Control of the glycolytic flux in *Saccharomyces cerevisiae* grown at low temperature: a multi-level analysis in anaerobic chemostat cultures

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Running title: Control of the glycolytic flux in yeast at low temperature

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Growth temperature has a profound impact on the kinetic properties of enzymes in microbial metabolic networks. Activities of glycolytic enzymes in *Saccharomyces cerevisiae* were up to 7.5-fold lower when assayed at 12 °C than at 30 °C. Nevertheless, the *in vivo* glycolytic flux in chemostat cultures (dilution rate; 0.03 h⁻¹) grown at these two temperatures was essentially the same. To investigate how the yeast maintained a constant glycolytic flux despite the kinetic challenge imposed by a lower growth temperature, a systems approach was applied that involved metabolic flux analysis, transcript analysis, enzyme activity assays and metabolite analysis. Expression of hexose-transporter genes was affected by the growth temperature, as indicated by differential transcription of five *HXT* genes and changed zero-trans-influx kinetics of ¹⁴C glucose transport. No such significant changes in gene expression were observed for any of the glycolytic enzymes. Fermentative capacity (assayed off-line at 30 °C), which was two-fold higher in cells grown at 12 °C, was therefore probably controlled predominantly by glucose transport. Massive differences in the intracellular concentrations of nucleotides (resulting in an increased adenylate energy charge at low temperature) and glycolytic intermediates indicated a dominant role of metabolic control as opposed to gene expression in the adaptation of glycolytic enzyme activity to different temperatures. In evolutionary terms, this predominant reliance on metabolic control of a central pathway, which represents a significant fraction of the organism’s cellular protein, may be advantageous to limit the need for protein synthesis and degradation during adaptation to diurnal temperature cycles.

Introduction

Changing ambient temperature, for example as a result of diurnal temperature cycling, is among the most common environmental changes that microorganisms have to contend with in nature. Temperature effects on microbial physiology are also relevant for the industrial exploitation of micro-organisms. For example, the temperature in industrial processes for production of alcoholic beverages with the yeast *Saccharomyces cerevisiae* (commonly 8 °C to 20 °C) is much
lower than the optimum temperature range for growth (25 – 30 °C) (1).

Much of the current knowledge on adaptation of the model eukaryote *S. cerevisiae* to suboptimal temperatures has been derived from studies on cold shock. These studies have identified cold-induced changes in membrane lipid composition (2), transport functions, translational efficiency, protein folding and nucleic acid structure (for a review see (3)). Transcriptome analysis of cold adaptation in *S. cerevisiae* has revealed several types of responses that depend on the temperature range applied, namely “cold shock” (between 20 and 10°C) (4;5) and “near freezing” (< 10°C) (6) conditions. Responses to low temperature are also affected by the exposure period to low temperature (early phase and late phase responses, (4;6). The early response in cold shock experiments encompasses upregulation of low-temperature marker genes such as *NSR1* (7), *TIR1*, *TIR2* (8), and *TIP1* (9), which are involved in *de novo* ribosome biogenesis, transcription and translation. The late phase is characterized by upregulation of genes involved in protein folding, trehalose synthesis and stress responses (e.g. *HSP12* and *HSP26* (10)), which suggests involvement of *MSN2* and *MSN4* in the regulatory circuit for cold adaptation (4;10). Recent reports have implicated the HOG pathway in the transduction of the low temperature signal, resulting in the production of glycerol and trehalose required for resistance to freezing but not for growth at 12°C (11).

Temperature also has a drastic effect on the catalytic properties of enzymes. The temperature dependency of enzyme kinetics on the catalytic rate constant, $k_{cat}$ of a reaction is partially governed by the Arrhenius equation [1]

\[
[1] \quad k_{cat} = A e^{\left(\frac{-E_a}{RT}\right)}.
\]

This equation dictates that increasing the temperature or decreasing the activation energy (for instance through the use of a catalyst) will result in an increase of $k_{cat}$ (12). Each enzyme has unique catalytic properties, including temperature optimum and specific binding to allosteric effectors that contribute to regulation of its *in vivo* activity (these effects are not taken into consideration by the equation [1]).

In natural environments, both specific growth rate and the fermentation rate are key parameters in determining evolutionary fitness of microorganisms. To optimize rates at lower temperatures, micro-organisms can, in principle, resort to different strategies. Firstly, the synthesis of rate controlling enzymes may be increased. This “vertical” regulation may be effected at the level of transcription, mRNA degradation, protein synthesis and degradation and/or post-translational modification. Alternatively or additionally, temperature-compensatory shifts of *in vivo* enzyme activity may be accomplished by “metabolic” regulation altering intracellular and extracellular concentrations of metabolites and effectors. The impact of metabolic control may also result from the temperature-dependent expression of (iso)enzymes with different kinetic properties (13).

The aim of the present study is to assess the contribution of vertical and metabolic regulation in the physiological adaptation of *S. cerevisiae* to lower temperature. In batch cultures, the tight coupling of temperature and specific growth rate makes it exceedingly difficult to dissect effects of these two parameters. For example, it is well documented that, even at a fixed growth temperature, different specific growth rates result in different gene expression patterns (14) as well as different intracellular metabolite concentrations (15). In the present study, we circumvent this problem by comparing growth of *S. cerevisiae* at 12 and 30°C in glucose-limited chemostat cultures. Since, in chemostat cultures, the specific growth rate is equal to the dilution rate, this cultivation technique enables investigation of the impact of temperature at a fixed specific growth rate. Our study is focused on glycolysis and alcoholic fermentation, a pathway that plays a central role in anaerobic growth and represents a substantial fraction of the yeast’s cellular protein (16). To dissect the regulation of *in vivo* glycolytic flux, data from physiological studies are integrated with *in silico* metabolic flux analysis, transcriptome analysis, enzyme-activity assays and intracellular metabolite analyses.

**Experimental procedures**

**Strain and growth conditions.** The *S. cerevisiae* prototrophic haploid reference strain CEN.PK113-7D (MATa) (17) was grown at a dilution rate (D)
of 0.03 h\(^{-1}\) at both 12°C or 30°C in 2-liter chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 liter as described in (18). A temperature probe connected to a cryostat controlled cultures grown at 12°C. Cultures were grown in a defined synthetic medium that was limited by glucose with all other growth requirements in excess as described by (18). The dilution rate was set at 0.03 hr\(^{-1}\) with pH measured online and kept constant at 5.0 by automatic addition of 2 M KOH using an Applikon ADI 1030 Biocontroller. The stirrer speed was set to 600 rpm. Anaerobic growth and steady state conditions were maintained as described (18). Biomass dry weight, metabolite, dissolved oxygen, and gas profiles were constant at least three volume changes prior to sampling.

To measure the specific maximal growth rate at 12°C and 30°C, anaerobic batch cultivations were performed in 2-litre chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1.0 litre. Precultures were grown in mineral medium with 2 % glucose until stationary phase in shake flask at 200 rpm, 30°C. Fermentors were inoculated with preculture at an OD\(_{660}\) of 0.1. Cultures were grown in a predefined synthetic medium for anaerobic growth (18) with 2 % glucose. pH, temperature and stirrer speed were maintained as previously described for chemostat anaerobic cultures.

**Analytical methods.** Culture supernatants were obtained with the method described in (19). For the purpose of glucose determination and carbon recovery, culture supernatants and media were analyzed by high performance liquid chromatography on an AMINEX HPX-87H ion exchange column using 5 mM H\(_2\)SO\(_4\) as the mobile phase. Ethanol evaporation from cultures was determined as described in (20). Residual ammonium concentrations were determined using cuvette tests from DRLANGE (Dusseldorf, Germany). Culture dry weights were determined as described in (21) while whole cell protein determination was carried out as described in (22).

**Trehalose & glycogen.** Trehalose and glycogen concentrations measurement were as according to Parrou et al. (1997) (23). Trehalose was determined in triplicate measurements for each chemostat. Glycogen was determined in duplicate for each chemostat. Glucose was determined using the UV-method based on Roche kit no. 0716251.

**Fermentative capacity assay.** Fermentative capacity assays were as previously described in (24). The fermentative capacity can be defined as the specific maximal production rate of ethanol per gram of biomass (mmol/g/h) under anaerobic and excess glucose conditions.

**Microarray analysis.** Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix Genechip® microarrays were performed as described previously in (25). RNA quality was determined using the Agilent 2100 Bioanalyzer. The results for each growth condition were derived from three independently cultured replicates. Data acquisition and analysis as well as statistical analysis were performed as described previously in (18). The microarray data have been deposited at Genome Expression Omnibus database ([http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) under the series number GSE6190.

**In vitro enzyme assays.** Each in vitro enzyme assays for the glycolytic pathway was performed as previously described in (24). All enzyme assays were performed at 12 and 30°C (with exception of enolase that has not been measured). Protein determinations of cell extracts were as previously described in (26).

**Metabolic flux distribution** Intracellular metabolic fluxes were calculated through metabolic flux balancing using compartmented stoichiometric model derived from the model developed in (27). The setup for the model was performed using the dedicated software (SPAD it, Nijmegen, The Netherlands). For both 12 and 30°C chemostats, the specific rates of growth, substrate consumption, carbon dioxide and ethanol production during steady-state cultivation were calculated from the measured concentrations and flow rates from three independent chemostats. Assumptions and the theory of the model were previously described in (28).

**Zero trans-influx uptake assays with labeled \(^{14}\text{C}-\text{glucose.** For both cultures from different temperatures, cells were harvested from duplicate fermentations by centrifugation at 4°C (5 min at 5000 rpm) and zero trans-influx uptake of labeled \(^{14}\text{C}-\text{glucose was determined at 30°C according to (29). The parameters of sugar transport were derived according to single component Michaelis-Menten kinetics.**
Intracellular metabolite determination

The procedure and equipment for rapid sampling of intracellular metabolites were based on (19) using the cold methanol quenching method. Two independent chemostats for each culture temperature were run and metabolite measurements were done in triplicate for each chemostat. Glycolytic intracellular metabolites were analyzed by ESI-LC-MS/MS according to (30) and the quantification was performed following the IDMS concept (31). Nucleotide concentration in the cell extract was analyzed by an ion pairing ESI-LC-MS/MS method and quantified following the IDMS concept. The adenylate charge (AC) was calculated as shown in equation [2]:

\[
AC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP]+[ADP]+[AMP]}
\]

**Results**

**Physiological and biochemical analysis of chemostat cultures grown at different temperatures**

The chemostat cultures were performed at 0.03 h⁻¹, a dilution rate compatible with the temperature of 12°C as the maximal specific growth rate of the *S. cerevisiae* CEN.PK113-7D strain was of 0.035 ± 0.002 h⁻¹ at 12°C (versus 0.34 ± 0.01 h⁻¹ at 30°C). Biomass yields, as well as specific rates of glucose consumption, ethanol and carbon dioxide production in anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* differed by less than 15% at 12°C and 30°C (Table 1a). Consistent with the similar culture kinetics (Table 1a), the glycolytic flux was not substantially different in cultures grown at 12°C and 30°C (Table 1b). Conversely, off-line measurements of the fermentative capacity in glucose-rich medium at 30°C yielded values that were 70% higher for cells grown at 12°C than for cells grown at 30°C (Table 1a, Supplemental Table S1).

The increased fermentative capacity of cells grown at 12°C suggested that an upregulation of key rate-controlling enzymes involved in fermentative glucose metabolism and/or glucose transport is part of the mechanism by which *S. cerevisiae* maintained a constant glycolytic flux in the chemostat cultures grown at 12°C and 30°C. We subsequently analyzed the contribution of different levels of cellular control to the regulation of glycolytic flux in more detail.

**Expression analysis of the fermentation pathway genes in low temperature chemostat cultivations**

DNA-microarrays were used for a genome-wide comparison of transcript levels in anaerobic, glucose-limited chemostat cultures grown at 12°C and 30°C. Here, we focus on transcripts that encode key enzymes involved in glucose transport, glycolysis and alcoholic fermentation (Fig. 1). The most striking differences were observed in the expression of several hexose-transporter (*HXT*) genes. Out of the seven *HXT* genes that were significantly expressed in at least one condition, five were differentially expressed at 12°C and 30°C (Table 2). *HXT2* (+2.6-fold), *HXT3* (+3.7), and especially *HXT4* (+33.5) were expressed at higher levels at 12°C while *HXT5* (-40.4) and *HXT16* (-10.0) were expressed at a lower level at 12°C. Interestingly, these changes did not seem to follow a simple logic that could be deduced from previous classification of the encoded hexose transporters as either low (*HXT3*),
high (HXT2) or moderate affinity carriers (HXT4) (33) were coordinately changed. Transcription of high affinity transporter genes, HXT6 and 7 was not significantly affected by the culture temperature.

Only four genes encoding enzymes of the glycolytic pathway were differentially expressed at 12°C compared to 30°C (Table 2). Three were downregulated (GPM2, PYK2, PDC6) while PDC5 was upregulated. Despite their significant change in expression, all four genes encode minor isoforms of the corresponding enzyme activities and were transcribed at low level compared to the genes encoding the main isoforms (GPM1, PYK1 and PDC1) (Table 2).

**Glucose uptake assay and glycolytic enzyme activities in low temperature chemostat cultivations**

To investigate whether the different transcript levels of the five HXT genes were reflected in glucose transport kinetics, zero trans-influx uptake assays with 14C-glucose were performed at 30°C. Cells grown at 12°C and 30°C exhibited high-affinity transport with substrate-saturation constants (K_m) of 1.1 mM and 0.8 mM, respectively (Fig. 2). The maximum transport capacity (V_max) was 60% higher at low temperature (Fig. 2), probably as a consequence of the increased transcript levels of several HXT genes (Table 2).

In anaerobic carbon-limited chemostat cultures, substrate-level phosphorylation via glycolysis is the main metabolic route for ATP supply. One mechanism to compensate for the decreased turnover numbers of glycolytic enzymes at low temperature might be an increased abundance (expression) of glycolytic enzymes. Hence, in vitro assays of the glycolytic and fermentative enzymes were carried out with cell extracts from chemostats cultures at 12°C and 30°C (Table 2). To gain insight in the effect of the temperature on enzymes, the assays were performed at both 12°C and 30°C.

Irrespective of the culture temperature, activities measured in cell extracts at 12°C were strongly reduced (2.1 to 7.5-fold) when comparing with in vitro measurements at 30°C, (Table 3). Fructose-1,6P aldolase (FBA) was most significantly affected by temperature with a seven-fold reduction for both growth temperatures. Hexokinase (HXK), Glucose-6P isomerase (PGI), G3P dehydrogenase (TDH) and 3P-glycerate kinase (PGK) showed a two to three-fold lower activity when measured at 12°C. Meanwhile phosphofructokinase (PFK), Triose-P isomerase (TPI), P-glycerate mutase (PGM), pyruvate kinase (PYK) and pyruvate decarboxylase (PDC) activities were three to seven-fold lower (Table 3). These observations show that the glycolytic enzymes have different temperature/activity relationships. To our surprise, the alcohol dehydrogenase activity did not show any difference in activity when assayed at 12°C and 30°C and this irrespective of the cultivation temperature. Addition of Zn^{2+}, Cu^{2+}, concentration of the cell extract by filtration on membrane with a cut-off of 10kDa did not yield any significant differences higher than 20% between 12°C and 30°C in vitro ADH activity.

A comparison of the in vitro enzymatic activities done at the same temperature revealed only minor differences between yeast cultures grown at 12°C and 30°C. Only the pyruvate decarboxylase activity was significantly higher (p-value<0.01) after growth at 12°C than after growth at 30°C (Table 3). Surprisingly, four activities were significantly lower at the low cultivation temperature (HXK, PGI, PFK and PGK) (Table 3), irrespective of the assay temperature. The capacity of the glycolytic and fermentative enzymes was thus generally kept constant or even decreased at low temperature. The observations are in good agreement with the transcript levels (Table 2).

The glycolytic enzyme activities of yeast cultures grown at 12°C and assayed at 12°C were much lower than those of cultures grown at 30°C and assayed at 30°C (Table 3). A comparison of estimated in vivo fluxes (Table1b) with the in vitro enzyme activities (Table 3) showed that for all reactions, except PFK, the enzyme capacity exceeded the in vivo fluxes (2 to 1000-fold, Fig. 3). The degree of in vivo saturation of PFK was well above 100 % (Fig. 3), indicating that this enzyme activity measured in vitro could not account for the estimated in vivo fluxes.

Since mRNA and enzyme activity measurements did not provide an indication for regulation of the in vivo activity of glycolytic enzymes at the level of enzyme synthesis (Vertical control), we subsequently analyzed the
intracellular concentration of metabolites of central carbon metabolism.

**Intracellular Metabolite concentrations at 12°C and 30°C**

The activities of glycolytic and fermentative enzymes are affected by their substrate and product concentrations and moreover regulated by allosteric effectors such as adenosine nucleotides (e.g. ATP, ADP and AMP), glycolytic intermediates (e.g. fructose-1,6 biphosphate, PEP) and pyridine nucleotide cofactors. The intracellular concentrations of most of the measured compounds were significantly and markedly different in the glucose-limited chemostat grown at 12°C and 30°C. The concentrations of the intermediates of both upper and lower glycolysis were concertedly increased by 1.5 to 5.7-fold (G6P, F6P, FBP, G3P, 2PG/3PG, PEP, PYR) (Table 4).

Adenine nucleotides act as allosteric effectors on several glycolytic enzymes (PFK (34), PYK (35)) but are also involved as substrates and products of glycolytic reactions (HXK, PFK, PGK, PYK). The intracellular ATP concentration was 2-fold higher at 12°C, while ADP and AMP levels were much lower (2.3 and 6.9-fold respectively), resulting in a higher adenylate sum ($\Sigma$AXP) and adenylate charge (AC) at 12°C than at 30°C (Table 4).

The concentration of trehalose-6-phosphate (T6P) dropped 5.7-fold at 12°C, concomitantly with the concentration of intracellular trehalose (Table 4). Besides its role in trehalose biosynthesis, T6P is a potent inhibitor of the hexokinase activity (36). Its lower concentration may participate in controlling the glucose phosphorylation and consequently the glycolytic flux (37). The increase in UTP and glucose-1-phosphate (G1P) concentrations were consistent with the coordinated increased concentration of glycogen at 12°C (Tables 1 and 3) (38).

**Discussion**

This study investigates how *S. cerevisiae* deals with the strong temperature dependence of key enzymes in glycolysis and alcoholic fermentation when it is grown at a sub-optimal temperature of 12°C. The activity assays of glycolytic enzymes, performed at 12°C and 30°C (Table 3), indicate that this temperature dependence is very strong. It should be realised that these assays were performed at a single concentration of substrates and effectors. Consequently, effects of temperature on the affinity for substrates and effectors may further increase the kinetic challenges posed by a low cultivation temperature (39;40).

Glucose transport across the plasma membrane was shown to be regulated at different levels. The higher residual glucose concentration at low temperature (Table 1) is likely to contribute to a higher degree of substrate saturation of hexose transporters and thereby compensate for a reduced capacity of transporters at 12°C. An additional level of regulation was observed by an analysis of mRNA levels for the *HXT* (hexose transporter) genes. While levels of the dominant *HXT* transcripts *HXT6* and *HXT7* (which encode transporters that, at 30°C, catalyse high-affinity glucose transport (33)) were not affected by temperature, other *HXT* genes, which exhibit lower affinities and as such would be less sensitive to an increased intracellular glucose concentration, showed clear transcriptional regulation. Since no kinetic data are available for the individual Hxt transporters at low temperature, it is not possible to interpret the significance of these changed transport levels. In addition to adjusting the overall capacity and/or affinity of hexose transport, this altered expression of *HXT* genes may represent an adjustment to temperature-dependent changes in membrane composition and/or fluidity (41). Kinetic analysis of glucose transport by cells pre-grown at 12°C and 30°C in chemostat cultures showed a clear increase of the capacity of transport. A plausible explanation for this increased capacity can be found in the differential expression of transcription of *HXT2, 3, 4, 5 and 16*. The fact that the maximum specific rates of glucose consumption (measured at 30°C) for cells pre-grown at 12°C and 30°C closely corresponded to the fermentative capacity of the same cultures (Table 1, Fig 2 and Supplemental Table S1) would support this hypothesis. However, the possibility cannot be excluded that glucose-transport activity was increased as a result of changes outside the hexose transporters, such as membrane composition. The change in glucose transport capacity, together with the minor changes in the levels of glycolytic enzymes (see below), strongly
suggests that glucose transport also controls fermentative capacity in these slowly growing chemostat cultures as well as at 30°C.

In contrast to sugar transport, there was virtually no evidence that regulation of glycolysis and alcoholic fermentation at the enzyme synthesis level (vertical regulation) contributed to the maintenance of in vivo glycolytic flux at low temperature. Indeed, the few glycolytic enzymes that did show a clear change at transcript levels and/or enzyme-activity level appear to show a lower level at low temperature, thus augmenting the effect of temperature on enzyme activity rather than compensating for it. The absence of a clear upregulation of the synthesis of glycolytic enzymes at low temperature is perhaps less surprising when it is taken into account that, in fermenting yeast cultures grown at 30°C, glycolytic enzymes already make up a significant fraction of the total cell protein (up to 21%, estimation extrapolated from (16)). It would take very significant increases in the concentrations of these glycolytic enzymes resulting in a significantly increased energetic demand to counteract the effects of the reduction in temperature.

The minor role of vertical control in the regulation of glycolytic flux at low temperature was in marked contrast to drastic differences in the intracellular concentrations of glycolytic intermediates and effectors. Several of the observed changes may contribute to a higher degree of saturation of the glycolytic capacity in the low-temperature cultures, thus compensating for the reduction of enzyme activity at low temperature. The lower intracellular concentration of T6P may have a similar effect, as this compound is a well-documented inhibitor of the S. cerevisiae hexokinases (42) that prevents “glucose-accelerated death” (36;43). The lower intracellular T6P concentration, an intermediate in trehalose biosynthesis, is also consistent with the lower trehalose concentrations in the cultures grown at 12°C.

Extensive changes were observed in the intracellular concentrations of adenine nucleotides. While the ATP concentration was higher at low temperature, the ADP and AMP concentrations were much lower, thus leading to an adenylate charge (AC) (see methods section equation [2]) of 0.94 in the chemostat cultures grown at 12°C (Table 4). This change seems counter-intuitive in a situation where the in vivo activity of glycolytic enzymes has to be boosted to compensate for low-temperature-induced reduction of their turnover numbers. An increased AC is generally correlated with a decrease of the activity of enzymes and/or pathways involved in ATP production (44) and in S. cerevisiae, intracellular ATP concentration is negatively correlated with glycolytic flux (45). This unexpected relation between growth temperature and AC may be related to strong changes of the kinetic and/or allosteric properties of enzymes at low temperature and to the changes of other relevant metabolites. For example, inhibition of PYK by the increased intracellular ATP concentration at 12°C may be compensated by the strongly increased intracellular concentration of its activator F1,6P (34;46) (Table 4). Similarly, a negative effect of increased ATP and decreased AMP on PFK activity (34) may be compensated for by changes of the positive allosteric regulator F2,6P (15), which could not be accurately measured in our experimental set-up.

An important factor in the interpretation of the intracellular metabolite data is that the kinetics of glycolytic enzymes and, in particular, the impact of low-molecular-weight effectors, has been extensively studied at 30°C, but not at 12°C. For example, if the kinetics of allosteric regulation are strongly temperature dependent, this may affect the impact of the AC on glycolytic flux by removing or reducing bottlenecks at the lower temperature. Indeed, several studies show that the affinity for allosteric effectors is reduced at low temperature (i.e. inhibition and activation constants are increased) (39;40;47). The apparent discrepancy between in vivo and in vitro PFK activities at 12°C represents a case in point (Fig. 3). In previous studies at 30 °C, the maximum capacity of PFK estimated in cell extracts was close to the in vivo flux through this enzyme (48). The difference between these parameters in cultures grown at 12°C may reflect suboptimal conditions in the enzyme assays due to temperature dependent changes in the complex allosteric regulation of this enzyme (49;50). A deeper understanding of the in vivo kinetics of glycolysis at low temperature, involving the application of kinetic modelling, will require quantitative data on the impact of temperature on
the kinetics and allosteric regulation of the entire glycolytic pathway in *S. cerevisiae*. In addition to providing ATP for anaerobic growth, glycolysis also is a key cellular supply line for biosynthesis. The temperature-dependent changes of the intracellular concentrations of glycolytic intermediates are therefore also likely to affect biosynthetic pathways. Clearly the relative demands placed on anaerobic glycolysis in terms of energetics and biosynthetic supplies are not the same at 12°C and 30°C. In addition to vital biomass components, the products derived from these biosynthetic pathways include important flavour compounds (51;52). Therefore, a further analysis of the mechanisms by which the entire yeast metabolic network adapts to temperature is highly relevant for yeast-based industrial fermentations that are performed at low temperature. Further insights will be gained from a similar study to this one using an industrial yeast strain, which is adapted to the low temperature conditions as opposed to a lab strain, which is probably adapted to the higher temperature conditions.

From an evolutionary perspective, a dominant role of metabolic regulation seems logical for a pathway that represents a substantial fraction of the cellular protein, in an organism that is subjected to circadian temperature cycles. In nature, *S. cerevisiae* is likely to encounter relatively exposed, and sugar-rich niches such as fruits and nectar. Moreover, growth is probably slow due to the low availability of nitrogen. In such environments, a preliminary vertical regulation of glycolytic activity would require wasteful cycles of massive synthesis (as temperature decreases in the evening) and degradation (in the morning) of glycolytic enzymes. This interpretation suggests that the high fermentative capacity of wild-type *S. cerevisiae* strains (assayed at 30 °C) may – at least in part – reflect an evolutionary adaptation to fluctuating temperatures in its natural environment.

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Footnotes
The research group of JTP is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative. We thank Koen de Graaf for his technical assistance during the fermentations.
Table 1: A-Physiological and biochemical characteristics of glucose-limited anaerobic chemostats grown at 12 °C and 30 °C

Values represent the mean ± S.D. of data from three independent steady-state chemostat cultures. B- *In silico* glycolytic fluxes

### A

| Culture temp (°C) | Physiological data | Biochemical data |
|------------------|--------------------|-----------------|
|                  | Y\textsubscript{Glc-X} | q\textsubscript{Glu} | q\textsubscript{ethanol} | q\textsubscript{CO2} | Carbon recovery | Residual glucose | Fermentative capacity | Trehalose | Glycogen | Whole cell proteins |
| °C               | g\textsubscript{glucose-DW}\textsuperscript{-1} | mmol.g\textsubscript{DW}\textsuperscript{-1}.h\textsuperscript{-1} | % | g/l | mmol.g\textsubscript{DW}\textsuperscript{-1}.h\textsuperscript{-1} | g\textsubscript{equivalent glucose-DW}\textsuperscript{-1} | g\textsubscript{protein-DW}\textsuperscript{-1} |
| 12               | 0.07 ± 0.01 | - 2.5 ± 0.2 | 3.8 ± 0.3 | 4.4 ± 0.3 | 100 ± 3 | 0.5 ± 0.2 | 19.3 ± 1.6 | < 0.005 | 0.06 ± 0.0 | 0.40 ± 0.0 |
| 30               | 0.07 ± 0.00 | - 2.3 ± 0.0 | 3.5 ± 0.0 | 3.8 ± 0.2 | 95 ± 1 | 0.05 | 11.4 ± 0.6 | 0.02 ± 0.0 | 0.04 ± 0.0 | 0.43 ± 0.0 |

### B

| Reaction number | Enzymes | *In silico* fluxes analysis (mmol.g\textsuperscript{-1}.h\textsuperscript{-1}) | 12 °C vs 30 °C |
|-----------------|---------|---------------------------------|----------------|
| 1               | Hexokinase | 2.4±0.1 | 2.1 ±0.1 | 1.1 |
| 2               | Glucose-6P isomerase | 2.3±0.1 | 2.0 ±0.1 | 1.1 |
| 3               | Phosphofructokinase | 2.3±0.1 | 2.0 ±0.1 | 1.1 |
| 4               | Fructose –1,6 P aldolase | 2.3±0.1 | 2.0 ±0.1 | 1.1 |
| 5               | Triose-P isomerase | 1.9±0.1 | 1.7 ±0.1 | 1.1 |
| 6               | G3P dehydrogenase | 4.3±0.2 | 3.8 ±0.1 | 1.1 |
| 7               | 3P-glycerate kinase | 4.3±0.2 | 3.8 ±0.1 | 1.1 |
| 8               | 3P-glycerate mutase | 4.3±0.2 | 3.8 ±0.1 | 1.1 |
| 9               | enolase | 4.3±0.2 | 3.8 ±0.1 | 1.1 |
| 10              | Pyruvate kinase | 4.3±0.2 | 3.8 ±0.1 | 1.1 |
| 11              | Pyruvate decarboxylase | 4.1±0.2 | 3.6±0.1 | 1.1 |
| 12              | Alcohol dehydrogenase | 4.1±0.2 | 3.6±0.1 | 1.1 |
Table 2: Hexose transporters and glycolytic pathway transcript levels in *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12 °C and 30 °C. Values represent the mean ± S.D. of data from three independent steady-state chemostat cultivations. Numbers at the last column correspond to the glycolytic pathway displayed in Figure 1.

*a* Fold change of transcription intensities of 12 °C over 30 °C.

*b* ORF not available on Affymetrix Genechip® YG-S98

n/a not applicable

| Gene          | Expression levels | Culture temperature | FC<sup>b</sup> |
|---------------|-------------------|---------------------|-----------------|
| Glucose       |                   |                     |                 |
| transport     |                   |                     |                 |
| (13)          |                   |                     |                 |
| HXT1          | 17 ± 8            | 12 ± 0              | 1.4             |
| HXT2          | 557 ± 160         | 214 ± 29            | 2.6             |
| HXT3          | 840 ± 240         | 230 ± 27            | 3.7             |
| HXT4          | 1020 ± 290        | 31 ± 2              | 33.5            |
| HXT5          | 12 ± 0            | 485 ± 100           | -40.4           |
| HXT6          | 3320 ± 510        | 3020 ± 480          | 1.1             |
| HXT7          | 2590 ± 310        | 2090 ± 360          | 1.2             |
| HXT8          | 19 ± 8            | 13 ± 1              | 1.4             |
| HXT9          | 23 ± 2            | 12 ± 0              | 1.9             |
| HXT10         | 12 ± 0            | 19 ± 6              | -1.6            |
| HXT11<sup>b</sup> | n/a                | n/a                 | -               |
| HXT12         | 43 ± 22           | 45 ± 10             | 1.0             |
| HXT13<sup>b</sup> | n/a                | n/a                 | -               |
| HXT14         | 12 ± 0            | 22 ± 3              | -1.8            |
| HXT15<sup>b</sup> | n/a                | n/a                 | -               |
| HXT16         | 34 ± 8            | 337 ± 96            | -10.0           |
| HXT17<sup>b</sup> | n/a                | n/a                 | -               |
| SLT1          | 25 ± 6            | 28 ± 4              | -1.1            |
| GAL2          | 15 ± 3            | 12 ± 0              | 1.3             |
| VSP73         | 82 ± 31           | 52 ± 2              | 1.6             |
| HXK (1)       |                   |                     |                 |
| HXK1          | 2050 ± 470        | 2960 ± 230          | -1.4            |
| HXK2          | 1660 ± 18         | 979 ± 130           | 1.7             |
| GLK1          | 1280 ± 37         | 1700 ± 170          | -1.3            |
| PGI (2)       |                   |                     |                 |
| PGI1          | 3120 ± 180        | 2840 ± 280          | 1.1             |
| PFK (3)       |                   |                     |                 |
| PFK1          | 1270 ± 140        | 1100 ± 94           | 1.2             |
| PFK2          | 1350 ± 40         | 1230 ± 91           | 1.1             |
| FBA (4)       |                   |                     |                 |
| FBA1          | 3480 ± 840        | 2700 ± 410          | 1.3             |
| TPI (5)       |                   |                     |                 |
| TPI1          | 4180 ± 540        | 3700 ± 380          | 1.1             |
| TDH (6)       |                   |                     |                 |
| TDH1          | 2520 ± 110        | 1810 ± 290          | 1.4             |
| TDH2          | 3670 ± 160        | 2750 ± 380          | 1.3             |
| TDH3          | 4190 ± 860        | 5410 ± 1210         | -1.3            |
| PGK (7)       |                   |                     |                 |
| PGK1          | 4310 ± 240        | 3750 ± 350          | 1.1             |
| GPM (8)       |                   |                     |                 |
| GPM1          | 3350 ± 100        | 3050 ± 230          | 1.1             |
| GPM2          | 91 ± 24           | 196 ± 35            | -2.1            |
| GPM3          | 127 ± 2           | 87 ± 8              | 1.5             |
| ENO (9)       |                   |                     |                 |
| ENO1          | 3420 ± 370        | 2880 ± 290          | 1.2             |
| ENO2          | 3490 ± 760        | 2840 ± 420          | 1.2             |
| PYK (10)      |                   |                     |                 |
| PYK1          | 2820 ± 210        | 2040 ± 157          | 1.4             |
| PYK2          | 47 ± 24           | 133 ± 34            | -2.8            |
| PDC (11)      |                   |                     |                 |
| PDC1          | 2190 ± 45         | 1410 ± 190          | 1.6             |
| PDC5          | 130 ± 12          | 56 ± 8              | 2.3             |
| PDC6          | 15 ± 2            | 66 ± 13             | -4.4            |
| ADH (12)      |                   |                     |                 |
| ADH1          | 4740 ± 180        | 3980 ± 590          | 1.2             |
| ADH2          | 40 ± 7            | 57 ± 17             | -1.4            |
| ADH3          | 749 ± 55          | 871 ± 110           | -1.2            |
| ADH4          | 237 ± 80          | 252 ± 35            | -1.1            |
| ADH5          | 937 ± 180         | 830 ± 130           | 1.1             |
Table 3: *In vitro* enzyme assays measurement in cell free extract of *S. cerevisiae* grown in glucose-limited anaerobic chemostat cultivations at 12°C and 30°C. Assay conditions were carried out at both 12°C and 30°C. Mean ± S.D. of data represented are from duplicate measurements from three independent chemostat cultures. Fold change (FC) of glycolytic enzymes with enzyme assays measured at 12 °C and 30 °C. *p*-values shown are from standard student T-test. In vitro enzyme activity of enolase was not measured. Numbers at the last column correspond to the glycolytic pathway displayed in Figure 1.

| Culture T | Assay T | Enzyme | A vs B | C vs D | B vs D | A vs C | Enzyme activities (umol/mg protein/min) |
|-----------|---------|--------|--------|--------|--------|--------|-----------------------------------------|
| 12 °C     | 30 °C   | HXK    | -2.9   | -2.8   | 1.6E-04| -2.7   | 1.6E-04                   |
| 12 °C     | 30 °C   | PGI    | 1.7    | 3.9    | 2.2    | 5.3    | -2.3                     |
| 12 °C     | 30 °C   | PFK    | -4.3   | 2.3E-03| -3.7   | 3.5E-04| -1.4                     |
| 12 °C     | 30 °C   | FBA    | -7.3   | 1.6E-03| -7.4   | 1.4E-05| -1.2                     |
| 12 °C     | 30 °C   | TPI    | -4.5   | 1.1E-03| -4.0   | 4.5E-06| 1.0                      |
| 12 °C     | 30 °C   | TDH    | -2.2   | 1.9E-02| -2.8   | 1.2E-02| 1.3                      |
| 12 °C     | 30 °C   | PGK    | -2.1   | 3.3E-04| -2.1   | 3.6E-03| -1.2                     |
| 12 °C     | 30 °C   | GMP    | -5.0   | 1.6E-03| -4.0   | 1.7E-03| -1.2                     |
| 12 °C     | 30 °C   | PYK    | -4.9   | 4.6E-03| -7.5   | 3.0E-03| 1.3                      |
| 12 °C     | 30 °C   | PDC    | -3.8   | 2.2E-03| -2.4   | 3.7E-03| 1.9                      |
| 12 °C     | 30 °C   | ADH    | 1.1    | 4.0E-01| 1.0    | 2.5E-01| 1.1                      |

*p<0.01*
Table 4  Intracellular metabolite concentrations. Values represent the mean ± S.D. of data from two independent steady-state chemostat cultivations measured in triplicates.

| Metabolite concentration | 12°C | 30°C | p value | Student T test | FCa |
|--------------------------|------|------|---------|----------------|-----|
| **G6P**                  | 21.6 ± 0.6 | 9.6 ± 0.2 | 1.1E-07 | 2.3 |
| **F6P**                  | 2.5 ± 0.4 | 1.1 ± 0.1 | 1.8E-04 | 2.3 |
| **F1,6P**                | 69.8 ± 5.5 | 30.3 ± 1.5 | 1.5E-06 | 2.3 |
| **G3P**                  | 3.9 ± 0.1 | 1.0 ± 0.1 | 1.5E-10 | 3.8 |
| **2PG/3PG**              | 1.1 ± 0.1 | 0.4 ± 0.0 | 1.9E-06 | 2.5 |
| **PEP**                  | 0.6 ± 0.1 | 0.1 ± 0.0 | 2.9E-06 | 5.7 |
| **PYR**                  | 3.0 ± 0.1 | 2.1 ± 0.2 | 6.8E-06 | 1.5 |
| **G1P**                  | 2.6 ± 0.1 | 1.2 ± 0.0 | 3.7E-09 | 2.2 |
| **ATP**                  | 51.0 ± 1.1 | 26.7 ± 1.5 | 1.1E-10 | 1.9 |
| **ADP**                  | 4.6 ± 0.7 | 10.4 ± 2.5 | 4.6E-04 | -2.3 |
| **AMP**                  | 0.9 ± 0.2 | 6.5 ± 1.3 | 1.0E-04 | -6.9 |
| **AC**                   | 0.94 | 0.73 | - | 1.3 |
| **UTP**                  | 10.3 ± 0.5 | 4.1 ± 0.1 | 3.7E-03 | 2.5 |
| **UDP**                  | 0.5 ± 0.1 | 1.4 ± 0.6 | 9.4E-03 | -2.7 |

a Fold change of metabolite concentrations of 12°C over 30°C

b UMP, CXP and GXP were also measured, however the concentration were too low to be accurately quantify

c Adenylate charge of the cell (see equation [2]).
Figure 2

\[ V_{\text{max}} = 8.9 \pm 0.5 \]
\[ K_m = 1.1 \pm 0.0 \]

\[ V_{\text{max}} = 5.6 \pm 0.3 \]
\[ K_m = 0.8 \pm 0.1 \]

\( v \) (mmol g\(^{-1}\) hr\(^{-1}\))

\( S \) (mM)
Figure 3
Legends

Figure 1
Central carbon metabolism in *S. cerevisiae* from external glucose to the production of ethanol.

Figure 2
Zero trans-influx uptake kinetics of labeled $^{14}$C-glucose of strain CEN.PK 113-7D measured at 30°C with chemostat cultures grown at 12°C (▲) and 30°C (■). Data and mean ± S.D. result from single component Michaelis-Menten kits of the averaged triplicates of two independent chemostat cultures, with $K_m$ in mM and $V_{max}$ in mmol.(g dwt)$^{-1}$.hr$^{-1}$. The dotted lines indicate the 95% confidence interval.

Figure 3
Degree of *in vivo* saturation of glycolytic enzymes, calculated from estimated *in vivo* fluxes and enzyme-capacity estimates from enzyme assays in cell extracts. ) 12°C - ■ 30°C. *In vitro* enzyme activities were expressed in g per g dry weight by assuming a soluble protein content of 33% in dry biomass.
Control of the glycolytic flux in Saccharomyces cerevisiae grown at low temperature: a multi-level analysis in anaerobic chemostat cultures
Siew Leng Tai, Pascale Daran-Lapujade, Marijke A. H. Luttik, Michael C. Walsh, Jasper A. Diderich, Gerard C. Krijger, Walter M. van Gulik, Jack T. Pronk and Jean-Marc Daran

J. Biol. Chem. published online January 24, 2007

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