Appl Microbiol Biotechnol (2010) 85:731–739
DOI 10.1007/s00253-009-2184-4

Enhancing the flux of D-glucose to the pentose phosphate pathway in *Saccharomyces cerevisiae* for the production of D-ribose and ribitol

Mervi H. Toivari · Hannu Maaheimo · Merja Penttilä · Laura Ruohonen

Received: 16 March 2009 / Revised: 31 July 2009 / Accepted: 4 August 2009 / Published online: 27 August 2009

Abstract Phosphoglucose isomerase-deficient (*pgi1*) strains of *Saccharomyces cerevisiae* were studied for the production of D-ribose and ribitol from D-glucose via the intermediates of the pentose phosphate pathway. Overexpression of the genes coding for NAD^+-specific glutamate dehydrogenase (*GDH2*) of *S. cerevisiae* or NADPH-utilising glyceraldehyde-3-phosphate dehydrogenase (*gapB*) of *Bacillus subtilis* enabled growth of the *pgi1* mutant strains on D-glucose. Overexpression of the gene encoding sugar phosphate phosphatase (*DOG1*) of *S. cerevisiae* was needed for the production of D-ribose and ribitol; however, it reduced the growth of the *pgi1* strains expressing *GDH2* or *gapB* in the presence of higher D-glucose concentrations. The CEN.PK2-1D laboratory strain expressing both *gapB* and *DOG1* produced approximately 0.4 g l^{-1} of D-ribose and ribitol when grown on 20 g l^{-1} (*w*/*v*) D-fructose with 4 g l^{-1} (*w*/*v*) D-glucose. Nuclear magnetic resonance measurements of the cells grown with ^{13}C-labelled D-glucose showed that about 60% of the D-ribose produced was derived from D-glucose. Strains deficient in both phosphoglucose isomerase and transketolase activities, and expressing *DOG1* and *GDH2* tolerated only low D-glucose concentrations (≤2 g l^{-1} (*w*/*v*)), but produced 1 g l^{-1} (*w*/*v*) D-ribose and ribitol when grown on 20 g l^{-1} (*w*/*v*) D-fructose with 2 g l^{-1} (*w*/*v*) D-glucose.

Keywords Sugar alcohols · Pentose sugars · *Saccharomyces cerevisiae* · Pentose phosphate pathway · D-ribose · Ribitol · NMR

M. H. Toivari (✉) · H. Maaheimo · M. Penttilä · L. Ruohonen
VTT Technical Research Centre of Finland,
P.O. Box 1000, FI-02044 Espoo, Finland
e-mail: Mervi.Toivari@vtt.fi

Introduction

The intermediates of the pentose phosphate pathway (PPP) D-ribulose 5-phosphate, D-ribose 5-phosphate, D-xylulose 5-phosphate, and D-erythrose 4-phosphate can serve as precursors for the production of D-ribulose, D-ribose, D-xylulose, D-erythrose, arabitol, ribitol, xylitol, and erythritol. These compounds, used, e.g., as sweeteners, are formed after dephosphorylation of the sugar phosphate and a possible reduction reaction in various either natural or engineered microbes (Sasajima and Yoneda 1989; De Wulf and Vandamme 1997; Ishizuka et al. 1989; Spencer and Sallans 1956; Povelainen and Miasnikov 2006a; Povelainen and Miasnikov 2006b).

The yeast *Saccharomyces cerevisiae* is one of the most exploited industrial organisms and an attractive production host because of the extensive knowledge of its physiology, genetics, and process behaviour and for its GRAS status (generally regarded as safe). We recently engineered *S. cerevisiae* to produce xylitol, ribitol, and ribose from D-glucose (Toivari et al. 2007). A transketolase- and xylulokinase-deficient strain expressing genes encoding xylitol dehydrogenase (*XYL1*) and a sugar phosphate phosphatase (*DOG1*) produced xylitol and ribitol from D-glucose with a yield of 3.6% (Toivari et al. 2007). This modest yield may be due to a limited flux of D-glucose to the PPP in *S. cerevisiae*, and thus means to increase this flux are needed.

Strains deficient in the phosphoglucose isomerase (Pgi1p) activity, converting D-glucose 6-phosphate to D-fructose 6-phosphate, metabolise D-glucose entirely via the PPP, offering a possibility to enhance the flux to the PPP. However, *pgi1*-deficient strains of *S. cerevisiae* are not able to grow on D-glucose, in contrast to the corresponding strains of *Kluyveromyces lactis* and *Escherichia coli* (Maitra 1971; Goffrini et al. 1991; Vinopal et al. 1975). The inability of *pgi1*

mutants of *S. cerevisiae* to grow on D-glucose has been suggested to be due to the accumulation of D-glucose 6-phosphate (Maitra 1971) and subsequent depletion of ATP (Ugarova et al. 1986; Ciriacy and Breitenbach 1979) and/or the lack of $NADP^+$ needed for the reactions of the oxidative PPP (Boles et al. 1993). Indeed, when introducing a NADPH-consuming reaction to the cells, e.g., by overexpression of the endogenous *GDH2* encoding NAD^+-dependent glutamate dehydrogenase, the growth of the *pgi1* strain of *S. cerevisiae* on D-glucose was restored. The $NADP^+$-dependent Gdh1p and NAD^+-dependent Gdh2p of *S. cerevisiae* possibly created a cycle of two reactions converting NADPH to NADH (Boles et al. 1993). Several other NADPH-dependent enzymes, such as the soluble transhydrogenase UdhA of *E. coli* (Fiaux et al. 2003; Heux et al. 2008), the alternative NAD(P)H:ubiquinone oxidoreductase KlNde1p, the glutathione reductase KlGlr1p and the NADPH-preferring glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of *K. lactis* (Tarrio et al. 2006; Verho et al. 2002, respectively), also enable growth of the *pgi1* mutants of *S. cerevisiae* on D-glucose. These enzymes could, thus, provide basis for enhanced D-glucose metabolism through the PPP in the *S. cerevisiae pgi1* strains and subsequently enable the production of 5-carbon sugars from D-glucose via the oxidative PPP intermediates with higher yields.

We generated phosphoglucose isomerase-deficient *S. cerevisiae* strains with two different NADPH-consuming reactions, the Gdh1p–Gdh2p cycle and a NADPH-dependent glycerol 3-phosphate dehydrogenase (Fig. 1). Because the activity of the GAPDH enzyme of *K. lactis* was relatively low when introduced into *S. cerevisiae* (Verho et al. 2002), we instead expressed a gene encoding a gluconeogenic, NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase (*gapB*) from *Bacillus subtilis* (Fillinger et al. 2000). The engineered strains were compared for their ability to produce the PPP-derived 5-carbon compounds ribitol and D-ribose from D-glucose. In order to increase the accumulation of the PPP sugar phosphates and to address the concept of a "closed production pathway" from D-glucose to 5-carbon compounds of the PPP, strains deficient in both transketolase and phosphoglucose isomerase activities were constructed, and the effect of the NADPH-consuming reaction by glyceraldehyde 3-phosphate dehydrogenase activity (GapB) or Gdh2p in the *pgi1 tkl1 tkl2* strain was studied. Enhancement of the dephosphorylation of the sugar phosphates D-ribose 5-phosphate and D-ribulose 5-phosphate was assessed by overexpression of the *S. cerevisiae DOG1* gene coding for a sugar phosphate phosphatase.

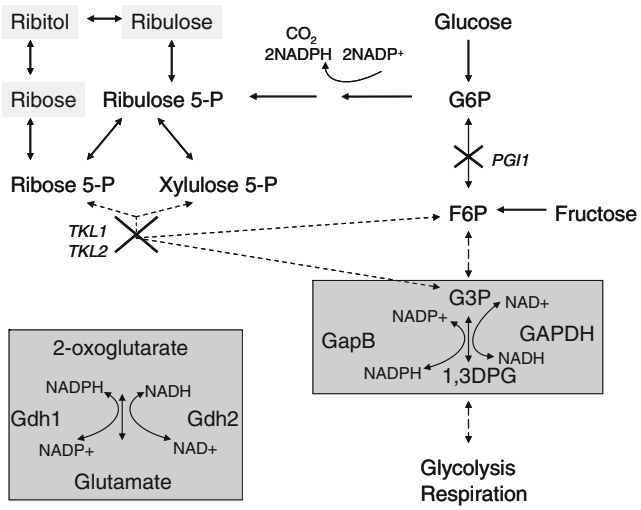

Fig. 1 A schematic representation of the pathways of cofactor regeneration by *gapB*- and *GDH2*-encoded activities and the route for the production of 5-carbon sugars and sugar alcohols. All compounds presented are in D-configuration

Materials and methods

Strains and strain constructions

The yeast strain CEN.PK2-1D (VW-1B; *MATα, leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8^c SUC2*; Boles et al. 1996) served as the parental strain for all strain constructions. The deletion of the *PGI1* gene coding for phosphoglucose isomerase was done by inserting the *HIS3* gene into the coding region of *PGI1* as described previously (Verho et al. 2002) resulting in strain H2493. In addition, a previously generated transketolase-deficient strain, constructed by inserting the *LEU2* gene into the coding region of *TKL1* and the geneticin resistance gene (*KMX*) into to the coding region of *TKL2* (unpublished, Dr. Kari Koivuranta, Espoo, Finland), was transformed with the *PGI1* deletion cassette in order to obtain a strain deficient in both phosphoglucose isomerase and transketolase activities. The insertions were verified with polymerase chain reaction (PCR) and Southern blots.

The *DOG1* gene coding for 2-deoxyglucose-6-phosphate (2-deoxy-glucose 6-P) phosphatase (Dog1p) was ligated previously (Toivari et al. 2007) into the YEplac195 vector (Gietz and Sugino 1988) between the modified *ADH1* promoter (Ruohonen et al. 1995) and *ADH1* terminator. The *ADH1* promoter–*DOG1*–*ADH1* terminator cassette was released with *Bam*HI and inserted into the *Bam*HI site of modified YEplac195 vector B1181. The B1181 (YEplac195+ *PGK1*PT) carried the *PGK1* promoter and terminator as *Hin*dIII fragment from the pMA91 vector (Mellor et al. 1983) at its *Hin*dIII site. The *gapB* gene was amplified by PCR from *B. subtilis* strain ATCC6633 by using oligos 5′-GGATCCAA CATGAAGGTAAAAGTAGCG-3′ and 5′-GGATCCTTATA CAGCAGACGGATG-3′. The 1-kb PCR fragment was

digested with *Bam*HI and ligated into the *Bam*HI site of vector B2159, which is the pYX212 vector modified by removing the ATG and HA tags from the multiple cloning site with *Eco*RI-*Xho*I digestion and by adding the *Eco*RI-*Sal*I linker region from pUC19 (Yanisch-Perron et al. 1985). The plasmid carrying the *gapB* gene was subsequently digested with *Bam*HI and the *gapB* fragment ligated into the *Bgl*II site between the *PGK1* promoter and terminator of YEplac195+*PGK1*PT or to the same vector additionally containing the *DOG1* gene in its *Bam*HI site. The *GDH2* gene of *S. cerevisiae* was ligated with its endogenous promoter from the plasmid YEpMSP3-T (Boles et al. 1993) as a 4.8-kb *Sst*I-*Xba*I fragment to the corresponding sites of YEplac195 resulting in vector denoted B1007. The *Bam*HI fragment containing *DOG1* with *ADH1* promoter and terminator was subsequently ligated to the *Sph*I site of the *GDH2*-YEplac195 vector resulting in vector denoted B1224. The bacterial cloning steps were carried out in *E. coli* DH5α.

The resulting plasmids, containing *gapB* (B2868), *GDH2* (B1007), *gapB*+*DOG1* (B2866), *GDH2*+*DOG1* (B1224), and *DOG1* (B1020) or the control plasmid YEplac195 with *PGK1* promoter and terminator (B1181), were subsequently introduced into the *pgi1* strain H2493 and into the *pgi1 tkl1 tkl2* strain MT64/06. Yeast transformations were carried out with the Gietz lab transformation kit (Medicorp Inc., Canada).

Yeast culture conditions

Yeast strains were cultured in 20 ml of growth medium in 100 ml Erlenmeyer flasks, with 250 rpm shaking at 30°C. Alternatively, the strains were cultured in 2 ml of growth medium in glass tubes (height 16 cm, Ø 1.4 cm). Modified yeast synthetic complete (YSC) medium (Sherman et al. 1983), with the amount of yeast nitrogen base (YNB) and the amino acids tripled, and D-fructose (20 g l^{-1}) and D-glucose (0.5 g l^{-1}) as the carbon source was used as the growth medium for the *pgi1* and *pgi1 tkl1 tkl2* stains. The amount of YNB and amino acids supplied was increased in order to support growth of the *pgi1 tkl1 tkl2* strains. Uracil and histidine were omitted from the growth medium for selection of the plasmids and the *HIS3* integration, respectively. Alternatively, YP medium, containing yeast extract (10 g l^{-1}) and bacto peptone (20 g l^{-1}) with D-glucose (20 g l^{-1}) as the carbon source was used as the growth medium. For nuclear magnetic resonance (NMR) analyses, the D-glucose was replaced with [U–^{13}C]-labelled D-glucose (99 atom% ^{13}C, Isotec, OH, USA).

Accumulation of biomass was measured as optical density (OD) of the cultures at 600 nm (OD_{600}). Biomass, expressed as cell dry weight, was based on the measurement of one OD_{600} unit being equal to 0.3 g l^{-1} cell dry weight. Samples of culture supernatant were collected for metabolite analyses and stored at −20ºC.

Analyses of metabolites

The high-performance liquid chromatography (HPLC) analyses were carried out using a Fast Acid Column (100× 7.8 mm) and a HPX-87H Ion Exclusion Column (300 mm× 7.8 mm, both Bio-Rad Laboratories, CA, USA) in series with 2.5 mM H_2SO_4 in water as the mobile phase at a flow rate of 0.3 ml min^{-1}, at 55ºC. This method enabled quantification of D-glucose, ethanol, glycerol, D-xylulose, ribitol, and xylitol. D-ribose, D-ribulose, and D-arabitol coeluted on the Aminex HPX-87H column. The CarboPac MA-1 column of Dionex ICS-3000 (Dionex Corporation, CA, USA) was used to analyze representative culture supernatant samples for the presence of arabitol and xylitol. Samples were run at column temperature of 30ºC with 480 mM NaOH at flow rate 0.4 ml min^{-1}. The CarboPac MA-1 column separated D-arabitol from D-ribose and D-ribulose, but the alkaline conditions degraded D-ribulose interfering with the quantification of D-ribose.

Enzyme activities

Yeast cells were disrupted with glass beads (Ø 425–600 μm, Sigma-Aldrich Corporation, MO, USA) in 100 mM sodium phosphate buffer pH 7.0 containing phenylmethylsulfonyl fluoride and pepstatin A in final concentrations of 0.17 mg ml^{-1} and 0.01 mg ml^{-1}, respectively.

The activity of NAD^+-dependent Gdh2p was measured in a reaction buffer of 0.5 M triethanol amine pH 7.7 and 2 mM NADH. After addition of the cell lysate, the reaction was started by adding a mixture of α-ketoglutarate (100 mM) and NH_4Cl (200 mM) to a final concentration of 2.4 mM and 4.9 mM, respectively. The GapB activity was measured as described previously (Verho et al. 2002). Shortly, the reaction mixture was 500 mM triethanol amine pH 7.8, 1 mM ATP, 2 mM $MgCl_2$, 200 μM NADPH, and 10 μg ml^{-1} of phosphoglycerate kinase (Boehringer, Germany). 3-phosphoglycerate was added to a final concentration of 5 mM to start the reaction. Activity measurements were performed with a Cobas Mira Plus automated analyzer (Roche, Switzerland).

NMR spectroscopy

Twelve millilitres of culture supernatant was lyophilized and dissolved in 1.2 ml of D_2O (Cambridge Isotope Laboratories, USA). After centrifugation, the supernatant was filtered through a 0.45-μm membrane filter directly to a 5-mm NMR tube.

The NMR spectra were recorded on a Varian Inova NMR spectrometer operating at proton frequency of 500 MHz. The total correlation spectroscopy (TOCSY) spectrum was recorded at 40ºC with the Clean TOCSY pulse sequence

(Griesinger et al. 1988). The spectrum width was 5,200 Hz, number of transients was 16, mixing time (MLEV-17) was 70 ms, and 2-ms trim pulses were used. WALTZ-16 decoupling was used during t_1 to remove the ^{13}C coupling from the proton signals in the indirect dimension. The residual water signal was suppressed by 1.5 s presaturation in the end of the relaxation delay. The total relaxation delay was 5 s, but no other attempts were made to correct the inaccuracy arising from the different relaxation times of ^{12}C- and ^{13}C-bound protons. A matrix of 2,048×256 complex data points was collected, and zero-filled once in F1 and a 90º shifted sine-bell weighting function was used in both dimensions prior to the Fourier transformation.

For the Heteronuclear Single Quantum Coherence (HSQC) spectrum, a matrix of 2,048×1,200 (F2×F1) complex data points was collected at 25ºC with 16 transients and zero-filled in F1 to 4,096 points. GARP ^{13}C decoupling was used during the acquisition time. The spectral width was 3,600 Hz and 4,500 Hz for ^{1}H and ^{13}C, respectively.

Results

In order to study the production of D-ribose and ribitol through the oxidative PPP, strains deficient in phosphoglucose isomerase activity (*pgi1* strains), as well as strains deficient in both phosphoglucose isomerase and transketolase activities (*pgi1 tkl1 tkl2* strains), were generated. Genes encoding either GapB (NADPH-utilising glyceraldehyde 3-phosphate dehydrogenase) or Gdh2p (NAD^+-dependent glutamate dehydrogenase) were expressed on a multicopy plasmid. The activity of GapB was 0.13 nkat mg^{-1} (soluble protein) in the *pgi1 gapB* strain (D-glucose as a sole carbon source), whereas in the *pgi1 tkl1 tkl2* strain, the GapB activity was not detected. Gdh2 activity was 6.2 nkat mg^{-1} (soluble protein) in both the phosphoglucose isomerase-deficient and *pgi1 tkl1 tkl2* strains. The *DOG1* gene, encoding a sugar phosphate phosphatase, was expressed alone or in combination with *gapB* or *GDH2* gene on a multicopy plasmid in the above-mentioned strains to enhance the formation of phosphate-free 5-carbon sugars. The strains used in this study are listed in Table 1.

Enhancing the flux to the oxidative PPP in a phosphoglucose isomerase-deficient strain by increasing the NADPH demand

The *pgi1* strains containing either *gapB* or *GDH2* grew on plates with 20 g l^{-1} (*w*/*v*) D-glucose as the carbon source, whereas growth of the strain with the empty vector was negligible. The *pgi1* strains, which also contained *DOG1* in addition to *gapB* or *GDH2*, grew as very small colonies.

Because the expression of *DOG1* seemed to inhibit growth on D-glucose, the strains were cultured on YSC medium with 20 g l^{-1} D-fructose and various concentrations of D-glucose as the carbon source. The strains showed clear variation in D-glucose tolerance (Fig. 2a, b). Strains overexpressing the *gapB* or *GDH2* gene alone grew in the presence of higher D-glucose concentrations compared to the control strain which only grew significantly in the presence of 0.5, 2, and 4 g l^{-1} (*w*/*v*) D-glucose (Fig. 2a). However, the presence of *DOG1* reduced the growth of these strains in D-glucose concentrations of 6 or 8 g l^{-1} (*w*/*v*; Fig. 2b), whereas in glucose concentrations of 0.5 and 2 g l^{-1}, the expression of *DOG1* appeared to slightly enhance the growth of the strain also harbouring *GDH2*.

Production of 5-carbon sugars from D-glucose in phosphoglucose isomerase-deficient strains

To study the effect of GapB or Gdh2 activities on the production of 5-carbon sugars or sugar alcohols, the growth medium samples of the cultures shown in Fig. 2a and b were analysed by HPLC. The amount of ribitol and D-ribose (potentially also D-ribulose; see Materials and methods) is reported as a sum describing the amount of 5-carbon compounds derived from the PPP precursors D-ribose 5-phosphate and/or D-ribulose 5-phosphate (Fig. 1). As quantification of D-ribulose was not possible, these compounds are referred to as ribitol and D-ribose hereafter. After 72 h of culturing, only the strains containing *DOG1* alone or together with *gapB* or *GDH2* had produced ribitol and D-ribose (Fig. 2c). Overexpression of *gapB* or *GDH2* in addition to *DOG1* increased the yield of D-ribose and ribitol in the presence of 4 g l^{-1} (*w*/*v*) D-glucose. With 0.5 g l^{-1} (*w*/*v*) D-glucose, the production was highest in the strain containing only *DOG1*. This strain produced at most approximately 0.4 g l^{-1} (*w*/*v*) of ribitol and D-ribose. The analysis of representative samples with Dionex MA-1 column showed no xylitol or D-arabitol (absent and below 24 mg l^{-1}, respectively), comparable amounts of ribitol and confirmed qualitatively the formation of D-ribose/D-ribulose. Additionally, in the NMR analyses, both D-ribose (see below) and ribitol were detected.

The effect of the overexpression of *gapB* or *GDH2* on the production of 5-carbon sugars and on the rate of D-glucose consumption was further investigated by culturing the strains containing *gapB* or *GDH2* together with *DOG1* and the strain with *DOG1* alone on 20 g l^{-1} (*w*/*v*) D-fructose with 4 g l^{-1} (*w*/*v*) D-glucose, the highest concentration tolerated by all the strains (Fig. 2a, b). The strain with *gapB* and *DOG1* grew fastest and consumed all the D-glucose provided in 33 h (Fig. 3). The other strains had still approximately 3.5 g l^{-1} residual D-glucose

Table 1 Yeast strains used in the study

Name (genotype of parental strain)	Gene(s) expressed from a plasmid(s)
H2493 (*leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8^c SUC2 pgi1::HIS3*)	–
H3435	*gapB*
H3437	*gapB, DOG1*
H3439	*GDH2*
H3441	*GDH2, DOG1*
H3443	*DOG1*
H2678	Control
MT64/06 (*leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8^c SUC2 pgi1::HIS3 tkl1::LEU2 tkl2::KMX*)	-
H3445	*gapB*
H3447	*gapB, DOG1*
H3449	*GDH2*
H3451	*GDH2, DOG1*
H3453	*DOG1*
H3455	Control

gapB encoding NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase of *B. subtilis*, *GDH2* encoding NAD^+-dependent glutamate dehydrogenase of *S. cerevisiae*, *DOG1* encoding 2-deoxy-glucose phosphate phosphatase of *S. cerevisiae*, *Control* strain with the empty expression vector

(around 90% of the total provided) after 60 h of cultivation (data not shown). The strain with *gapB* and *DOG1* overexpressions produced a total of 0.4 g l^{-1} ribitol and D-ribose.

The D-ribose production originates both from D-glucose and D-fructose

In the *pgi1*-deficient strain, the products D-ribose and ribitol, in addition to D-glucose, may also be derived from D-fructose through the action of transaldolase, transketolase, D-ribulose-phosphate 3-epimerase, and D-ribose-5-phosphate ketol-isomerase reactions. To clarify from which of the two carbon sources the D-ribose and ribitol were produced, we cultured the most efficient strain, the *pgi1* strain expressing *gapB* and *DOG1* genes, with 0.4 g l^{-1} of uniformly ^{13}C-labelled D-glucose and 20 g l^{-1} of unlabelled D-fructose, and measured the labelling of the produced D-ribose from the culture supernatant with NMR. Since ^1H NMR signals are split to doublets (so-called ^{13}C satellites) when the proton is bound to a ^{13}C atom, it is possible to determine the fractional ^{13}C labelling of a given carbon position by ^1H NMR. Although the ^1H NMR spectrum of the culture supernatant was very crowded, the β-ribose cross peaks of a 2D TOCSY spectrum were well resolved and could be used to determine the fractional labelling of ribose C1, C2, and C3 (Fig. 4). Since the carbons corresponding to the carbons 3 to 5 of ribose are never split to different molecules in PPP, C4 and C5 are assumed to originate from the same carbon source molecule as C3 and thus, have similar fractional ^{13}C enrichment. In order to make the TOCSY spectrum less crowded, the ^{13}C couplings were removed from the second dimension by employing broadband decoupling during t_1, resulting in a spectrum where the ^{13}C satellites are present only in the first dimension.

Figure 4a shows cross sections of the TOCSY cross peaks corresponding to protons 1–3 of the beta pyranosidic form of D-ribose. The fractional ^{13}C labelling of C1 and C2 was identical (71%), but clearly different from that of C3 (60%). The different labelling of C2 and C3 suggests that the C2–C3 bond of the carbon backbone of some of the D-ribose molecules has been cleaved during the biosynthesis. To confirm this, we therefore used the ^{13}C fine structure of an HSQC spectrum to determine the intact carbon backbone fragments originating from a single molecule of D-glucose (Szyperski 1995). In such molecules, the whole carbon backbone is labelled, and the ^{13}C signals appear as quartets (carbons in the middle of the chain, which couple to two neighbouring carbons) or as doublets (terminal carbons). Cross sections along ^{13}C dimension of the relevant signals are shown in Fig. 4b. The C1 signal, a doublet with a coupling constant of 47 Hz, indicated that when C1 is labelled, C2 is also always labelled. If only C1 were labelled, the signal would be a singlet. The C2 signal consisted of a 47 Hz doublet and a quartet. The doublet arises from the molecules having C2 and C1, but not C3 labelled, while the quartet corresponds to the situation where all three carbons are labelled. Together with the result from C1, this shows that C1 and C2 always originate from the same carbon substrate, which, according to the TOCSY spectrum, is D-glucose in 71% of the molecules. However, the presence of the doublet in C2 signal indicates that in about 10% of these D-ribose molecules having labelled C1 and C2, C3 originates from fructose, which explains its lower labelling degree. Interestingly, the C3 signal is an almost pure triplet with a coupling constant of 37 Hz. This indicates that in all molecules having

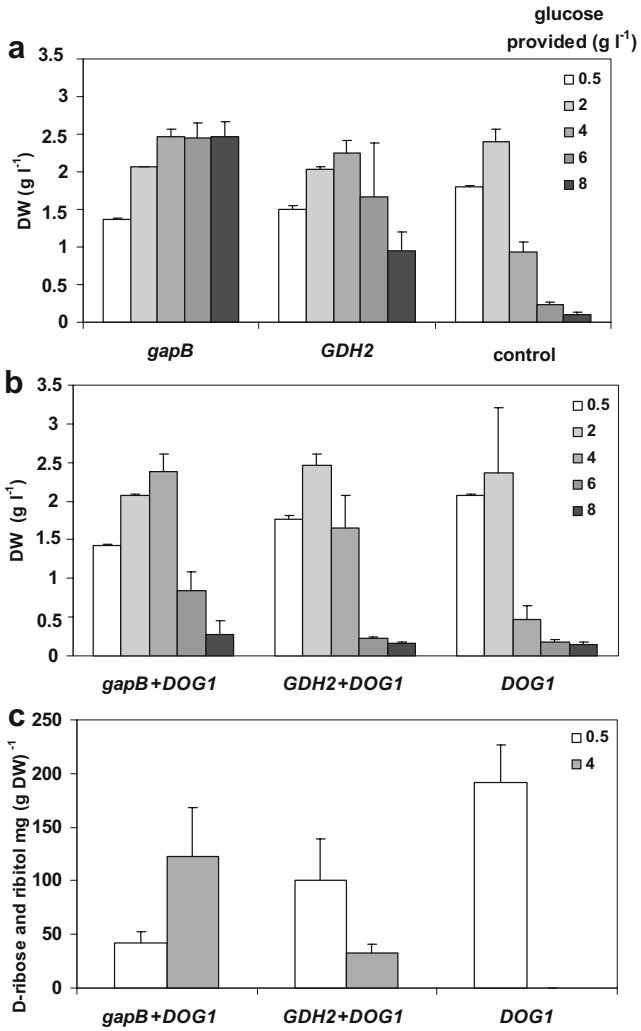

Fig. 2 Tolerance of D-glucose of the *pgi1* strains expressing the *gapB* or *GDH2* gene or the empty vector (**a**), and in addition, harbouring the *DOG1* gene (**b**). Biomass yield (dry weight (DW) gram per litre) is shown after 72 h of culture on 20 g l^{-1} (*w*/*v*) D-fructose with 0.5 to 8 g l^{-1} (*w*/*v*) D-glucose as indicated in the legends. Concentration of ribitol plus D-ribose and D-ribulose (milligram (gram per DW)) after 72 h by strains expressing *gapB* and *DOG1*, *GDH2* and *DOG1*, or *DOG1* alone in cultures described above (**c**). Results are from two to three individual experiments. *Error bars* represent variation (standard deviation(SD)). Fast Acid and HPX-87H columns in series were used for the quantification of ribitol plus D-ribose and D-ribulose

labelled C3, both C2–C3 and C3–C4 bonds are always intact, and C2 and C4 are also labelled. Consistently, the carbons 3–5 are never separated in the reactions of glycolysis or the PPP and thus, should all have an identical labelling degree.

In conclusion, the NMR results indicate that three different labelling patterns were present in the excreted D-ribose. About 60% of the molecules were fully labelled, and all their carbons, thus, originated from D-glucose. In about 10%, only C1 and C2 originated from D-glucose, and the C3–C5 fragment originated from D-fructose. In the remaining 30% of D-ribose, all carbons originated from D-fructose.

Production of 5-carbon sugars from D-glucose in a strain deficient in phosphoglucose isomerase and transketolase activities

We showed previously that D-xylulose 5-phosphate accumulated in the transketolase-deficient strain and thus, probably facilitated the formation of 5-carbon compounds D-ribose, D-ribulose, D-xylulose, and their derivatives (Toivari et al. 2007; Teleman et al. 1999). Subsequently, we deleted the phosphoglucose isomerase gene in the transketolase-deficient CEN.PK2-1D and expressed the *gapB* or *GDH2* gene, with or without the *DOG1*, to determine whether the flux of D-glucose towards 5-carbon compounds would be increased. As D-glucose can no longer enter glycolysis through the nonoxidative part of the PPP, D-fructose is needed for growth and maintenance.

The *pgi1 tkl1 tkl2* strain expressing the *gapB* or *GDH2* gene with or without the *DOG1* gene, as well as *DOG1* alone, and a control strain were cultured on YSC medium with 20 g l^{-1} (*w*/*v*) D-fructose and various concentrations of D-glucose (*w*/*v*). The strains expressing *gapB* produced less biomass in the presence of more than 0.5 g l^{-1} D-glucose compared to the other strains (data not shown). In general, biomass production decreased with increasing D-glucose concentration in the medium (Fig. 5a). The strains expressing *GDH2*, *DOG1*, or *GDH2* and *DOG1* or containing the control plasmid grew alike on all D-glucose concentrations, except on 2 g l^{-1} D-glucose; after 96 h of culturing the strain expressing only *DOG1* produced clearly more biomass than the other strains (Fig. 5a). HPLC analysis showed that the strains produced at maximum, a total of 1 g (g dry weight)$^{-1}$ of D-ribose and ribitol (Fig. 5b) with a yield of 50% (*w*/*w*) on D-glucose consumed. Again on 2 g l^{-1} glucose, the strain expressing both *GDH2* and *DOG1* produced about 40% to

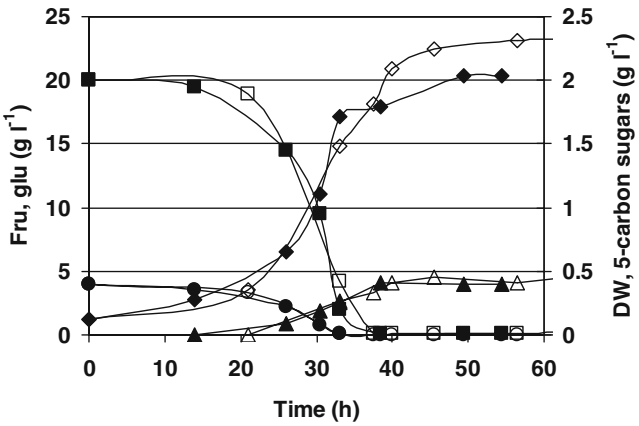

Fig. 3 Accumulation of biomass (gram per litre), consumption of D-fructose and D-glucose (gram per litre), and production of D-ribose and ribitol (gram per litre) of the *pgi1* strain containing *gapB* and *DOG1*. D-fructose (*Fru*; *squares*), D-glucose (*Glu*; *circles*), biomass (*DW*; *diamonds*) and 5-carbon sugars (*triangles*). *Open and closed symbols* represent two separate experiments

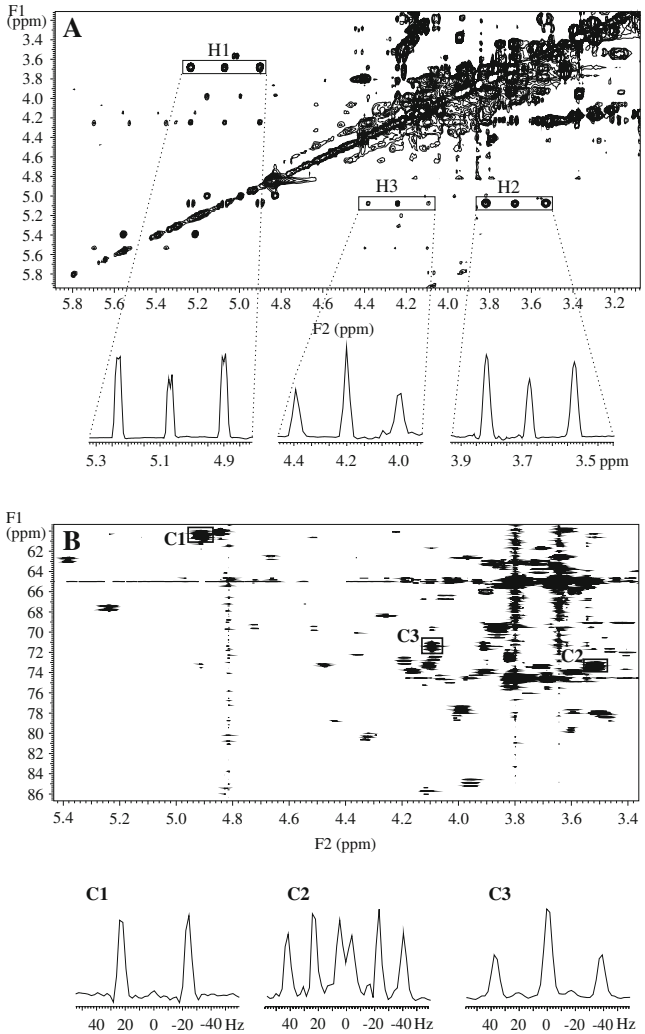

Fig. 4 **a** An expansion of a TOCSY spectrum of the culture supernatant of the *pgi1* strain expressing *gapB* and *DOG1* genes grown on [U–^{13}C] D-glucose and unlabelled D-fructose. Cross sections of the D-ribose β-pyranose signals used for determination of the fractional ^{13}C enrichments are shown under the 2D spectrum. The fractional enrichment of each carbon position can be determined directly from the ratio of the satellite signals to the total intensity of each proton signal, because the ^{13}C coupling was decoupled from the indirect dimension of the spectrum. **b** Carbohydrate signal region of an HSQC spectrum of the same sample and cross sections along the ^{13}C dimension showing the ^{13}C–^{13}C coupling fine structure of the D-ribose β-pyranose signals. Because rather narrow spectral width was used in ^{13}C dimension in order to maximise the point resolution, the C1 signals appear as *fold-back peaks* at around 62 ppm

60% higher concentration of D-ribose and ribitol compared to the other strains studied.

Discussion

The modification of the intracellular redox balance by introducing the NADPH-consuming reaction of Gdh2p or GapB into the *pgi1 DOG1* strain increased D-glucose tolerance and the amount of D-ribose and ribitol produced on higher D-glucose concentrations. The overexpression of *GDH2* (as described previously by Boles et al. 1993) or *gapB* genes enabled the growth of *S. cerevisiae pgi1*-deficient strain on glucose. The strains deficient in both phosphoglucose isomerase and transketolase activities produced D-ribose and ribitol even without the Dog1 phosphatase, whereas the effect of the NADPH-consuming reactions on product formation was moderate (Gdh2p) or negligible (GapB).

The expression of *DOG1* encoding the sugar phosphate phosphatase was essential for ribitol and D-ribose to be produced in the *S. cerevisiae pgi1* strain. However, Dog1p activity resulted in very slow or no growth and correspondingly, in poor production of 5-carbon sugars on D-glucose alone. In addition, tolerance of D-glucose in the presence of D-fructose was decreased by expression of *DOG1* in the *pgi1* strains. *DOG1* and *DOG2* genes were originally characterised as enabling growth in the presence of 2-deoxyglucose, most likely by dephosphorylating the toxic 2-deoxyglucose 6-phosphate and thus, preventing its accumulation. Dog1p is also able to dephosphorylate D-glucose 6-phosphate,

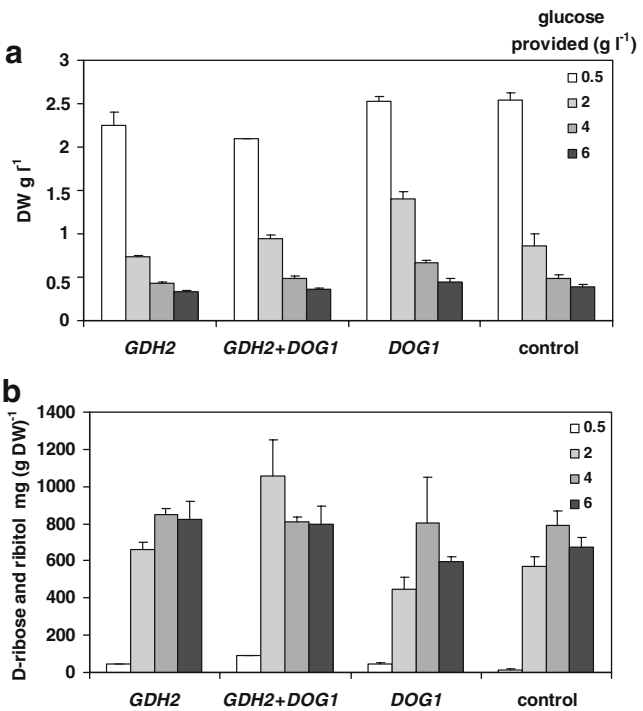

Fig. 5 Accumulation of biomass (gram per litre; **a**) and production of D-ribose and ribitol (milligram (gram per DW); **b**) after 96 h of culturing of the strain deficient in both phosphoglucose isomerase and transketolase activities with plasmids carrying *GDH2*, *GDH2* and *DOG1*, and *DOG1* or the control strain with the empty vector. The strains were grown on 20 g l^{-1} (*w*/*v*) D-fructose with 0.5 to 6 g l^{-1} (*w*/*v*) D-glucose as indicated in the legends. Results are from three individual experiments. *Error bars* represent variation (SD). Fast Acid and HPX-87H columns in series were used for the quantification of ribitol plus D-ribose and D-ribulose

although the relative activity on D-ribose 5-phosphate was reported to be about twofold higher than on D-glucose 6-phosphate (Randez-Gil et al. 1995). The reduced growth may be due to ATP depletion caused by the sequential phosphorylation and dephosphorylation of D-glucose and D-glucose 6-phosphate by the hexokinases and the Dog1 phosphatase, respectively.

When the *pgi1* mutant grows on a mixture of D-glucose and D-fructose, part of the produced D-ribose and ribitol may originate from D-fructose 6-phosphate and glyceraldehyde 3-phosphate by the reversible reactions of transaldolase and transketolase. Maaheimo and coworkers (Maaheimo et al. 2001) have reported that when grown on D-glucose, 30% (relative flux) of the carbon in D-ribose 5-phosphate was derived through the transketolase reaction in *S. cerevisiae*. In the present study, ^{13}C labelling and subsequent NMR spectroscopic analysis of product labelling directly from the culture supernatant proved to be a very efficient method for analysing the origin of carbon in D-ribose. The observed labelling pattern is in accordance with the so-called ping-pong mechanism proposed for transketolase (Cleland 1963; Kleijn et al. 2005). According to this mechanism, the keto group (corresponding to D-ribose C1–C2) remains bound to the enzyme until it is linked to an acceptor sugar phosphate. In the transketolase reaction producing D-xylulose 5-phosphate, the acceptor is glyceraldehyde 3-phosphate. Since most of the glyceraldehyde 3-phosphate pool must be unlabelled, when the yeast is growing on unlabelled D-fructose, the product D-xylulose 5-phosphate, in most cases, has an unlabelled C3–C5 fragment, while the C1–C2 fragment may or may not be labelled. Thus, in addition to the oxidative PPP, D-ribose was also formed through the nonoxidative part of PPP via nonlabelled glyceraldehyde 3-phosphate and D-fructose 6-phosphate.

The deletion of both phosphoglucose isomerase and the two transketolase isoenzyme encoding genes led to a very different phenotype compared to that of the *pgi1* strain as the strain is no longer able to channel D-glucose via PPP. Already 2 g l^{-1} (*w*/*v*) D-glucose with D-fructose reduced the yield of biomass significantly compared to D-fructose with 0.5 g l^{-1} (*w*/*v*) D-glucose. The expression of *DOG1* together with *gapB* or *GDH2* did not inhibit growth in the *pgi1 tkl1 tkl2* strain, and in the presence of 2 g l^{-1} glucose, the *DOG1* strain produced more biomass than the other strains. However, expression of the *gapB* reduced the growth with a mechanism still to be resolved. D-Ribose and ribitol were produced with the *pgi1 tkl1 tkl2* strains even without *DOG1*, suggesting that an unknown endogenous phosphatase was active in these strains, as also observed previously in the transketolase-deficient strains (Toivari et al. 2007). However, on 2 g l^{-1} D-glucose concentration, *DOG1* when expressed together with *GDH2* increased the yield of D-ribose and ribitol.

Based on the results of the present study, the use of the *pgi1* and *pgi1 tkl1 tkl2* strains of *S. cerevisiae* for the production of D-ribose and ribitol from D-glucose is possible, and introduction of a NADPH-consuming reaction of Gdh2p or GapB together with Dog1p was beneficial. However, for an efficient production strain, better tolerance for D-glucose is needed.

Acknowledgements Dr. Peter Richard is acknowledged for providing the *gapB* plasmid. Dr. Kari Koivuranta is acknowledged for providing the transketolase-deficient strain H2926. M.Sc. Ritva Verho is thanked for the strain H2493. Prof. Eckhard Boles is thanked for providing the *GDH2* plasmid YEpMSP3-T. Dr. Marilyn Wiebe is acknowledged for helpful discussions and critical reading of the manuscript. Pirjo Tähtinen, Outi Könönen, and Merja Helanterä are thanked for excellent technical assistance. Helena Simolin and Marita Ikonen are thanked for the Dionex HPLC analysis.

The financial support from Danisco sweeteners and Tekes, the Finnish Funding Agency for Technology and Innovation (project 4007/96), and Finnish Academy (Centre of Excellence in White Biotechnology—Green Chemistry, project 118573) is gratefully acknowledged.

References

Boles E, Lehnert W, Zimmermann FK (1993) The role of the NAD-dependent glutamate dehydrogenase in restoring growth on glucose of a *Saccharomyces cerevisiae* phosphoglucose isomerase mutant. Eur J Biochem 217:469–477

Boles E, Gohlmann HW, Zimmermann FK (1996) Cloning of a second gene encoding 5-phosphofructo-2-kinase in yeast, and characterization of mutant strains without fructose-2, 6- bisphosphate. Mol Microbiol 20:65–76

Ciriacy M, Breitenbach I (1979) Physiological effects of seven different blocks in glycolysis in *Saccharomyces cerevisiae*. J Bacteriol 139:152–160

Cleland WW (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim Biophys Acta 67:104–137

De Wulf P, Vandamme EJ (1997) Production of D-ribose by fermentation. Appl Microbiol Biotechnol 48:141–148

Fiaux J, Cakar ZP, Sonderegger M, Wüthrich K, Szyperski T, Sauer U (2003) Metabolic-flux profiling of the yeasts *Saccharomyces cerevisiae* and *Pichia stipitis*. Eukaryot Cell 2:170–180

Fillinger S, Boschi-Muller S, Azza S, Dervyn E, Branlant G, Aymerich S (2000) Two glyceraldehyde-3-phosphate dehydrogenases with opposite physiological roles in a nonphotosynthetic bacterium. J Biol Chem 275:14031–14037

Gietz RD, Sugino A (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74:527–534

Goffrini P, Wesolowski-Louvel M, Ferrero I (1991) A phosphoglucose isomerase gene is involved in the Rag phenotype of the yeast *Kluyveromyces lactis*. Mol Gen Genet 228:401–409

Griesinger C, Otting G, Wüthrich K, Ernst RR (1988) Clean TOCSY for ^{1}H spin system identification in macromolecules. J Am Chem Soc 110:7870–7872

Heux S, Cadiere A, Dequin S (2008) Glucose utilization of strains lacking *PGI1* and expressing a transhydrogenase suggests differences in the pentose phosphate capacity among *Saccharomyces cerevisiae* strains. FEMS Yeast Res 8:217–224

Ishizuka H, Wako H, Kasumi T, Sasaki T (1989) Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. J Ferment Bioeng 68:310–314

Kleijn RJ, van Winden WA, van Gulik WM, Heijnen JJ (2005) Revisiting the ^{13}C-label distribution of the non-oxidative branch of the pentose phosphate pathway based upon kinetic and genetic evidence. FEBS J 272:4970–4982

Maaheimo H, Fiaux J, Cakar ZP, Bailey JE, Sauer U, Szyperski T (2001) Central carbon metabolism of *Saccharomyces cerevisiae* explored by biosynthetic fractional ^{13}C labeling of common amino acids. Eur J Biochem 268:2464–2479

Maitra PK (1971) Glucose and fructose metabolism in a phosphoglucoseisomeraseless mutant of *Saccharomyces cerevisiae*. J Bacteriol 107:759–769

Mellor J, Dobson MJ, Roberts NA, Tuite MF, Emtage JS, White S, Lowe PA, Patel T, Kingsman AJ, Kingsman SM (1983) Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. Gene 24:1–14

Povelainen M, Miasnikov AN (2006a) Production of D-arabitol by a metabolic engineered strain of *Bacillus subtilis*. Biotechnol J 1:214–219

Povelainen M, Miasnikov AN (2006b) Production of xylitol by metabolically engineered strains of *Bacillus subtilis*. J Biotechnol 128:24–31

Randez-Gil F, Blasco A, Prieto JA, Sanz P (1995) *DOGR1* and *DOGR2*: two genes from *Saccharomyces cerevisiae* that confer 2-deoxyglucose resistance when overexpressed. Yeast 11:1233–1240

Ruohonen L, Aalto MK, Keränen S (1995) Modifications to the *ADH1* promoter of *Saccharomyces cerevisiae* for efficient production of heterologous proteins. J Biotechnol 39:193–203

Sasajima K, Yoneda M (1989) Production of D-ribose by microorganisms. In: Vandamme EJ (ed) Biotechnology of vitamins, pigments and growth factors. Elsevier, New York, pp 167–197

Sherman F, Fink G, Hicks JB (1983) Methods in yeast genetics. Cold Springs Harbor Laboratory, Cold Springs Harbor, NY

Spencer JFT, Sallans HR (1956) Production of polyhydric alcohols by osmophilic yeasts. Can J Microbiol 2:72–79

Szyperski T (1995) Biosynthetically directed fractional ^{13}C-labeling of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism. Eur J Biochem 232:433–448

Tarrio N, Becerra M, Cerdan ME, Gonzalez Siso MI (2006) Reoxidation of cytosolic NADPH in *Kluyveromyces lactis*. FEMS Yeast Res 6:371–380

Teleman A, Richard P, Toivari M, Penttilä M (1999) Identification and quantitation of phosphorus metabolites in yeast neutral pH extracts by nuclear magnetic resonance spectroscopy. Anal Biochem 272:71–79

Toivari MH, Ruohonen L, Miasnikov AN, Richard P, Penttilä M (2007) Metabolic engineering of *Saccharomyces cerevisiae* for conversion of D-glucose to xylitol and other five-carbon sugars and sugar alcohols. Appl Environ Microbiol 73:5471–5476

Ugarova NN, Romay C, Garcia I, Pascual C (1986) Intracellular ATP in a glucosephosphate isomerase mutant of *Saccharomyces cerevisiae*. Folia Microbiol (Praha) 31:113–119

Verho R, Richard P, Jonson PH, Sundqvist L, Londesborough J, Penttilä M (2002) Identification of the first fungal NADP-GAPDH from *Kluyveromyces lactis*. Biochemistry 41:13833–13838

Vinopal RT, Hillman JD, Schulman H, Reznikoff WS, Fraenkel DG (1975) New phosphoglucose isomerase mutants of *Escherichia coli*. J Bacteriol 122:1172–1174

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119