Effect of Low-concentration Furfural on Sulfur Amino Acid Biosynthesis in Saccharomyces cerevisiae

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Ethanol produced from lignocellulosic biomass (bioethanol) is a promising alternative fuel to gasoline. Production of bioethanol from lignocellulose requires various steps, including pretreatment, enzymatic hydrolysis and fermentation. However, many fermentation inhibitors, including furfural and 5-hydroxymethyl furfural, are generated during the hydrothermal pretreatment of lignocellulose. Recent studies have identified techniques for removing fermentation inhibitors from lignocellulosic hydrolysates. The present study focused on the effect of low-concentration furfural on ethanol production by Saccharomyces cerevisiae. Specifically, gene expression of furfural-inducible genes was analyzed using a S. cerevisiae DNA microarray. The expression of most sulfur amino acid biosynthesis genes increased in response to furfural. To determine whether furfural induces the depletion of sulfur-containing amino acids, the effect of the addition of methionine on yeast growth was investigated. However, exogenous addition of methionine did not compensate for the inhibitory effect. The findings of this study show that furfural affects amino acid synthesis, even at low concentrations, and may be important in the development of high-efficiency processes for large-scale bioethanol production from lignocellulosic biomass.

Keywords
Saccharomyces cerevisiae, Fermentation inhibitor, Biomass, Sulfur amino acid

1. Introduction

Production of bioethanol from lignocellulosic biomass requires pretreatment of the substrate material. For example, hydrothermal pretreatment, one of the most cost effective methods, increases the enzymatic digestibility of lignocellulosic biomass, such as bamboo1) and wheat straw2), into fermentable sugars. Typically, the hydrothermal pretreatment of lignocellulose in wheat straw is first used to alter the structure of the plant cell wall and increase water accessibility3). However, hydrothermal pretreatment also generates a number of compounds, including furfural, 5-hydroxymethyl furfural (5-HMF), acetic acid, and formic acid4), which adversely affect yeast fermentation. High concentrations of furfural have inhibitory effects on yeast growth5) and fermentation6), but the mechanism of inhibition is still under investigation. For example, the addition of furfural is shown to induce the generation of reactive oxygen species in Saccharomyces cerevisiae7).

Comprehensive analyses of the inhibitory mechanisms of furfural, 5-HMF, and acetic acid on yeast growth and fermentation8)−10) have shown that more genes are down-regulated than up-regulated in the presence of these compounds8). In addition, the translational activity of furfural-treated S. cerevisiae cells is lower than that of untreated cells11). The genes down-regulated in the presence of furfural are involved in the expression of metabolic enzymes8). For example, SPE2 and PHR2 encode for the biosynthesis of spermidine, spermine and glycerol, respectively. However, many oxidative response genes are up-regulated in the presence of furfural8).

Such adverse effects of furfural have encouraged research to decrease the concentration of fermentation inhibitors in lignocellulosic hydrolysates12), which is expected to increase the efficiency of bioethanol fermentation by yeast. S. cerevisiae can convert furfural to less-toxic compounds13), so identifying the factors related to furfural tolerance is important for increasing the efficiency of bioethanol production.

The present study investigated the effect of low concentrations of furfural on yeast cell growth. In addition, to investigate new mechanisms of furfural tolerance, we analyzed gene expression by furfural using a custom microarray chip for S. cerevisiae. Our findings showed that furfural induced sulfur metabolic genes but the addition of amino acids did not compensate for the decrease of growth rate induced by furfural. These findings might be important for effective ethanol production.
from lignocellulosic biomass.

2. Materials and Methods

2.1. Yeast Strain and Growth Analysis

Saccharomyces cerevisiae type 2, a diploid industrial strain of yeast, was purchased from Sigma Aldrich. For the analysis of growth, cells were precultured in 5 mL YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose; Difco, Becton, Dickinson and Co., Japan) at 30°C for 18 h, and 100 μL of the preculture was added to 5 mL YPD medium with and without 10 mM (1 M = 1 mol L⁻¹) furfural in a test tube. Cell concentration was measured periodically over a 24-h period in the cell culture diluted 10-fold with YPD medium using a spectrophotometer measuring light absorption at 600 nm. For testing of the effect of addition of amino acids, L-methionine and L-cysteine were added to the YPD medium after preculture of yeast and the growth rate with added furfural was analyzed.

2.2. RNA Extraction, Microarray Analysis, and Real-time PCR

Culture samples with and without furfural were collected 24 h after the start of incubation. Cells were pelleted by centrifugation and total RNA extraction was performed using an RNeasy mini kit (Qiagen, Tokyo, Japan). The amount and quality of RNA was analyzed by measuring the O.D. 260/280 ratio using a UV spectrophotometer.

Labelling and hybridization of cDNA were performed at the Dragon Genomics Center (Takara Bio Inc., Shiga, Japan) using a Yeast Gene Expression Microarray 4×44 K V1 hybridization chip (Agilent Technologies). The microarray included more than 6256 ORFs of S. cerevisiae S288C. Real-time PCR was performed with a One Step SYBR® PrimeScript™ RT-PCR Kit (Takara) and the following primer pairs: MET3-forward 5'-TGGATTATTACGTCGGTGTC-3' and MET3-reverse 5'-TAGCTTCTCTGCGGCCCTTC-3'; MET14-forward 5'-GGGAGGGTGTAATCAAGGAGTTT-3' and MET14-reverse 5'-GGGAGGGTGTAATCAAGGAGTTT-3'; and ACT1-forward 5'-CACACCCTGCTGCTCAATCCTTCT-3' and ACT1-reverse 5'-GGGGACTCTGAATCTTTCG-3'. Gene expression changes were determined by comparing the Cq values of the target genes with that of the ACT1 gene as a reference.

3. Results and Discussion

Measurement of the growth rate of S. cerevisiae cells in the presence of 10 mM furfural found that furfural slightly inhibited log-phase growth, but had little effect on growth in the stationary phase compared to control cultures (Fig. 1). Investigation of the expression of furfural inducible or repressible genes using a microarray, particularly the expression of furfural detoxification genes including ZWF1, GND1, RPE1 and TKL1 of the pentose phosphate pathway found that expression of none of these genes showed any significant change in response to the presence of furfural (Table 1). A previous report suggested that these genes might be involved in cell damage repair, rather than in detoxification of furfural, and that the growth rate of a yeast strain with single or multiple deletions of these genes was lower than that of the wild type. Moreover, furfural detoxification is known to be catalyzed by NADPH-dependent aldehyde reductases, such as YGL157W, whose expression is induced by furfural. However, the present study found that furfural only slightly induced the expression of YGL157W (data not shown). Therefore, the mechanism underlying furfural tolerance in S. cerevisiae might be mediated by several pathways. The concentration of furfural used in previous studies ranged from 20-150 mM, which is slightly higher than the 10 mM furfural used in the present study. Such differences in the concentration of furfural may have influenced the expression and type of furfural tolerance-related genes.

To identify novel pathways that provide tolerance to low concentrations of furfural, this study analyzed upregulated genes, particularly those related to sulfur metabolism, after 24-h culture in medium with 10 mM furfural (Fig. 2). Furthermore, changes in gene expression of MET3 and MET14 were confirmed using real time PCR (Fig. 3). Although the changes in expression were slightly different from those found by microarray analysis, expression of both genes were in-
creased in the presence of furfural after 24 h. Yeast cells use exogenous sulfate for the biosynthesis of sulfur-containing amino acids\textsuperscript{15).} A previous study showed that the sulfur metabolism pathway in \textit{S. cerevisiae} is induced by acetaldehyde\textsuperscript{16).} Acetaldehyde is produced during ethanol fermentation, so furfural might inhibit ethanol production by promoting the accumulation of intermediate compounds, such as acetaldehyde and glycerol\textsuperscript{17).} Furfural also induces the expression of sulfur-assimilation genes in an ethanologenic strain of \textit{Escherichia coli} (LY180)\textsuperscript{18). Notably, the inhibitory effect on ethanol production can be reversed by adding sulfur-containing amino acids to cultures of \textit{E. coli}\textsuperscript{18). Although the sulfur metabolism pathway in \textit{E. coli} LY180 differs from that in \textit{S. cerevisiae}, the up-regulation of sulfur-related genes in yeast may be induced by the depletion of sulfur-containing amino acids. Therefore, the present study attempted to compensate for the inhibitory effect of furfural on yeast growth by supplementing cultures with sulfur amino acids. However, the exogenous addition of 1 mM methionine did not compensate for the inhibitory effect (Fig. 4). Therefore, we investigated the effect of 10 mM methionine and cysteine on yeast growth with furfural. However, addition of sulfur amino acids did not resolve the inhibitory effect of furfural. Further study is needed to analyze the effects of various concentrations of different amino acids on yeast growth.

The present study revealed that furfural, even at low concentration, induces the depletion of amino acids in \textit{S. cerevisiae}, resulting in the expression of sulfurmetabolism genes and biosynthesis of sulfur-containing amino acids.

### 4. Conclusions

The present study investigated changes in gene expression in \textit{S. cerevisiae} in response to exogenous furfural and showed that many sulfur-metabolism genes were up-regulated. This finding indicates that furfural induces the expression of sulfur-metabolism genes, even at low concentrations. These findings may be important in the development of high-efficiency processes for large-scale bioethanol production from lignocellulosic biomass.
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