Metabolic Engineering of *Saccharomyces cerevisiae* to Improve Glucan Biosynthesis

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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 -glucan biosynthesis pathway in *S. Cerevisiae* was engineered to enhance β -glucan accumulation. A newly identified bacterial β -1, 6-glucan 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 glucan 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 -glucan biosynthesis in *Saccharomyces cerevisiae*.

Keywords: *Saccharomyces cerevisiae*, β -glucan, synthase, enzyme overexpression, cell resistance

Introduction

β -glucan is widely distributed in bacteria, algae, mushrooms, and unicellular fungi such as *Saccharomyces cerevisiae* and *Candida albicans* and higher plants [1] as a main constituent of cell wall structure. β -Glucan has many reported biological functions such as immune modulation and enhancement of resistance against infections and cancer development [2]. Moreover, β -glucans can also provide protection against oxidation [3] and radiation, and lower the level of cholesterol and blood lipids. Therefore, β -glucans, especially those extracted from *Saccharomyces cerevisiae*, have been widely used in the medical, health food and cosmetics industries [4–6].

The cell wall of *S. cerevisiae* consists of a bilayered structure of β -1, 3-glucan, mannoprotein and β -1, 6-glucan [7, 8]. β -1, 3-Glucan and β -1, 6-glucan account for approximately 50–55% and 10–15% of the total cell wall polysaccharide, respectively [8, 9]. β -1, 3-Glucan as a major constituent forms a network by attaching to the heavily branched β -1, 6-glucan [10, 11]. To date, three membrane-associated β -1, 3-glucan synthases from *S. cerevisiae* have been identified.

The major β -1, 3-glucan synthase Fks1 has been well studied [12]. In particular, Fks1 was found in association with Rho1, a GTPase for activating glucan synthesis [13, 14]. In contrast, the β -1, 6-glucan synthase activity has not been characterized and the regulatory mechanism remains unclear, although many *KRE* genes, such as *KRE5*, *KRE6*, and *KRE9* have been repeatedly demonstrated to be involved in β -1, 6-glucan synthesis [15].

The β -glucan biosynthesis pathway in *S. cerevisiae* is shown in Fig. 1. After transportation and phosphorylation, glucose-6-phosphate is converted to glucose-1-phosphate by phosphoglucomutase. Then, the polymer precursor UDP-glucose is synthesized from UTP and glucose-1-phosphate by UDP-glucose pyrophosphorylase. Finally, the glucan synthases Fks1, Fks2, and Fks3 in association with Rho1 polymerize β -glucan from UDP-glucose. Although many efficient strategies for extracting *S. cerevisiae* β -glucan have been developed, no efforts have been spent on strengthening β -glucan accumulation from the perspective of metabolic pathway engineering. In this study, we engineered the β -glucan biosynthesis pathway in *S. cerevisiae* for the first time.

Fig. 1. Biosynthetic pathway of β -glucan from glucose in *S. cerevisiae*.

1, Glucan synthases Fks1, Fks2, and Fks3 (only one is shown in the figure) synthesize β -1, 3-glucan from UDP-glucose; 2, Exogenous β -1, 3-glucan synthase Gtf, Crd synthesize β -1, 3-glucan from UDP-glucose; 3, Exogenous β -1, 6-glucan synthase GsmA synthesizes β -1, 6-glucan from UDP-glucose.

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 μ g/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 (2.5×10^6 cells) and harvested by centrifugation ($10,000 \times g$, 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) H_2SO_4 at room temperature for 4 h and hydrolyzed with 2 M H_2SO_4 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:

$$\text{Glucan content} = M_{(\text{polysaccharide})} / \text{CDW},$$

where $M_{(\text{polysaccharide})}$ is the amount (mg) of polysaccharides while cell dry weight (CDW) is expressed in g.

Table 1. Plasmids and strains used in this study.

Plasmids and strains	Genotype	References
Plasmids		
pRS305	<i>E. coli/S. cerevisiae</i> shuttle vector, <i>ScLEU2</i> , 2 μ m, Ap ^r	[29]
pRS305- <i>gtf</i>	<i>P</i> _{TEF} - <i>gtf</i> - <i>T</i> _{CYC1} in pRS305	This work
pRS305- <i>crd</i>	<i>P</i> _{TEF} - <i>crd</i> - <i>T</i> _{CYC1} in pRS305	This work
pRS305- <i>gsmA</i>	<i>P</i> _{TEF} - <i>gsmA</i> - <i>T</i> _{CYC1} in pRS305	This work
pY26	<i>E. coli/S. cerevisiae</i> shuttle vector, <i>ScURA3</i> , 2 μ m, Ap ^r	This work
pY26- <i>RHO1</i>	<i>P</i> _{GAP} - <i>RHO1</i> - <i>T</i> _{CYC1} in YE351	This work
pY26- <i>HXX1</i>	<i>P</i> _{GAP} - <i>HXX1</i> - <i>T</i> _{CYC1} in YE351	This work
pY26- <i>PGM2</i>	<i>P</i> _{GAP} - <i>PGM2</i> - <i>T</i> _{CYC1} in YE351	This work
pY26- <i>UGP1</i>	<i>P</i> _{GAP} - <i>UGP1</i> - <i>T</i> _{CYC1} in YE351	This work
Strains		
<i>E. coli</i> JM109	<i>F'</i> <i>traD36 proA</i> ⁺ <i>B</i> ⁺ <i>lacIq</i> Δ (<i>lacZ</i>) <i>M15</i> / Δ (<i>lac-proAB</i>) <i>glnV44e14- gyrA96 recA1 relA1 endA1 thi hsdR17</i>	Invitrogen
<i>S. cerevisiae</i>	<i>MATa</i> ; <i>ura3-52</i> ; <i>trp1-289</i> ; <i>leu2-3,112</i> ; <i>his3?</i> 1; <i>MAL2-8</i> ;	Lab stock
CEN.PK2-1C	<i>SUC2</i>	This work
S1	<i>S. cerevisiae</i> CEN.PK2-1C/ pRS305	This work
S2	<i>S. cerevisiae</i> CEN.PK2-1C/ pY26	This work
S3	<i>S. cerevisiae</i> CEN.PK2-1C/ pRS305, pY26	This work
S- <i>gtf</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pRS305- <i>gtf</i>	This work
S- <i>crd</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pRS305- <i>crd</i>	This work
S- <i>gsmA</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pRS305- <i>gsmA</i>	This work
S- <i>HXX1</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pY26- <i>HXX1</i>	This work
S- <i>PGM2</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pY26- <i>PGM2</i>	This work
S- <i>UGP1</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pY26- <i>UGP1</i>	This work
S- <i>RHO1</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ pY26- <i>RHO1</i>	This work
G-pY26	<i>S. cerevisiae</i> CEN.PK2-1C/ <i>gsmA</i> , pY26	This work
G- <i>HXX1</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ <i>gsmA</i> , pY26- <i>HXX1</i>	This work
G- <i>PGM2</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ <i>gsmA</i> , pY26- <i>PGM2</i>	This work
G- <i>UGP1</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ <i>gsmA</i> , pY26- <i>UGP1</i>	This work
G- <i>RHO1</i>	<i>S. cerevisiae</i> CEN.PK2-1C/ <i>gsmA</i> , pY26- <i>RHO1</i>	This work

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) acetone, and acetone 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- μ l 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

Table 2. Primers used in this study.

Primers	Sequences (5'-3')
gtf-F	CGGGATCCATGTTGAACGATAACGATTCTG
gtf-R	AACTGCAGTTAATCGTTCCAATCAACAGTCTCAG
crd-F	CGGGATCCATGTACTTTTCTGCTGAAGGAG
crd-R	AACTGCAGTTAACCGAAAGCTCTAGCCAAC
gsmA-F	CGGGATCCATGAAGAAAAACAGAACGTTTTCATCTTCGTG
gsmA-R	AACTGCAGTTATTTCTGCTGCGCGC
RHO1-F	CGGGATCCATGTCAACAACAAGTTGG
RHO1-R	AACTGCAGCTATAACAAGACACACTTC
HXK1-F	CGGGATCCATGGTTCATTTAGGTCCAAAGAAACCACAGGC
HXK1-R	AACTGCAGTTAAGCGCCAATGATACCAAGAGAC
PGM2-F	CGGGATCCATGTCAATTCAAATTGAAACGGTCCCCACC
PGM2-R	AACTGCAGTTAAGTACGAACCGTTGGTCTTCAGTTCC
UGP1-F	CGGGATCCATGTCCACTAAGAAGCACACC
UGP1-R	AACTGCAGTCAATGTTCCAAGATTTCG

Bold letters represent the restriction enzyme sites.

biosynthesis in *S. cerevisiae* are strictly regulated and influenced by environmental conditions [17]. In order to increase the overall polymerization capability, three heterologous β -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 β -glucan than the wild-type strain. In particular, overexpression of GsmA resulted in

43% increase of β -glucan content. The result here confirmed that GsmA from *M. agalactiae* is a functional β -1, 6-glucan synthase [18]. Moreover, the results demonstrate that introduction of heterologous β -1, 3-glucan synthases can also strengthen the biosynthesis of β -1, 3-glucan in *S. cerevisiae*. Although many KRE genes have been reported to participate in β -1, 6-glucan biosynthesis in *S. cerevisiae*, no specific β -1, 6-glucan synthase has been determined experimentally [9]. Here, functional expression of the *M. agalactiae* β -1, 6-glucan synthase GsmA in *S. cerevisiae*

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.

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.

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

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 μ l) of 10-fold serial dilutions of mutant strains and parental strain cells were plated onto the designed plates.

Fig. 5. The content of polysaccharides in the cell wall of strain G-RHO1.

Cells were cultured in SD medium with 10, 20, 40, and 60 g/l glucose and 50 g/l NaCl respectively. The data shown are mean values from triplicates with error bars indicating the standard deviation.

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.

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