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Metabolomics approach to reduce the Crabtree effect in continuous culture of *Saccharomyces cerevisiae*

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The budding yeast *Saccharomyces cerevisiae* is an important microorganism for fermentation and the food industry. However, during production, *S. cerevisiae* commonly uses the ethanol fermentation pathway for glucose utilization if excess sugar is present, even in the presence of sufficient oxygen levels. This aerobic ethanol fermentation, referred to as "the Crabtree effect" is one of the most significant reasons for low cell yield. To weaken the Crabtree effect in fed-batch and continuous culture, sugar flow should be limited. In addition, in continuous culture, the dilution rate must be reduced to avoid washing out cells. However, under such conditions, production speed might be sacrificed. It is difficult to solve this problem with the tradeoff between cell yield and production speed by using conventional tactics. However, a metabolomics approach may be an effective way to search for clues regarding metabolic modulation. Therefore, the purpose of this study was to reduce ethanol production in continuous culture of *S. cerevisiae* at a higher dilution rate through a metabolomics approach. We used a metabolomics analysis to identify metabolites that were drastically increased or decreased in continuous culture when the dilution rate shifted from biomass formation to ethanol fermentation. The individual addition of two of the selected metabolites, fumaric acid and malic acid, reduced ethanol production at a higher dilution rate. This result demonstrates the potential for using metabolomics approaches to identify metabolites that reduce ethanol production in continuous culture at high dilution rates.

[**Key words:** Metabolomics; Crabtree effect; Continuous culture; *Saccharomyces cerevisiae*; Ethanol reduction]

The budding yeast *Saccharomyces cerevisiae* is an important microorganism for fermentation and in the food industry, as it is used in the production of various goods, such as beer and yeast extract. However, during culture with *S. cerevisiae*, the "Crabtree effect" is often observed when excess glucose is present, even under aerobic conditions. This effect, which represses respiration and leads to ethanol production, results in low cell yield (1,2). However, the detailed mechanism underlying the Crabtree effect is unclear. Therefore, to weaken the Crabtree effect in fed-batch and continuous cultures, sugar flow should be limited, which unfortunately further reduces cell yield. Various attempts have been made to weaken the Crabtree effect, and most studies have been performed in *S. cerevisiae*. For example, increasing glycerol production by overexpressing glycerol-3-phosphate dehydrogenase 1 and 2 was reported to reduce ethanol levels (3). In addition, because ethanol and glycerol are produced as a result of the disrupted redox balance of NADH/NAD^+ induced by the Crabtree effect, overexpression of NADH oxidase, to produce H_2O, also reduced ethanol levels (4,5). However, it is difficult to use these strategies for fermentation and food industry applications because they require genetic modifications of the yeast. In other studies, metabolic flux analyses were performed to elucidate the mechanism underlying the Crabtree effect in continuous culture by taking advantage of the shift from biomass formation to ethanol fermentation when the dilution rate was increased (6,7); however, these approaches did not lead to a reduction in ethanol levels. These results show that it has been difficult to ameliorate the low cell yield and ethanol production caused by the Crabtree effect using the above mentioned conventional methods.

Metabolomics is the comprehensive analysis of metabolites obtained from genome information, which closely reflects an organism's contiguous macro phenotypes. Because the production yield and rate in microorganisms are regarded as quantitative phenotypes, metabolomics can be used to obtain information to improve microbial capacity. Thus, metabolomics is thought to be a promising technique through which biological performance can be drastically improved (8,9).

We previously reported that metabolome analysis under various conditions revealed the replicative lifespan of yeast (10) and improved 1-butanol and ethanol tolerance (11,12) as well as poly (γ-glutamic acid) and 1-butanol production (13–15). Therefore, we surmised that metabolome analysis may be useful for reducing the Crabtree effect in continuous culture of *S. cerevisiae*.

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In this study, we carried out a metabolome analysis by sampling from continuous cultures with different dilution rates. Then, based on this analysis, we selected metabolites predicted to affect ethanol production.

MATERIALS AND METHODS

Strain and cultivation *S. cerevisiae* NBRC101557 was purchased from Biological Resource Center, NITE (Chiba, Japan). Pre-cultivation was performed at 30 °C for 18 h in 100 mL of YPD medium (10 g/L yeast extract, 20 g/L D-glucose, and 20 g/L D-glucose, Wako Pure Chemical Industries, Ltd., Osaka, Japan). For batch and continuous culture, a synthetic medium was used, containing 10 g/L glucose, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.06 g/L $CaCl_2$, 5 g/L $(NH_4)_2SO_4$, 0.4 g/L K_2SO_4, 0.1 mg/L biotin, 1.5 mg/L D-pantothenic acid hemicalcium salt, 60 mg/L myo-inositol, 3 mg/L pyridoxine hydrochloride, 14 mg/L thiamine hydrochloride, 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 4 mg/L $ZnSO_4 \cdot 7H_2O$, 10 mg/L $FeSO_4 \cdot 7H_2O$ (all these chemicals were from Wako Pure Chemical Industries, Ltd. or Sigma, St. Louis, MO, USA), and 30 mg/L BIOSPUMEX 36K (Cognis, Manheim, Germany). To assess the effects of metabolite addition, trehalose dihydrate, L-ornithine monohydrochloride, fumaric acid, and malic acid were added to this medium at a final concentration of 100 mg/L. All chemicals were purchased from Wako Pure Chemical Industries, Ltd. or Sigma, unless otherwise indicated. The main culture was performed in a 2-L jar fermentor (Mitsuwa Frontech, Osaka, Japan) with a working volume of 1 L at 30 °C. The pH was maintained at 5.0 by adding 4 M NaOH. The aeration rate and agitation speed were maintained at 1.0 L/min and 700 rpm, respectively. The initial density of the batch culture was $OD_{600} = 0.1$. When the cells reached the late log phase, continuous cultures were started at various dilution rates (D). Then, cells were harvested from the cultures during steady state.

Analysis of biomass and ethanol concentration Cell dry mass (CDM) was determined gravimetrically after washing and centrifugation (twice) at 4 °C and 6000 ×*g* for 5 min and drying at 105 °C overnight. Ethanol concentration was determined using an ethanol sensor (BF-7; Oji Scientific Instruments, Hyogo, Japan).

Sample preparation for gas chromatography/mass spectrometry analysis Sample collection and metabolite extraction were performed as described previously (16), with minor modifications. When the continuous culture reached steady state, an appropriate volume of culture was rapidly collected onto a 47-mm diameter and 0.45-μm pore size mixed cellulose ester membrane filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) by vacuum filtration and washed with distilled water. The volume of culture broth collected was adjusted based on the OD_{600} using the following formula: sampling volume (mL) × $OD_{600} = 80$. After the filter paper was lyophilized overnight, 5 mg of cells was measured gravimetrically and transferred into a 2-mL microcentrifuge tube (Eppendorf, Hamburg, Germany) containing one zirconia ball. After the cells in the tube were frozen in liquid nitrogen, they were disrupted using a Mixer Mill MM400 (Verder-Scientific, Newtown, PA, USA) at 20 Hz for 5 min. Next, 1.0 mL of mixed solvent (methanol/H_2O/chloroform, 5/2/2 [v/v/v]) and 60 μL of ribitol solution (0.2 mg/mL; Wako Pure Chemical Industries, Ltd.), which was included as an internal standard, were added to the tube and vortexed (VORTEX-2 GENIE; Scientific Industries, Inc., Bohemia, NY, USA). Metabolites were extracted from the cells using a Mixer Mill MM400 (20 Hz, 5 min). After centrifugation at 4 °C and 16,000 ×*g* for 5 min, 900 μL of solution was transferred to a new tube, and 400 μL of Ultrapure Water (Wako Pure Chemical Industries, Ltd.) was added before vortexing. After centrifugation at 4 °C and 16,000 ×*g* for 5 min, 500 μL of the upper polar phase was transferred to a new tube. The samples were centrifugally dried for 2 h and lyophilized overnight. Next, 100 μL of methoxyamine hydrochloride (20 mg/mL-pyridine, Wako Pure Chemical Industries, Ltd.) was mixed with the lyophilized sample and incubated at 30 °C and 1200 rpm for 90 min using a thermal mixer (Thermomixer Comfort; Eppendorf). Next, 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (GL Sciences, Kyoto, Japan) was added and incubated in the thermal mixer at 30 °C and 1200 rpm for 30 min.

Gas chromatography/mass spectrometry analysis Gas chromatography/mass spectrometry (GC/MS) analysis was performed as described previously (17). The analysis employed a GC/MS-QP2010Ultra (Shimadzu Corporation, Kyoto, Japan) gas chromatograph and a mass spectrometer combined with an AOC-20s autosampler (Shimadzu) and AOC-20i auto injector (Shimadzu). GCMS solution ver. 4.20β software (Shimadzu) was used to acquire the GC and MS data.

A 30 m × 0.25 mm i.d. DF: 0.25 μm InertCap 5MS/NP (GL Science) was used as the GC column. The inlet temperature was 230 °C, and the column flow rate was 1.12 mL/min. High purity helium (Air Liquide Kogyo Gas Ltd., Tokyo, Japan) was used as the carrier gas. The column temperature was maintained at 80 °C for 2 min and then increased to 320 °C at a rate of 15 °C/min and held for 6 min. The transfer line and source temperature were 250 °C and 200 °C, respectively. Electron ionization was performed at 70 V. Twenty scans per second were recorded over a mass range of 85–500 *m/z*.

Data processing The GC/MS analysis data were exported in net CDF format, and peak detection and alignment were performed using MetAlign Ver. 041012 (18). Aloutput ver. 1.29 was used for peak annotation and principal component analysis (PCA) (19). Automatically annotated peaks were manually confirmed by automated mass spectral deconvolution and identification system. Pareto scaling was used as the scaling method, and 1/4 root transformation was performed.

RESULTS

Continuous culture and ethanol production To investigate the metabolites related to the Crabtree effect, continuous cultures were performed under aerobic conditions at different dilution rates (between 0.05 h^{-1} and 0.30 h^{-1}; Fig. 1). Because the Crabtree effect was inhibited at lower dilution rates, *S. cerevisiae* NBRC101557 did not produce ethanol below $D = 0.20\ h^{-1}$. However, at $D = 0.30\ h^{-1}$, the Crabtree effect was induced, and ethanol production was confirmed. Similar results were previously reported in continuous cultures of *S. cerevisiae* (6,20). While the CDM and oxygen consumption were increased at dilution rates below $D = 0.20\ h^{-1}$, they were lower at $D = 0.30\ h^{-1}$ than that at $D = 0.20\ h^{-1}$.

Metabolome analysis using GC/MS Forty-nine metabolites, including amino acids, organic acids, and sugars, were identified by GC/MS analysis (Table S1). To investigate the metabolites related to the Crabtree effect, PCA was performed on the metabolome data obtained from fermentation with different dilution rates. According to the PCA, the samples were clustered based on dilution rate along PC1 (Fig. 2A). The metabolites that contributed to the separation of PC1 are shown in Fig. 2B. Several metabolites, including trehalose, valine, 4-aminobenzoic acid, and fructose 6-phosphate, accumulated in cells cultured at a lower dilution rate. In contrast, other metabolites, including glycerol, *N*-α-acetyl-L-ornithine, and ornithine, accumulated at the higher dilution rate.

The results of metabolome analysis are shown in Fig. 3. At $D = 0.30\ h^{-1}$, glycerol accumulated in the cells. Oura reported that glycerol is a by-product of ethanol production in *S. cerevisiae* (21). At dilution rates below $D = 0.20\ h^{-1}$, malic acid, fumaric acid, and succinic acid accumulated in response to an increase in the dilution rate. This shows that enzymes in the TCA cycle were activated by increased influx from glucose. Therefore, the rate of oxygen consumption increased with the dilution rate. In contrast, the cellular concentration of malic acid, fumaric acid, and succinic acid were lower at $D = 0.30\ h^{-1}$ than at $D = 0.20\ h^{-1}$. Because

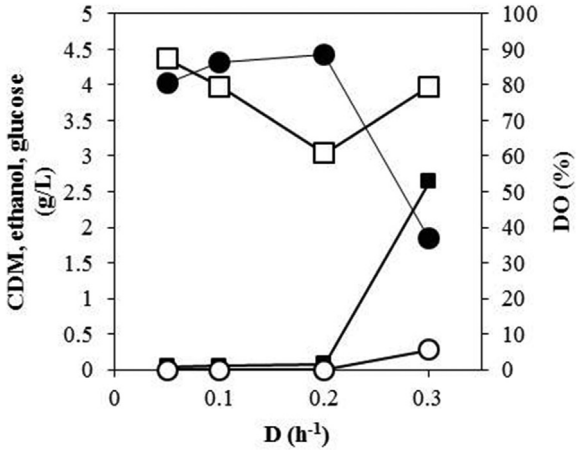

FIG. 1. Continuous culture results at different dilution rates: filled circles, CDM; filled squares, ethanol concentration; open circles, glucose concentration; open squares, dissolved oxygen (DO). All data are the mean of triplicate experiments. The error bar indicates the standard deviation.

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