Metabolic Engineering of *Saccharomyces cerevisiae* to Improve Glucan Biosynthesis

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Introduction

β-Glucan is a chief structural polymer in the cell wall of yeast. β-Glucan has attracted intensive attention because of its wide applications in health protection and cosmetic areas. In the present study, the β-glucon biosynthesis pathway in *S. cerevisiae* was engineered to enhance β-glucon accumulation. A newly identified bacterial β-1, 6-glucon synthase GsmA from *Mycoplasma agalactiae* was expressed, and increased β-glucan content by 43%. In addition, other pathway enzymes were investigated to direct more metabolic flux towards the building of β-glucan chains. We found that overexpression of Pgm2 (phosphoglucomutase) and Rho1 (a GTPase for activating glucon synthesis) significantly increased β-glucan accumulation. After further optimization of culture conditions, the β-glucan content was increased by 53.1%. This study provides a new approach to enhance β-glucon biosynthesis in *Saccharomyces cerevisiae*.

**Keywords:** *Saccharomyces cerevisiae*, β-glucon, synthase, enzyme overexpression, cell resistance
Materials and Methods

Plasmid and Strain Construction

All strains and plasmids used in this study are listed in Table 1. *Saccharomyces cerevisiae* CEN.PK2-1C was used as the wild-type strain. Glucan synthesis pathway genes RHO1, HXK1, PGM2 and UGP1 were amplified from the genome of *S. cerevisiae* S288c with designated oligonucleotides (Table 2). PCR fragments of the five genes were digested with enzymes *Bam*HI and *Pst*I, and subcloned into YEp351. The genes *gtf*, *crd*, and *gsmA* were synthesized by Genscript (China), and inserted into pRS305 after digestion with enzymes *Bam*HI and *Pst*I. These recombinant plasmids (Table 1) were transformed into *S. cerevisiae* CEN.PK2-1C by LiAc/SSDNA/PEG and the transformants were screened on synthetic dropout plate and further confirmed with PCR.

Medium and Culture Conditions

LB medium (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl, pH 7.0) was used for *E. coli* cultures and for all DNA manipulations. Ampicillin (100 μg/ml) was added to provide selective pressure during growth when necessary. Recombinant yeast cells were grown in YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose). Synthetic dropout (SD) medium [0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose] media with appropriate supplemental amino acids were used to select yeast transformants.

Analysis of the Polysaccharides of Different Strains

The contents of polysaccharides in yeast cell wall were measured to examine the influences of overexpressed genes on yeast cell wall structure. Cells were grown to the early exponential phase (2.5 × 10⁶ cells) and harvested by centrifugation (10,000 × g, 1 min). Cells were incubated in 50 ml of 0.1 M acetic acid-sodium acetate (pH 5.0), at 55°C for 24 h to induce cell autolysis. The autolyzed cells were further lysed by cryogenic grinding. The isolated cell wall was treated with 72% (v/v) H₂SO₄ at room temperature for 4 h and hydrolyzed with 2 M H₂SO₄ at 100°C for 4 h. After adjusting pH to 7.0, the composition of monosaccharides (mannose and glucose) was analyzed and quantified with high-performance anionic exchange chromatography. The content of polysaccharides in the cell wall of strains was calculated according to the following equation:

\[ \text{Glucan content} = \frac{M_{\text{polysaccharide}}}{\text{CDW}}, \]

where \( M_{\text{polysaccharide}} \) is the amount (mg) of polysaccharides while cell dry weight (CDW) is expressed in g.
Transmission Electron Microscopy (TEM) Analysis
The cell wall structure of yeast strains was analyzed with transmission electron microscopy (TEM). Yeast cells were cultured on plate at 30°C for 24 h and then harvested for TEM analysis. Yeast cell samples were fixed as described [16]. The samples were fixed in 2.5% (w/v) glutaraldehyde-PBS buffer (pH 7.2) overnight at 4°C, and then rinsed with the same buffer for six times and post-fixed with 1% (w/v) osmium tetroxide for 2 h at 4°C. The samples were dehydrated stepwise with 30%, 50%, 70% and 90% (v/v) of ethanol, 1: 1 mixture of 90% (v/v) ethanol and 90% (v/v) aceton, and aceton for three times. The samples were then embedded in Epon812 and polymerized. Ultrathin sections of the samples were prepared and examined with a transmission electron microscope.

Stress Resistance Assays
The resistances of mutants to different stresses were tested on SD plate contain 1 M sorbitol and 5% (w/v) NaCl, respectively. Yeast cells were grown in 5 ml SD at 30°C for 24 h and harvested. Cells were washed twice with distilled water and suspended with 0.9% (w/v) saline. Finally, a 10-fold serial dilution of this culture was made and a 5-μl aliquot of each dilution was spotted onto appropriate solid medium and incubated at 30°C for 3 days.

Results and Discussion
Overexpression of Heterologous β-glucan Synthases to Increase β-glucan Biosynthesis
The expression of β-glucan synthase and β-glucan
Improvement of Glucan Biosynthesis

In order to increase the overall polymerization capability, three heterologous \( \beta \)-glucan synthases, GsmA, Gtf and CrdS, from *Mycoplasma agalactiae* 5632 [18], *Oenococcus oeni* [19] and *Agrobacterium sp.* [20], were overexpressed in *S. cerevisiae* and the effects on the composition of cell wall polysaccharides were investigated. As shown in Fig. 2A, all recombinant strains accumulated more \( \beta \)-glucan than the wild-type strain. In particular, overexpression of GsmA resulted in 43\% increase of \( \beta \)-glucan content. The result here confirmed that GsmA from *M. agalactiae* is a functional \( \beta \)-1, 6-glucan synthase [18]. Moreover, the results demonstrate that introduction of heterologous \( \beta \)-1, 3-glucan synthases can also strengthen the biosynthesis of \( \beta \)-1, 3-glucan in *S. cerevisiae*. Although many *KRE* genes have been reported to participate in \( \beta \)-1, 6-glucan biosynthesis in *S. cerevisiae*, no specific \( \beta \)-1, 6-glucan synthase has been determined experimentally [9]. Here, functional expression of the *M. agalactiae* \( \beta \)-1, 6-glucan synthase GsmA in *S. cerevisiae*

**Table 2. Primers used in this study.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5′–3′)</th>
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<tbody>
<tr>
<td>gtf-F</td>
<td>CGGGATCCATGTTGAACGATAACGATTCTG</td>
</tr>
<tr>
<td>gtf-R</td>
<td>AACTGCAATATCGTTCCAAATCAACAGTTCAG</td>
</tr>
<tr>
<td>crd-F</td>
<td>CGGGATCCATGTTTTTCTGCTGAAGGAG</td>
</tr>
<tr>
<td>crd-R</td>
<td>AACTGCAATTAACCGAAAGCTCTAGGCAAC</td>
</tr>
<tr>
<td>gsmA-F</td>
<td>CGGGATCCATGAAGAAAAACAGAAAGCTTTTCTTCTGTG</td>
</tr>
<tr>
<td>gsmA-R</td>
<td>AACTGCAATTTTCTGCTGCCAGC</td>
</tr>
<tr>
<td>RHO1-F</td>
<td>CGGGATCCATGTCACAAACAGTTGG</td>
</tr>
<tr>
<td>RHO1-R</td>
<td>AACTGCAAGCTATAAACAAGACACACTTC</td>
</tr>
<tr>
<td>HXK1-F</td>
<td>CGGGATCCATGTTCCATTAGGTCAAAGAAACACAGG</td>
</tr>
<tr>
<td>HXK1-R</td>
<td>AACTGCAATGACGGCAAATGATACCAAGAG</td>
</tr>
<tr>
<td>PGM2-F</td>
<td>CGGGATCCATGCTATTCAAATTGAAACGTCGCT</td>
</tr>
<tr>
<td>PGM2-R</td>
<td>AACTGCAATGACGGAAACGTCGCTTCCAGT</td>
</tr>
<tr>
<td>UGP1-F</td>
<td>CGGGATCCATGTCACACTAAGAAGACACACC</td>
</tr>
<tr>
<td>UGP1-R</td>
<td>AACTGCAATGTCTTTCAAGATTGTC</td>
</tr>
</tbody>
</table>

Bold letters represent the restriction enzyme sites.

**Fig. 2.** (A) The content of polysaccharides in the cell wall of strains S1, S-gtf, S-crd, S-gsmA. Yeast cells were cultured in YPD medium. The data shown are mean values from triplicates with error bars indicating the standard deviation; (B) TEM photographs of *S. cerevisiae* strains S1, S-gtf, S-crd, S-gsmA.
could help characterize the specific function of the KRE proteins. In bacteria, β-1, 3-glucan is synthesized and secreted in the medium [20]. However, no β-glucan was detected in the culture medium of S. cerevisiae (data not shown). The results suggested that all the produced β-1, 6-glucan and β-1, 3-glucan by the overexpression of β-glucan synthases were used as building blocks for cell wall construction.

In addition to β-glucan, the mannan content of the recombinant S. cerevisiae strains S-gtf, S-crd and S-gsmA was also investigated. Interestingly, the mannan content decreased after the overexpression of heterologous β-glucan synthases, suggesting competition for substrates (such as glucose-6-phosphate) might be ascribed to biosynthesis of β-glucan and mannan in S. cerevisiae. To observe the change in cell wall structure, TEM analysis was also carried out. According to previous studies, the electron-transparent inner layer is mainly composed of β-glucan while the electron-dense outer layer consists of mannoprotein [14]. Consistently, overexpression of β-glucan synthases, especially S-gsmA, increased the thickness of the electron-transparent inner layer (Fig. 2B), suggesting an increased accumulation of β-glucan.

**Optimization of β-Glucan Biosynthesis Pathway to Enhance Glucan Production**

To direct more UDP-glucose towards β-glucan biosynthesis, the pathway enzymes Hxk1, Pgm2, Ugp1 and guanosine triphosphatase (Rho1) were separately overexpressed in S. cerevisiae. As shown in Fig. 3A, upregulation of PGM2 and RHO1 increased β-glucan to 37.3 mg/g CDW and 35.5 mg/g CDW, respectively. In contrast, no obvious effect was observed when overexpressing HXK1 and UGP1. Similar results were also observed when overexpressing

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**Fig. 3.** (A) The content of polysaccharides in the cell wall of strains S2, S-HXK1, S-PGM2, S-UGP1, S-RHO1; (B) The contents of polysaccharides in the cell wall of strains G-pY26, G-HXK1, G-PGM2, G-UGP1, G-RHO1. Yeast cells were cultured in SD medium. The data shown are mean values from triplicates with error bars indicating the standard deviation.

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**Fig. 4.** Assay for sensitivities to different stresses. (A) Resistance of strains S3, G-pY26, G-PGM2, G-RHO1 to sorbitol; (B) Resistance of strains S3, G-pY26, G-PGM2, G-RHO1 to NaCl. Aliquots (5 μl) of 10-fold serial dilutions of mutant strains and parental strain cells were plated onto the designed plates.
these enzymes in recombinant strain S-gsmA (Fig. 3B). In fact, it has been reported that Pgm2 is a rate-limiting enzyme [21] and its overexpression can significantly improve galactose metabolism in *S. cerevisiae* [22–24]. Moreover, previous studies have also demonstrated that Rho1 acts as molecular switches to multiple stress conditions [25] and is involved in regulation of cell wall biogenesis in *S. cerevisiae* [13, 14]. Thus, our results here confirmed that Pgm2 and Rho1 are the committed enzymes for β-glucan biosynthesis. Furthermore, the resistance ability of the recombinant strains to osmotic stress was increased with β-glucan accumulation (Fig. 4), demonstrating the significant role of β-glucan in resistance of yeast cells to environmental stresses.

**Optimization of Culture Medium to Increase β-Glucan Biosynthesis**

The medium composition affects cell growth and generates significant effects on the cell wall structure. Moreover, when encountering environmental stresses, the cell wall remodels into a highly regulated and polarized morphology [13]. For this reason, different concentrations of glucose and NaCl (high osmotic pressure) were added and the effects on β-glucan biosynthesis were analyzed. As shown in Fig. 5, no obvious effects were detected when adding different concentrations of glucose. The results indicate that under normal conditions, the β-glucan content should be constant even in the presence of excess glucose. In contrast, when supplementing 50 g/l NaCl in SD medium, the β-glucan content was increased by 53.1% while no obvious increase was found for mannan. The results suggest that when yeast cells encounter high osmotic pressure, the β-glucan biosynthesis is strengthened and as a result, higher content of β-glucan can be found responsible for cell wall integrity [13].

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**


