After five serial subcultures, the cells were spread onto YP medium containing agar and 20 g/L galactose. Four strains with the ability to form colonies rapidly compared to the parent strain were isolated (Supplementary Fig. 2).

2.2. Culture conditions

For inoculation, cells were propagated in 10 mL of YP medium containing 20 g/L glucose at 30 °C and 250 rpm. After harvesting cells at the mid-exponential phase, cell pellets were suspended in fresh YP medium and used to inoculate new cultures at an initial OD_{600} of ~0.5. Batch cultures were carried out using 100 mL of YP medium containing galactose or a mixture of galactose and glucose in a 500 mL-scale baffled flask (Nalgene Co., Rochester, NY, USA) at 30 °C and 80 rpm. For repeated-batch fermentation, the yeast cells were grown in YP medium with 53 g/L galactose and 47 g/L glucose, harvested, and cultivated in fresh YP medium with the same composition. These processes were repeated four times. For batch cultures using hydrolysates of red algae, the yeast cells were inoculated into 100 mL YP medium containing the hydrolysates and cultured at 30 °C, 80 rpm and an initial pH of 5.5.

2.3. Preparation of red algal hydrolysates

The red alga *G. amansii* collected from Jeju island (Korea) was hydrolyzed as a previous study with some modifications (Park et al., 2011). Briefly, *G. amansii* was washed with distilled water, dried at 40 °C for 1 day and ground to a powder with a hammer mill. Pieces larger than 300 μm were removed with a sieve. Dried powder was suspended in water at a solid to liquid ratio of 10% and mixed with H_2SO_4 to obtain final concentration of 2% (w/v). This slurry was subjected to 1.3 kg_f/cm^2 of pressure at 150 °C for 15 min, and neutralized to pH 5.5 with calcium carbonate. After centrifugation of the slurry, the supernatant was collected and mixed with activated carbon powder (Junsei, Japan) at a concentration of 10% (w/v), and incubated at 25 °C for 1 h, with shaking at 150 rpm. This mixture was subjected to centrifugation, and the resulting supernatant was used as the *G. amansii* hydrolysate carbon source for batch culture of *S. cerevisiae*.

2.4. Analysis

Cell growth was monitored by measuring the optical density (OD) at 600 nm of wavelength by using a spectrophotometer (Ultrospec 8000, GE Healthcare Co., USA), and the dry cell weight (DCW) was calculated by multiplying the OD with a conversion factor of 0.4 g/L/OD. Metabolites in the culture broth and red algal hydrolysates were analyzed by high performance liquid chromatography (1200 series, Agilent Technologies, Santa Clara, CA, USA) with a carbohydrate analysis column (Rezex ROA organic acid, Phenomenex Inc., Torrance, CA, USA). The column was heated to 60 °C and eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Detection was made with a refractive index (RI) detector (1200 series, Agilent Technologies Inc, USA).

2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

To analyze the transcription levels of the *GAL2*, *GAL1*, *GAL7*, *GAL10* and *PGM2* genes, DNA primers specific to each gene were designed to amplify 220–250 bp of the corresponding RT-PCR products (Table 1). The *TDH3* gene was used as an internal control. The yeast strains were grown in YP medium containing 52 g/L

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