0273-2289/01/91–93/0161/$12.25

Overexpression of Glucose-6-Phosphate Dehydrogenase in Genetically Modified *Saccharomyces cerevisiae*

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) is an abundant enzyme in *Saccharomyces cerevisiae*. This enzyme is of great interest as an analytical reagent because it is used in a large number of quantitative assays. A strain of *S. cerevisiae* was genetically modified to improve G6PD production during aerobic culture. The modifications are based on cloning the G6PD sequence under the control of promoters that are upregulated by the carbon source used for yeast growth. The results showed that *S. cerevisiae* acquired from a commercial source and the same strain produced by aerobic cultivation under controlled conditions provided very similar G6PD. However, G6PD production by genetically modified *S. cerevisiae* produced very high enzyme activity and showed to be the most effective procedure to obtain glucose-6-phosphate dehydrogenase. As a consequence, the cost of producing G6PD can be significantly reduced by using strains that contain levels of G6PD up to 14-fold higher than the level of G6PD found in commercially available strains.

Index Entries: Glucose-6-phosphate dehydrogenase; *Saccharomyces cerevisiae*; aerobic culture; molecular biology.

Introduction

Glucose 6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49), which is an abundant enzyme in *Saccharomyces cerevisiae*, is the first enzyme of the

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pentose phosphate pathway and shows a wide distribution in nature, being found in almost all animal tissues and microorganisms. This enzyme presents great interest as an analytical reagent because it is used in many quantitative assays, including the measurement of hexokinase and creatine kinase activities, adenosine triphosphate and hexose concentration, and as a marker for enzyme immunoassays *(1)*. The use of G6PD for measuring glucose in the presence of fructose constitutes an important tool for detecting illegal sugar addition in the final products of the wine and fruit juice industries. Thus, studies related to G6PD production by *S. cerevisiae* should become an important matter. This yeast has been demonstrated to be a very useful organism for the expression of various genes for protein purification and analysis, and for studies of control of gene expression.

As a eukaryotic organism, *S. cerevisiae* is a suitable host for high-level production of both secreted and soluble cytosolic proteins. Most vectors used to overexpress proteins in yeast are based on the multicopy 2μ natural plasmid, but vectors containing centromeric regions that determine the presence of a single copy per cell have also been developed. These plasmids contain sequences for replication and selection in *Escherichia coli* and *S. cerevisiae*, as well as yeast promoters and terminators for transcription by RNA polymerase II (RNA pol II) *(2)*. The *S. cerevisiae* phosphoglycerate kinase 1 (PGK1) gene encodes one of the most abundant mRNA and protein species in the cell, accounting for between 1 and 5% of the total cellular mRNA and protein *(3)*. Therefore, the PGK1 promoter is an attractive option for obtaining high levels of protein expression. Sequences encoding proteins of interest have also been cloned under the control of the GAL1 promoter, which is one of the most powerful tightly regulated promoters of *S. cerevisiae (4)*. Expression of the yeast GAL1 gene is undetectable in cells grown in the absence of galactose and is induced by more than 1000-fold when galactose becomes available *(5,6)*. Fusion of different promoters has also been successfully used for expression of proteins in yeast, such as the GAL1-PGK1 fusion *(7,8)*.

The present work is a comparative study of the overexpression of *S. cerevisiae* G6PD using genetically modified strains, which carry plasmid constructs containing the G6PD coding sequence under the control of the PGK1 and GAL1-PGK1 (GPF) promoters.

Materials and Methods

DNA Analysis Methods and Plasmid Construction

DNA cloning and electrophoresis analysis were performed as described by Sambrook et al. *(9)*. Both the G6PD coding sequence and the PGK1 promoter were amplified from yeast genomic DNA by using polymerase chain reaction (PCR). Oligodeoxynucleotides that served as primers for PCR reactions were purchased from Bio-Synthesis (Lewisville, TX). The primers used to PCR-amplify the G6PD coding sequence were

5'ggctggatccacagaaagagtaa3' (GPD-Bam5'), and 5'gtgatctagacgataaatgaatg3' (GPD-Xba3'), which contain sites for the restriction enzymes *Bam*HI and *Xba*I, respectively (underlined). The primers used to PCR-amplify the PGK1 promoter were 5'caccctcgagctattatcagggc3' (PGK-Xho5'), and 5'aattggatcc ttgatgatctgta3' (PGK-Bam3'), which contain sites for the restriction enzymes *Xho*I and *Bam*HI, respectively. PCR amplification reactions were performed according to instructions provided by the supplier (Gibco-BRL). Vectors YCpSUPEX1 *(8)* and YEplac181 *(10)* were used to construct plasmids YCpGPF-G6PD and YEpPGK-G6PD, which were used for expression of G6PD in yeast.

Media, Yeast Strains, and Genetic Techniques

Different carbon sources were added to yeast extract/peptone medium (YP) and synthetic medium (YNB). YPD and YNB-Glu contained 2% of glucose and YNB-Gal contained 2% of galactose as the carbon source. Yeast strains were incubated at 30°C as described by Sherman et al. *(11)*. *S. cerevisiae* W303-1a (MATa ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100) was used as the host strain for the plasmids YCpGPF-G6PD and YEpPGK-G6PD. YCpGPF-G6PD contains the URA3 gene, which is a genetic marker for yeast. Cells carrying this plasmid (named W303/YCpGPF-G6PD) were grown in YNB-Glu/Gal medium, containing 2% glucose; 2% galactose; 20 µg/mL of adenine, histidine, and tryptophan; and 30 µg/mL of leucine. YEpPGK-G6PD plasmid contains the LEU2 genetic marker, allowing for the cells (W303/YEpPGK-G6PD) to grow in YNB-Glu medium, containing 20 µg/mL of adenine, uracil, histidine, and tryptophan. Plasmids were transformed into yeast cells by using the lithium acetate method, as described previously *(11)*.

RNA Isolation

Total yeast RNA was isolated by using the hot phenol method *(12)* from 50-mL cultures. Briefly, cells were harvested by centrifugation, washed with cold water, and resuspended in 500 µL of 50 m*M* sodium acetate buffer, pH 5.0. Cells were lysed by vortexing, following the addition of 100 µL of 10% sodium dodecyl sulfate (SDS), 0.4 g of glass beads (0.4-mm diameter), and 1.0 mL of prewarmed (65°C) phenol. The aqueous phase was extracted with 1.0 mL of phenol-chloroform and precipitated with 1.5 mL of ethanol. RNA pellets were resuspended in 100 µL of diethylpyrocarbonate-treated water, and RNA concentration was determined by measuring the optical density (OD) at 260 nm.

Northern Hybridization

RNA electrophoresis was performed as described by Sambrook et al. *(9)*. Typically, a 30-µL sample was prepared by mixing 20 µg of total yeast RNA (up to 15 µL) with 6 *M* glyoxal (6 µL); DMSO (8 µL); and 0.1 *M* sodium phosphate buffer, pH 7.0 (3 µL). Samples were incubated at 50°C for 1 h and

chilled on ice. Subsequently, 4 µL of stop solution (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanolFF) was added to the RNA samples, which were loaded onto a 1.3% agarose gel prepared with 10 m*M* sodium phosphate buffer, pH 7.0. Following electrophoresis, the RNA was blotted onto nylon membranes (Hybond-N; Amersham) and crosslinked by ultraviolet irradiation for 2 min (Ultra-Lum Electronic Dual Light Transillumination). DNA probes were labeled by random priming with ^{32}P-α-dATP by using the Gibco-BRL labeling system. Hybridization was performed at 42°C using a hybridization solution containing 5X SSPE, 5X Denhardt's solution, 50% formamide, 0.1% SDS, and 50 µg/mL of herring sperm DNA *(9)*. The DNA fragments used as probes corresponded to the yeast G6PD and ACT1 (actin 1) coding sequences. Radioactive bands were quantitated using a Molecular Dynamics Phosphorimager.

Aerobic Culture Process

To prepare the inoculum, *S. cerevisiae* (isolated from pressed yeast cake) was maintained in agar slant tubes containing 23 g/L of nutrient-agar (Difco, Detroit, MI) and 1.0 g/L of glucose, at 4°C. The cells were transferred to 250-mL Erlenmeyer flasks containing 50 mL of growth medium (15 g/L of glucose; 2 g/L of sucrose; 5.0 g/L of peptone; and 3.0 g/L of yeast extract, pH 4.5) and incubated for 10 h at 35°C on a rotary shaker (175 rpm) (New Brunswick). A volume of 0.45 L of inoculum (4.7 $g_{dry\ cell}$/L) was poured into a 5-L NBS-MF 105-New Brunswick bench fermentor (coupled with NBS dissolved oxygen controller, DO-81) containing 2.55 L of culture medium. The composition of the culture medium was 20 g/L of glucose, 5.0 g/L of peptone, 3.0 g/L of yeast extract, 2.4 g/L of $Na_2HPO_4 \cdot 12H_2O$, 0.075 g/L of $MgSO_4 \cdot 7H_2O$, and 5.1 g/L of $(NH_4)_2SO_4$ at pH 4.0. The culture was grown batchwise at 35 ± 0.5°C and pH 4.0 ± 0.1. Foaming was controlled by the addition of a mixture containing silicone emulsion (10%) and water (90%) (Thomas Scientific, Swedesboro, NJ) (addition dropwise when needed). Agitation and aeration rates were, respectively, 800 rpm and 2.3 vvm (oxygen transfer volumetric coefficient, $K_La = 230\ h^{-1}$). Dissolved oxygen tension was measured by polarographic electrode (Ingold). Airflow was measured by an in-line rotatometer and was set using a needle valve. The pH of the medium during the aerobic culture was measured by combination electrode (Ingold) at the desired value by automatic addition of 0.5 *M* NaOH and 0.5 *M* H_2SO_4.

Measurement of Cell Concentration and Protein Determination

The cell concentration values of the cultures were obtained by using a calibration curve to correlate OD with dry weight (grams/liter). The amount of total protein was determined according to the Coomassie blue method described by Bradford *(13)* using bovine serum albumin as a protein concentration standard.

G6PD Activity Assays

Precultures of control strains and of strains containing either plasmid YCpGPF-G6PD or YEpPGK-G6PD were incubated for 18 h at 30°C in 2 mL of YNB or YPD medium. Cells from the preculture were transferred to a 50-mL culture of the same medium and incubated for 18 h at 30°C up to the OD_{600} of approx 1.0. Subsequently, cells were harvested, immediately frozen, and stored at –20°C. For extract isolation, cells were resuspended in 50 m*M* Tris-HCl buffer, pH 7.5; 5 m*M* $MgCl_2$; 0.2 m*M* EDTA; 10 m*M* β-mercaptoethanol; 2 m*M* aminocaproic acid; and 1 m*M* phenylmethylsulfonyl fluoride. The cells were disrupted by submitting to a vortex (PHOENIX AT56) in the presence of glass beads (0.5-mm diameter). Cell debris and glass beads were removed by centrifugation (2880*g* for 10 min at 1°C), and the G6PD activity of the supernatant was measured by spectrophotometric quantitation of reduced NADP at 30°C, as described by Bergmeyer *(1)*. One G6PD unit was defined as the amount of enzyme catalyzing the reduction of 1 µmol of NADP/min under the assay conditions.

Results and Discussion

Construction of Plasmids YCpGPF-G6PD and YEpPGK-G6PD

One of the aims of this work was to obtain yeast strains expressing G6PD protein at levels higher than the level of G6PD of the commercially available wild-type strains. Therefore, plasmids were constructed in which the G6PD coding region is under the control of two strong RNA pol II transcription promoters: the PGK1 promoter and the GPF promoter that was constructed by making a fusion between the GAL1 and PGK1 promoters *(8)*. The GPF promoter has the advantage of maintaining the features of the inducible GAL1 promoter and of having a unique transcription start site, which is determined by the PGK1 portion of GPF *(8)*. The yeast G6PD coding sequence was isolated by PCR amplification using genomic DNA as template and a pair of primers complementary to its 5' and 3' ends. The PCR reaction yielded a product showing an electrophoretic mobility of 1.5 kb pairs on agarose gel, which is the expected size of the G6PD coding sequence. This DNA fragment was submitted to restriction digestion with the enzymes *Bam*HI and *Xba*I and ligated with plasmid YCpSUPEX1, which had been digested with the same restriction enzymes. The ligated DNA fragments were transformed into *E. coli* cells. Plasmid DNA was isolated from the transformants and screened for clones containing the G6PD insert by means of restriction digestion with the enzymes *Hind*III, *Xba*I, and *Bam*HI. The resulting plasmid was named YCpGPF-G6PD (Fig. 1A). The PGK1 promoter was PCR-amplified from genomic DNA using a pair of primers complementary to its 5' and 3' ends that contain sites for the restriction enzymes *Xho*I and *Bam*HI, respectively. The DNA fragment obtained showed the correct size (0.6 kb) and was submitted to restriction digestion

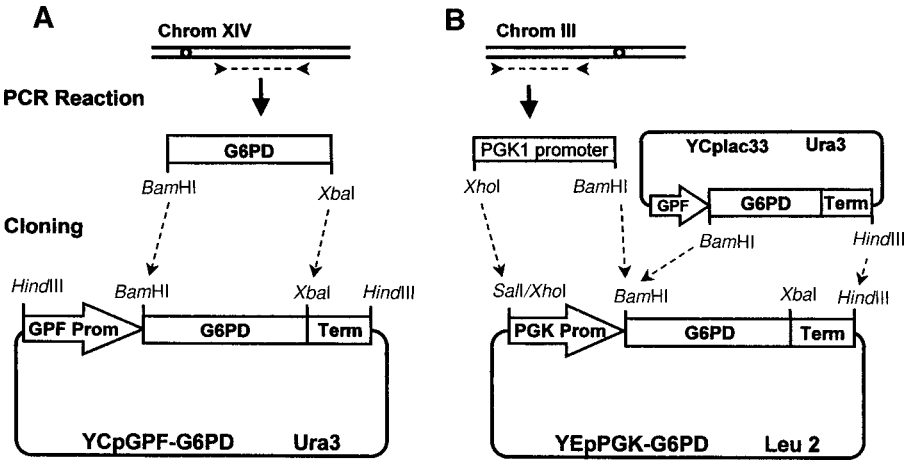

Fig. 1. Construction of plasmids YCpGPF-G6PD and YEpPGK-G6PD. **(A)** The G6PD coding sequence was PCR-amplified from genomic DNA and inserted into the *Bam*HI and *Xba*I sites of vector YCpSUPEX1 to construct plasmid YCpGPF-G6PD. **(B)** The PGK1 promoter was amplified by using PCR from genomic DNA. A 1.8-kb *Bam*HI-*Hind*III fragment containing the G6PD coding sequence and the PGK1 transcription terminator was isolated from YCpGPF-G6PD. This fragment was ligated to PGK1 promoter digested with *Xho*I and *Bam*HI, and plasmid YEplac181 digested with *Sal*I and *Hind*III. Tha *Sal*I and *Xho*I sites are compatible for ligation, although none of them is regenerated. The resulting plasmid was named YEpPKG-G6PD.

with *Xho*I and *Bam*HI. This fragment was cloned into the vector YEplac181 *(10)* at the same time as the G6PD coding sequence and the PGK1 transcription terminator, which were isolated from the YCpGPF-G6PD plasmid as a *Bam*HI-*Hind*III 1.8-kb fragment (Fig. 1B). The new plasmid was named YEpPGK-G6PD.

Quantitation of G6PD mRNA

The gene copy number per cell and the strength of the transcription promoter are major factors that determine the level of a given protein in the cell, provided that there is no posttranscriptional regulatory mechanism. In this work, we used both strong promoters and, in the case of YEpPGK-G6PD, a high copy vector. This should lead to an increase in both G6PD mRNA and protein levels in the cell. We performed Northern analysis to determine the steady-state level of G6PD mRNA in control cells and in cells carrying plasmids YCpGPF-G6PD and YEpPGK-G6PD (Fig. 2). Actin 1 (ACT1) encodes a constitutively expressed protein that has been widely used as an internal control. Interestingly, W303-1a cells grown in synthetic medium (YNB) showed an increased amount of both G6PD and ACT1 mRNAs compared to W303-1a cells incubated in rich medium (YPD). Quantitation of G6PD mRNA revealed a fourfold increase in G6PD mRNA in cells containing the YCpGPF-G6PD plasmid, when incubated in media

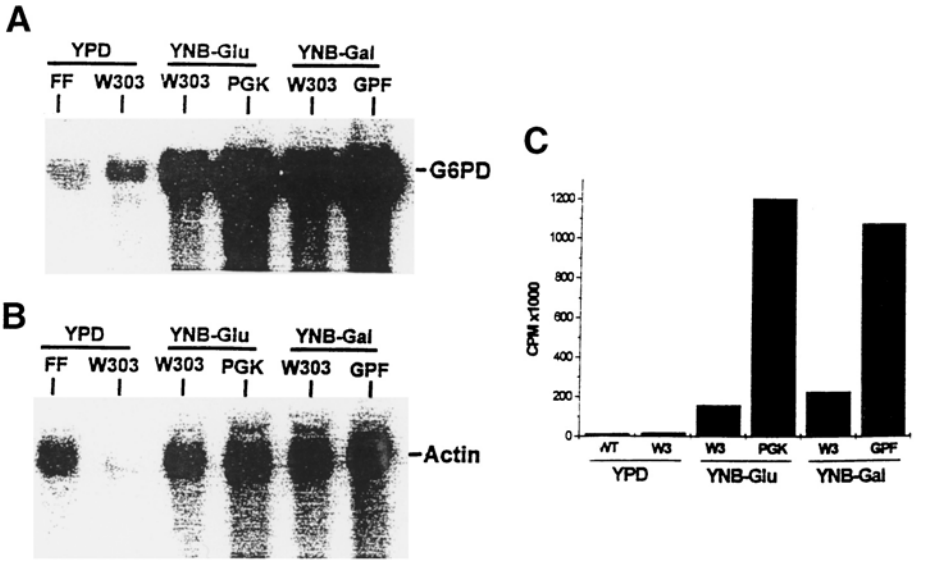

Fig. 2. Quantitation of G6PD mRNA steady-state level. **(A,B)** Northern blot analysis of G6PD and actin mRNAs, respectively; **(C)** graph showing quantitation of Northern blots (G6PD/actin ratio). WT, commercially available strain; W303, W303-1a; PGK, W303-1a cells carrying plasmid YEpPKG-G6PD; GPF, W303-1a cells carrying plasmid YCpGPF-G6PD. YPD, YNB-Glu, and YNB-Gal indicate the medium in which cells were incubated.

containing galactose, compared to the W303-1a parental strain (Fig. 2C). In cells carrying plasmid YEpPGK-G6PD, the level of G6PD mRNA was increased fivefold relative to the W303-1a strain.

Although the GAL1 promoter has been demonstrated to be stronger than the PGK1 promoter, the results obtained in this work can be explained because the GPF-G6PD construct is in a single-copy plasmid, whereas the PGK-G6PD clone is present in a multicopy plasmid. The advantage of plasmid YEpPGK-G6PD is that expression can be performed in medium containing glucose as the carbon source, which is a reagent less expensive than galactose.

G6PD Production

Table 1 shows the G6PD activities obtained from the different *S. cerevisiae* strains. The genetically modified strains produced higher (up to ~40 times $U/mg_{protein}$ and up to ~108 times U/g_{cell}) enzyme-specific activities compared with those of the wild types. Specifically, the strains carrying plasmids YCpGPF-G6PD and YEpPGK-G6PD showed a 6- and 14-fold increase in G6PD activity, respectively, compared with the parental strains grown under the same conditions. Consistent with the results obtained from G6PD RNA quantitation, cells carrying vector YEpPGK-G6PD showed a higher G6PD activity than cells carrying vector YCpGPF-

Table 1
G6PD Activities Obtained from Different Sources

Enzyme source		G6PD activity U/mg_{prot}	G6PD activity U/g_{cell}
Baker's yeast (commercial source)		0.22	50
S. cerevisiae produced by aerobic cultivation		0.30	49
Strain[a]	Medium		
W303	YPD	0.36	191.8
W303GAL	SC-Gal	0.28	140.8
GAL-G6PD[b]	SC-Gal	1.72	1437.9
W303GLU	SC-Glu	0.61	211.7
PGK-G6PD[b]	SC-Glu	8.90	5303.8

[a]W303GAL, W303 grown in galactose medium; GAL-G6PD, W303/YCpGPF-G6PD in galactose medium; W303GLU, W303 grown in glucose medium; PGK-G6PD, W303/UEpPGK-G6PD in glucose medium.
[b]Genetically modified *S. cerevisiae*.

G6PD. This results clearly show that the technique employed in this work to improve G6PD production is efficient and promising.

Conclusion

S. cerevisiae acquired from a commercial source and the same strain produced by aerobic cultivation under controlled conditions provided very similar G6PD productivities. However, G6PD production by *S. cerevisiae* can be improved by using the techniques of recombinant DNA. Its production can be influenced by substituting the native transcription promoter by promoters that are upregulated specifically by the carbon source of the culture medium used to grow the cells. Therefore, the genetically modified *S. cerevisiae* produced very high enzyme activity and proved to be the most effective procedure to obtain G6PD.

The results presented here show that the cost of producing G6PD can be significantly reduced by using strains that contain levels of G6PD up to 14-fold higher than the level of G6PD found in commercially available strains.

Acknowledgments

We acknowledge receipt of fellowships from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP)/Brazil. We also acknowledge the financial support of FAPESP and Conselho Nacional de Desenvolvimento Científico e Tecnologico (CNPq)/Brazil and Prof. Dr. José Abrahão-Neto.

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