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Research Article

Simultaneous overexpression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of *Saccharomyces cerevisiae*

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Abstract

Recombinant *S. cerevisiae* strains, with elevated levels of the enzymes of lower glycolysis (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, enolase, pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase) were physiologically characterized. During growth on glucose the enzyme levels in the recombinant strains (YHM4 and YHM7) were 1.1–3.4-fold higher than in the host strain (CEN.PK.K45). The recombinant strains were grown in aerobic or anaerobic batch cultures on glucose or a mixture of glucose and galactose. The specific ethanol production rates in the recombinant strains were the same as for the host strain and the physiological behaviour of the recombinant strains and the host strain was similar. When the cellular demand for ATP was increased by means of glucose pulses (final concentrations of 3.9 g/l or 2.0 g/l, respectively) to aerobic chemostat cultures maintained at a dilution rate of 0.08/h, the specific carbon dioxide production rate (qCO_2) of CEN.PK.K45 accelerated at 6×10^{-3} mmol/g/min^2 during the first 15 min, whereas during the same time period the qCO_2 of YHM7 accelerated twice as fast at 12×10^{-3} mmol/g/min^2, indicating a higher fermentative capacity in the recombinant strain. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: *Saccharomyces cerevisiae*; lower glycolysis; glycolytic flux; fermentative capacity; glyceraldehyde-3-phosphate dehydrogenase; phosphoglycerate kinase; pyruvate kinase; pyruvate decarboxylase

Introduction

Saccharomyces cerevisiae is an industrially important microorganism that is used as baker's yeast and for the production of ethanol. Furthermore, it is presently used as host for production of several important heterologous proteins, such as human insulin and hepatitis vaccines (Walker, 1998). In the classical applications of this yeast, a key issue is the glycolytic flux. When used as baker's yeast, the fermentative capacity is an important quality parameter, and in the production of ethanol—either as distillers' alcohol or as fuel alcohol—the rate of conversion of sugar to ethanol is of utmost importance. Several attempts have been made to increase the flux through the glycolytic pathway and, based on kinetic modelling of the glycolytic pathway, it has been suggested that flux control is at the point of sugar uptake, at the hexokinase, at the phosphofructose kinase (PFK), or at the pyruvate kinase (Galazzo and Bailey, 1990; Cortassa and Aon, 1994). However, glycolytic enzymes, including those mentioned above, have been overexpressed individually and in combinations without

increasing the glycolytic flux (Schaaff *et al.*, 1989). This points to a very tight control of the individual enzymes in the glycolytic pathway, and a better strategy for increasing the glycolytic flux has been to increase the drain of ATP, i.e. to pull the flux through the pathway that leads to ATP generation (Cornish-Bowden *et al.*, 1995). Increased glycolytic flux in *S. cerevisiae* has been observed when a proton decoupling component, such as a weak acid, was added to the medium (Verduyn *et al.*, 1992), when the enzyme fructose-1,6-bisphosphatase was constitutively expressed (Navas *et al.*, 1993; Navas and Gancedo, 1996), and when the plasma membrane ATPase was overexpressed in *Escherichia coli* (Jensen *et al.*, 1993). The concept has also been illustrated in *S. cerevisiae* overexpressing phosphofructokinase (PFK) (Davies and Brindle, 1992), in which a flux increase was observed under aerobic conditions in resting cells, where the contribution of respiration to ATP generation is larger (100%) than in growing cells (20%) (Lagunas *et al.*, 1982). In growing cells, the ATP demand is already fully fulfilled by glycolysis, and thus overproduction of PFK did not lead to an increase in glycolytic flux.

Although there is an understanding of how the glycolytic flux might be increased, the correlation between metabolite levels and glycolytic flux still needs to be established. Only recently has the activation of PFK by fructose-2,6-biphosphate been demonstrated not to be a major event in the control of the glycolytic flux (Müller *et al.*, 1997). Unravelling the relationship between metabolite levels and flux may lead to an understanding of the regulatory structures in the pathway, and should eventually permit deregulation of key enzymes in the glycolysis and design of strains with increased glycolytic flux.

In the present study, two novel recombinant strains of *S. cerevisiae*, YHM4 and YHM7 (Hauf *et al.*, 2000), were physiologically characterized. In these strains—contrary to previous studies—all enzymes of the lower glycolysis (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, enolase, pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase) were simultaneously overexpressed. In these recombinant strains, a drain of sugar phosphates and a concomitant deregulation of the upper glycolytic enzymes could be expected. The two glycolytic enzymes that are most tightly biochemically regulated, hexokinase and PFK, belong to the upper part of the pathway (Figure 1). The intracellular concentrations of the products of hexokinase and PFK, glucose 6-phosphate and fructose 1,6-bisphosphate, and the glycolytic flux were determined at low and high glucose concentrations in batch and continuous cultures to gain insight into the regulatory structure of glycolysis. Additionally, the recombinant strains' capacity to respond to an enhanced cellular demand for ATP was investigated by administrating glucose pulses to aerobic chemostat cultures at low dilution rate.

Materials and methods

Strain construction

Strains *S. cerevisiae* YHM4 and YMH7 were constructed as described in Hauf *et al.* (2000). Briefly, a truncated 390 bp fragment of the *S. cerevisiae HXT7* promoter has been shown to cause up to 2.5-fold stronger expression in glucose medium than the strongly expressed glycolytic genes (Hauf *et al.*, 2000). This truncated promoter was used to overproduce the lower glycolysis enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, enolase, pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase (Figure 1), which are encoded by the structural genes *TDH3*, *PGK1*, *GPM1*, *ENO2*, *PYK1*, *PDC1* and *ADH1*. The open reading frames were amplified by PCR and fused to the constitutively expressed *HXT7* promoter fragment. Transcription was terminated by the *PGK1*-termination region, which was fused to the respective ORF.

The overexpression constructs were cloned pairwise into modified, integrative yeast vectors, containing prototrophic markers. The resulting constructs, *TDH3*/*ENO2* (*URA3*), *PYK1*/*GPM1* (*LEU2*) and *PDC1*/*ADH1* (*TRP1*), were integrated into *S. cerevisiae* strain CEN.PK.K45. The *PGK1* construct was integrated using a dominant *kanMX* marker. Among the transformants, two were selected for further analysis, and these were designated YMH4 and YMH7.

Preparation of inoculum for strain characterization

The strains were kept at -80°C in YPD medium (Rose *et al.*, 1990) containing 200 ml glycerol/l. They were spread onto YPD agar plates, and one colony was taken to inoculate a shake flask.

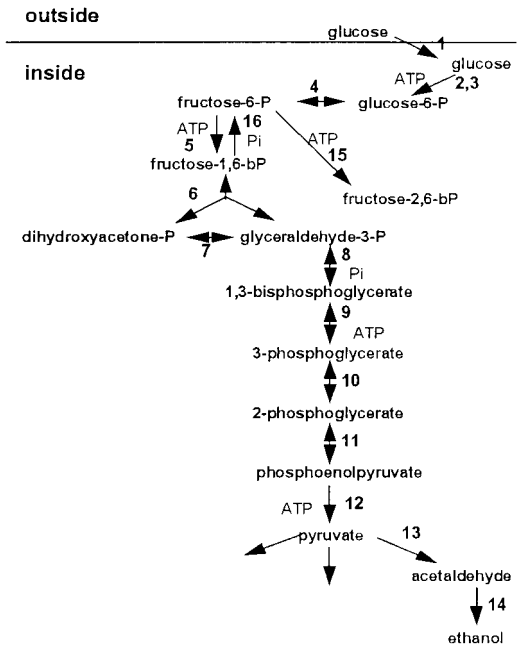

Figure 1. Overview of the main reactions of glycolysis in *S. cerevisiae*. Main enzymes are denoted by numbers: **1** = hexose transporters; **2** = hexokinase PI and PII; **3** = glucokinase; **4** = phosphoglucose isomerase; **5** = 6-phosphofructo-1-kinase; **6** = fructose-1,6-bisphosphate aldolase; **7** = triosephosphate isomerase; **8** = glyceraldehyde-3-phosphate dehydrogenase; **9** = phosphoglycerate kinase; **10** = phosphoglycero mutase; **11** = enolase; **12** = pyruvate kinase; **13** = pyruvate decarboxylase; **14** = alcohol dehydrogenase; **15** = 6-phosphofructo-2-kinase; **16** = fructose-1,6-bisphosphatase

The shake flasks were baffled, cotton-stoppered, 500 ml Erlenmeyer flasks, each containing 100 ml medium (see below). Cells were grown at 30°C at 120 rpm until the cell mass had reached 1.5 g/l dry weight (dw) and were harvested in the exponential growth phase. The bioreactors were inoculated to give 1.50 mg dw/l of cell mass.

Culture media and culture conditions

Precultures were grown in 100 ml of medium adjusted to pH 5.5 and with the following composition: $(NH_4)_2SO_4$, 7.5 g/l; KH_2PO_4, 14 g/l; $MgSO_4 \cdot 7H_2O$, 0.74 g/l; trace element solution, 10 ml/l; vitamin solution 1 ml/l; Synperonic antifoam (Sigma A 8436), 50 µl/l; histidine, 50 mg/l; and glucose, 10 g/l. Sugars were autoclaved separately from the minerals in order to prevent the Maillard reaction.

The medium used in the batch cultures contained: $(NH_4)_2SO_4$, 7.5 g/l; KH_2PO_4, 3.5 g/l; $MgSO_4 \cdot 7H_2O$, 2.7 g/l; trace element solution, 20 ml/l; vitamin solution, 2 ml/l; Synperonic antifoam, 50 µl/l; histidine, 50 mg/l; glucose, 20 g/l, or a mixture of glucose, 10 g/l, and galactose 10 g/l. For anaerobic batch cultures, 1 ml/l of an ergosterol/Tween 80 solution (2 g ergosterol and 84 g Tween 80 in 200 ml ethanol) was added.

For aerobic carbon-limited continuous cultures, the medium was composed of: $(NH_4)_2SO_4$, 7.5 g/l; KH_2PO_4, 3.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.74 g/l; trace element solution, 10 ml/l; vitamin solution 1 ml/l; Synperonic antifoam, 50 µl/l; histidine, 50 mg/l and glucose, 5.5 g/l.

The trace element solution had the following composition: EDTA, 3.00 g/l; $CaCl_2 \cdot 2H_2O$, 0.90 g/l; $ZnSO_4 \cdot 7H_2O$, 0.90 g/l; $FeSO_4 \cdot 7H_2O$, 0.60 g/l; H_3BO_3, 200 mg/l; $MnCl_2 \cdot 2H_2O$, 156 mg/l; $Na_2MoO_4 \cdot 2H_2O$, 80 mg/l; $CoCl_2 \cdot 2H_2O$, 60 mg/l; and KI, 20 mg/l. The trace element solution was autoclaved together with the minerals. The vitamin solution contained the following components: D-biotin, 50 mg/l; *p*-aminobenzoic acid, 200 mg/l; nicotinic acid, 1.00 g/l; calcium pantothenate, 1.00 g/l; pyridoxine hydrochloride, 1.00 g/l; thiamine hydrochloride, 1.00 g/l and *m*-inositol, 25 g/l. The vitamin solution was sterilized by filtration through a sterile 0.2 µm filter.

Bioreactor conditions

For batch cultivations, baffled 5 l reactors were used with 3.5 litres working volume and fitted with two Rushton turbines, rotating at 800 rpm for aerobic cultivations, and at 450 rpm for anaerobic cultivations. The temperature was kept at 30 ± 1°C, and the pH was kept at 5.0 ± 0.2 by automatic addition of 4 M NaOH. The gasflow was set to 1 vvm (3.5 l/min) and the off-gas was led through a condenser and analysed for O_2 and CO_2 (Model 1308, Brüel&Kjær, Nærum, Denmark). The concentration of oxygen and carbon dioxide in the off-gas were determined with an accuracy of 1%.

For continuous cultivations, 3.0 l bioreactors (MBR), using a working volume of 2.0 l equipped with four baffles and two Rushton six-blade turbines, were used. The temperature, airflow (1 vvm), stirring and pH control were identical to the parameters in the batch-cultivations. The dilution rate was precisely controlled at D = 0.08/h, on a constant weight basis. The continuous culture was

regarded to be in steady state when both the dilution rate and off-gas signal had not changed for at least five residence times, and when the metabolite concentrations in two successive samples taken at a time interval of three to five residence times deviated by less than 3%.

The continuous cultures were pulsed with glucose by adding 20 ml of a concentrated glucose solution through a sterile filter. The final glucose concentration was between 2–4 g/l. The feed was stopped while the pulse was consumed.

Determination of cell mass concentration

Dry weight was determined using nitrocellulose filters with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI). The filters were pre-dried in a microwave oven (Moulinex Optiquick Compact Y51) at 150 W for 10 min. A volume of 5 ml cell culture was filtered and the filter pellet was washed three times with 5 ml distilled water, after which the filters were dried for 15 min at 150 W. The cell mass concentration was determined with an accuracy of 2%.

Sugar and metabolite analysis

For measurements of sugars and extracellular metabolites, the cultivation medium was sampled, immediately filtered through a 0.45 μm pore size cellulose acetate filter (Sartorius AG, Göttingen, Germany), frozen and stored at −20°C until analysis. Glucose, galactose, ethanol, glycerol, acetate and pyruvate were separated on an Aminex HPX-87H ion-exclusion column (Bio-Rad, Hercules, CA) at 65°C, using 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 ml/min. The sugars, ethanol and glycerol were detected by a refractometer (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA), whereas acetate and pyruvate were monitored spectrophotometrically (Waters 486 Tunable Absorbance Detector) at 219 nm. The two detectors were connected in series. The concentrations were determined by HPLC with an accuracy better than 5%.

Extraction and analysis of intracellular sugar-phosphates

The method for the determination of intracellular sugar phosphates has been described previously (Smits *et al.*, 1998). An aliquot of 10 ml cultivation medium was quenched in cold methanol (−40°C), such that the final methanol concentration was 50% (v/v). The cells were separated from the quenching medium by centrifugation ($770 \times g$, 20 min). Cells were suspended in 100% methanol and buffer (3 mM Pipes, 3 mM EDTA, pH 7), whereupon extraction of the metabolites was initiated by adding chloroform. The solution was shaken vigorously (500 rpm) on a platform shaker for 45 min at −20°C. Separation of the chloroform and the aqueous phase was induced by centrifugation ($770 \times g$, 20 min). The upper aqueous phase, containing the metabolites, was collected without disturbing the pellet of cell debris on top of the lower chloroform phase. A second extraction was started by adding buffer and methanol to the chloroform and the cell pellet and the aqueous phases from both extractions were pooled. The extract was cleaned and concentrated by solid phase extraction, and was analyzed for its content of sugar phosphates by using anion exchange chromatography with pulsed amperometric detection. The analytical system used was a DX 500 HPLC system (Dionex, Sunnyvale, CA) consisting of a Dionex GP40 gradient pump and a Dionex ED40 electrochemical detector. Samples were separated on a 250×4 mm CarboPac PA1 column (Dionex, Sunnyvale, CA). For each sample point, three independent extractions were performed. The standard deviation varied from 4% to 15%.

Determination of enzyme activities

Cells were grown in 5 ml YPE or YPD (Rose *et al.*, 1990) cultures overnight and specific enzyme activities were determined in crude extracts prepared by shaking the cells with glass beads (Ciriacy and Breitenbach, 1979) and then diluting in 50 mM imidazol buffer (50 mM imidazol, 10 mM $MgCl_2$, 100 mM KCl), pH 7.0. The crude extracts for the pyruvate decarboxylase assay were diluted in 50 mM imidazol buffer, pH 7.0, without $MgCl_2$ and KCl. The protein concentrations were estimated by the microbiuret method (Zamenhoff, 1957), with BSA as reference. The following enzyme activities were determined: glyceraldehyde-3-phosphate, phosphoglycerate kinase, phosphoglyceromutase, enolase, pyruvate kinase (Maitra and Lobo, 1971), pyruvate decarboxylase (Schmitt and Zimmermann, 1982) and alcohol dehydrogenase (Bergmeyer, 1974). The enzyme activities are reported as U/mg protein, with 1 Unit (U) defined as the conversion of 1 μmol

Table 1. Specific enzyme activities (mU/mg protein) in CEN.PK.K45 (wild-type), YHM4 and YHM7 grown on 20 g/l glucose or 20 g/l ethanol

Strain	Carbon source	Tdh1	Pgk1	Gpm1	Eno2	Pyk1	Pdc1	Adh1
CEN.PK.K45	Glucose	1879	1178	1299	1784	4390	712	1625
YHM4	Glucose	2529	1567	4342	2453	10860	802	2120
YHM7	Glucose	2845	1672	4483	2647	11072	795	2052
CEN.PK.K45	Ethanol	1231	675	496	273	1060	175	1198
YHM4	Ethanol	1750	1155	7698	1027	11055	208	1745
YHM7	Ethanol	1921	1593	7982	1078	10248	213	1539

Abbrevations: tdh1 = glyceraldehyde-3-phosphate dehydrogenase; pgk1 = phosphoglycerate kinase; gpm1 = phosphoglyceromutase; eno2 = enolase; pyk1 = pyruvate kinase; pdc = pyruvate decarboxylase; adh1 = alcohol dehydrogenase.

substrate/min. All given values for specific enzyme activities are the calculated average values obtained from the measured values in the assays. Each enzyme activity was assayed at least twice, with a standard deviation $\leq 5\%$.

Results

Enzyme activities

In the recombinant strains, elevated activities of all enzymes were observed during both glucose growth and ethanol growth (Table 1). When grown on glucose, the enzyme with the greatest activity increase was phosphoglyceromutase (3.4-fold) and the enzyme with the least activity increase was pyruvate decarboxylase (1.1-fold), both in YHM4 and YHM7 (Table 1). When grown on ethanol, both recombinant strains showed enhanced enzyme activity levels ranging from 1.2-fold for pyruvate decarboxylase, up to 16-fold for phosphoglyceromutase (Table 1).

Generally, it was found that the enzyme activity was higher in strains transformed only with one plasmid compared to a strain transformed with several or all plasmids (Hauf *et al.*, 2000). Strains YHM4 and YHM7 showed the highest enzyme activities of a total of seven transformants having all seven enzyme overexpression constructs integrated (data not shown). Although all seven enzymes of lower glycolysis were brought under the control of the same constitutive promoter, their activities varied greatly in comparison to those determined in the host strain, which reflects differences in the expression of the native genes, post-transcriptional regulation and turnover rates.

Growth and metabolite production in batch cultivations on glucose

During aerobic batch growth on glucose, the recombinant strains YHM4 and YHM7 exhibited lower specific growth rates (0.27 and 0.38/h) than the CEN.PK.K45 strain (0.41/h) (Table 2). The ethanol productivity was approximately 1.0 g/g/h under aerobic conditions and 1.5–1.6 g/g/h for all strains under anaerobic conditions (Table 2). Besides ethanol, cell mass was produced with a yield of 0.12–0.13 g/g under aerobic conditions and 0.07–0.08 g/g under anaerobic conditions (Table 2). The main by-products were acetate and glycerol. Under aerobic conditions 0.04–0.05 g/g glycerol was produced, compared to 0.1 g/g under anaerobic conditions. Generally, the results show that differences in product pattern mainly reflected the difference between aerobic and anaerobic growth

Table 2. Growth characteristics determined in batch cultivations grown on glucose under aerobic and anaerobic conditions

	CEN.PK.K45		YHM4		YHM7	
Aerobic/anaerobic	+	−	+	−	+	−
μ_{max}	0.41	0.34	0.27	0.35	0.38	0.30
Y_{sx}	0.13	0.08	0.12	0.08	0.12	0.07
Y_{seth}	0.33	0.35	0.33	0.36	0.36	0.36
Y_{sac}	0.03	0.01	0.02	0.01	0.02	0.01
Y_{sglyc}	0.05	0.1	0.05	0.1	0.04	0.1
r_s	3.0	4.4	2.1	4.1	2.9	4.1
q_{eth}	1.0	1.6	0.6	1.5	1.0	1.5

Abbrevations: μ_{max} = specific growth rate (/h); Y_{sx} = cell mass yield (g/g); Y_{seth} = ethanol yield (g/g); Y_{sac} = acetate yield (g/g); Y_{sglyc} = glycerol yield (g/g); r_s = substrate consumption rate (g/g/h); q_{eth} = specific ethanol production rate (g/g/h); + = aerobic; − = anaerobic.

conditions, rather than between the host and the recombinant strains.

The effect of over-expression of the lower glycolytic enzymes on the regulatory network was evaluated by measuring the levels of two metabolites of the upper glycolysis. Metabolite levels were measured at biomass concentrations of 0.5–2.5 g/l for both CEN.PK.K45 and YHM7. Glucose-6-phosphate and fructose-1,6-diphosphate levels were relatively constant during the time span of the batch growth in both aerobic (Figure 2a) and anaerobic conditions (Figure 2b). Moreover, the levels of the two sugar phosphates were comparable in the two strains. Thus, the overexpression of the lower glycolytic enzymes did not drain metabolites from the upper glycolysis, suggesting that even though the level of the enzymes in the lower glycolysis was increased, there was not an enhanced glycolytic flux in the recombinant strain YHM7.

Growth and metabolite production in anaerobic batch cultivations using a mixture of glucose and galactose

During anaerobic batch cultivation, glucose was utilized before galactose consumption started (results not shown). The ethanol yield on glucose (0.35–0.37 g/g) was slightly higher than on galactose (0.32–0.35 g/g) (Table 3). However, the yields of ethanol and other metabolites were not significantly different in the CEN.PK.K45 strain, as compared with the recombinant strains. The main difference between growth on glucose and galactose was the lower specific growth rate on galactose, and the consequently lower ethanol productivity; 0.04–0.14 g/g/h on galactose compared with 1.2 g/g/h on glucose. Under all conditions the extracellular levels of pyruvate, which is the end point of glycolysis, were low and comparable in the CEN.PK.K45 strain and the recombinant strains (data not shown), demonstrating that the overproduction of lower glycolytic enzymes did not lead to an increase in the end product of glycolysis.

Table 3. Growth characteristics determined in anaerobic batch cultivations grown on glucose–galactose mixtures

	CEN.PK.K45	YHM4	YHM7
μ_{max} on glucose	0.37	0.39	0.35
μ_{max} on galactose	0.07	0.02	0.06
Y_{sx} on glucose	0.10	0.10	0.10
Y_{sx} on galactose	0.10	0.10	0.09
Y_{seth} on glucose	0.37	0.36	0.35
Y_{seth} on galactose	0.34	0.35	0.32
Y_{sac} on glucose	0.005	0.005	0.007
Y_{sac} on galactose	0.002	0.006	0.001
Y_{sglyc} on glucose	0.023	0.022	0.025
Y_{sglyc} on galactose	0.011	0.014	0.010
$r_{glucose}$	3.3	3.4	3.3
$r_{galactose}$	0.68	0.19	0.53
q_{eth} on glucose	1.2	1.2	1.22
q_{eth} on galactose	0.04	0.07	0.14

Abbrevations: μ_{max} = specific growth rate (/h); Y_{sx} = cell mass yield (g/g); Y_{seth} = ethanol yield (g/g); Y_{sac} = acetate yield (g/g); Y_{sglyc} = glycerol yield (g/g); $r_{glucose}$ = glucose consumption rate (g/g/h); $r_{galactose}$ = galactose consumption rate (g/g/h); q_{eth} = specific ethanol production rate (g/g/h).

Glucose consumption rates were lower (3.3–3.4 g/g/h) (Table 3) when the strains were grown on a glucose–galactose mixture than when grown on glucose solely, where the glucose consumption rate was estimated to be 4.1–4.4 g/g/h (Table 2). This might indicate that, although galactose is not metabolized, the sugar competes with glucose for binding sites of the glucose transporters, leading to

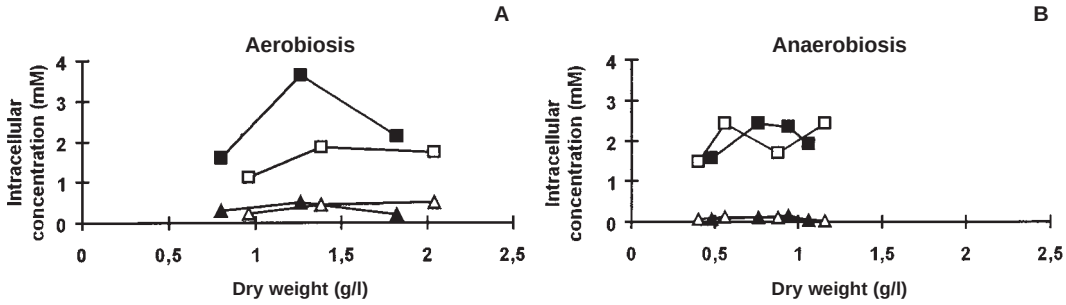

Figure 2. Intracellular metabolites in CEN.PK.K45 (-△-, glucose-6-phosphate, and -□-, fructose-1,6-diphosphate) and YHM7 (-▲-, glucose-6-phosphate, and -■-, fructose-1,6-diphosphate), grown aerobically (A) and anaerobically (B) on glucose

reduced glucose consumption rates (Liang and Gaber, 1996; Reifenberger *et al*., 1997).

Growth and metabolite production in continuous cultures and pulse experiments

Cornish-Bowden *et al*. (1995) hypothesized that the flux of a metabolic pathway is controlled by the demand for its products, which was tested in the present study by experiments with continuous cultures and addition of glucose pulses. Thus, the strategy of using resting cells, as in the work of Davies and Brindle (1992), was avoided, since in resting cells the cellular make-up can differ considerably from that of growing cells, which may result in an altered regulation of glycolysis. Under conditions of nitrogen starvation glucose transporter proteins that have high turnover rates (2–7 h) are inactivated, which decrease the glucose transport activity by 80% (Lagunas *et al*., 1982; Busturia and Lagunas, 1986). More specifically, it has been shown that the high-affinity hexose transporters Hxt6 and Hxt7 in *S. cerevisiae* are subject to catabolite inactivation (Krampe *et al*., 1998). In the present study, a glucose pulse to slow-growing cells in continuous culture was used to avoid resting cells and to prevent glycolysis from working at its maximum capacity. The cells were maintained in a carbon-limited continuous culture at a low specific growth rate of 0.08/h, so that a relatively high fraction of the produced ATP went to maintenance.

CEN.PK.K45 and YHM7 did not show substantially different growth and metabolite production patterns when grown in continuous culture (Table 4). Both continuous cultures were operated at a low dilution rate of $D = 0.08$/h, resulting in a slow glucose consumption rate of approximately 0.17 g/g/h for both strains (Table 4). The respiratory coefficient (RQ) was close to one (Figures 3a and 3b) and CO_2 and biomass were formed as the sole detectable products. These observations indicate that glucose was fully respired by both strains. The continuous cultures were pulsed with glucose to a final concentration of 3.9 g/l for CEN.PK.K45 and 2.0 g/l for YHM7. The pulse was consumed within 100 min by the CEN.PK.K45 strain and within 75 min by YHM7. After the pulse, an instantaneous rise in the specific CO_2 production rate (qCO_2) was observed in both strains (Figures 3a, b). However, the O_2 consumption rate (qO_2), did not increase to the same extent as qCO_2, so the RQ increased above one. The glycolytic capacity in both strains can be evaluated by determining the increase in the acceleration rate of product formation, moments after the pulse was given. The CO_2 formation was measured with high frequency before and after addition of the glucose pulse and the acceleration rate of qCO_2 was calculated. qCO_2 gives a good measure of the glycolytic flux, as carbon dioxide is produced not only during ethanol production but also in the other reactions following glycolysis under aerobic conditions. Moreover, carbon dioxide was, in this case, the most frequently and accurately measured metabolite. During the first 15 min, qCO_2 of CEN.PK.K45 accelerated at 6×10^{-3} mmol/g/min^2, whereas in the same time period qCO_2 of YHM7 accelerated twice as fast at 12×10^{-3} mmol/g/min^2.

Table 4. Effects of a glucose pulse to an aerobic C-limited continuous culture

	CENPK.K45		YHM7	
	Steady state	**Pulse**	**Steady state**	**Pulse**
c_f	4.49	4.49	4.49	4.49
Pulse (g/l)	–	3.86	–	2.09
D	0.08	0	0.08	0
q_s	0.17	1.06	0.18	0.83
Y_{sx}	0.44	n.m.	0.45	n.m.
Y_{seth}	n.d.	0.27	n.d.	0.28
Y_{spyr}	n.d.	0.01	n.d.	0.01
Y_{sac}	n.d.	0.12	n.d.	0.18
Y_{sglyc}	n.d.	0.07	n.d.	0.05
q_{eth}	n.d.	0.29	n.d.	0.23
q_{ac}	n.d.	0.08	n.d.	0.15

Abbrevations: D = dilution rate (/h); c_f = glucose concentration in feed (g/l); Y_{sx} = cell mass yield (g/g); Y_{seth} = ethanol yield (g/g); Y_{sac} = acetate yield (g/g); Y_{sglyc} = glycerol yield (g/g); q_s = substrate consumption rate (g/g/h); q_{eth} = specific ethanol production rate (g/g/h); q_{ac} = specific acetate production rate (g/g/h); n.d. = not detectable with HPLC; n.m. = not measured.

After the pulse, the glucose consumption rate increased to 1.1 g/g/h in CEN.PK.K45 and to 0.8 g/g/h in YHM7 (Table 4). Both strains started to produce ethanol and small amounts of glycerol and pyruvate, indicative of a switch from respirative metabolism to respiro-fermentative metabolism (Figures 3c, d). The ethanol production rate was 0.29 g/g/h in CEN.PK.K45 and 0.23 g/g/h in YHM7 during the pulse. In addition, acetate was formed in both strains, with a production rate of 0.08 g/g/h for CEN.PK.K45 and 0.15 g/g/h for YHM7 (Table 4, Figures 3c, d).

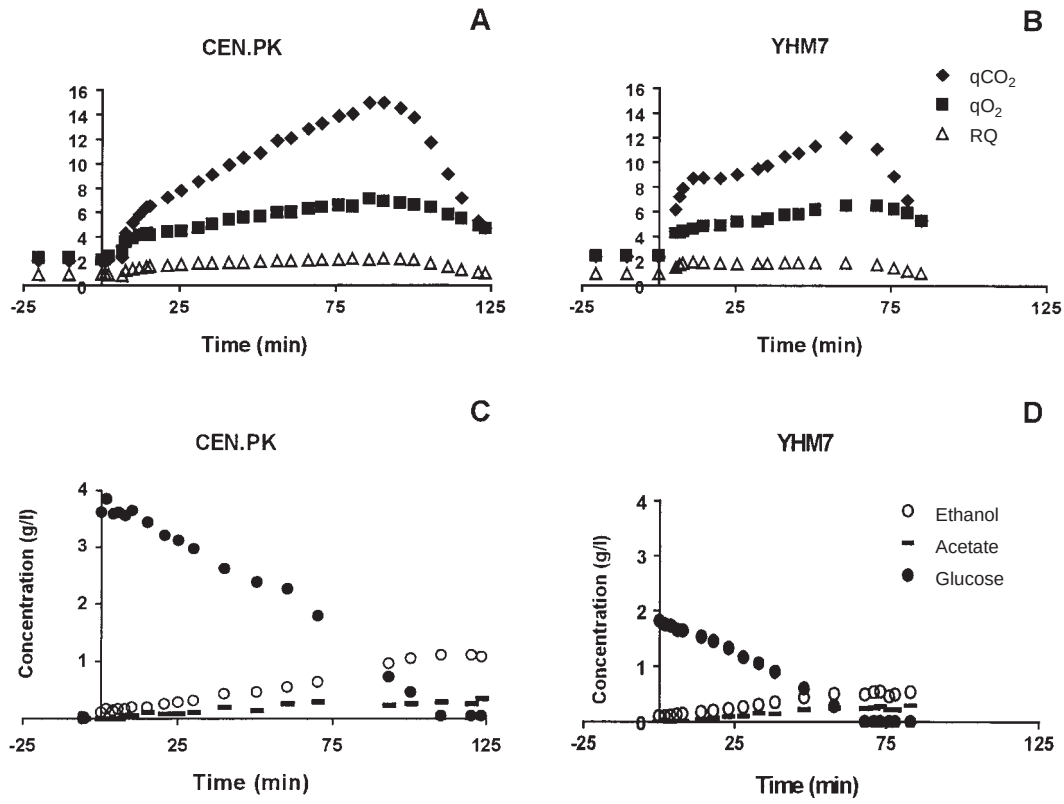

Figure 3. Product formation before and after a glucose pulse (added at 0 min) in CEN.PK.K45 (3.9 g/l) (A and C) and YHM7 (2.0 g/l) (B and D), grown aerobically on glucose in a carbon-limited continuous culture at D = 0.08/h. (A) and (B) show specific gas production rates (mmol/g/h) (qCO_2 and qO_2) and RQ (qCO_2/qO_2). (C) and (D) show consumption (g/l) of glucose and formation (g/l) of acetate and ethanol

Discussion

The present study was conducted to investigate whether overexpression of a set of consecutive glycolytic enzymes could lead to enhanced glycolytic flux. In pulse experiments, the specific CO_2 production rate accelerated two-fold faster in the recombinant strain than in the CEN.PK.K45 strain, indicating that enzyme overexpression permitted the glycolytic flux in the recombinant strain to respond more quickly. It has been demonstrated that after a glucose pulse to *S. cerevisiae* cultures grown at glucose limited conditions, i.e. in chemostat cultures similar to those used in this study, there is a rapid decrease in ATP for sugar phosphorylation (Rizzi *et al.*, 1997). Thus, the recombinant strain with overexpression of lower glycolytic enzymes may respond quicker, due to this ATP demand. In contrast, the flux did not increase in batch cultivations and in continuous cultures, where no increased ATP demand was imposed. Consequently, an increase in glycolytic capacity by overproduction of its enzymes may only lead to a flux increase when the cell needs the extra capacity, which supports the proposal of Cornish-Bowden *et al.* (1995), that the flux of a metabolic pathway is controlled by the demand for its products.

Our strategy in this study was to overproduce an entire sequence of enzymes, in contrast to an earlier study where at most two enzymes were overproduced at the same time (Schaaff *et al.*, 1989). The flux control in a given pathway is distributed throughout the enzymes in that pathway, and the distribution of flux control amongst the enzymes might change upon overexpression of a single enzyme (Kell and Westerhoff, 1986; De Hollander, 1994). According to the theory of metabolic control analysis, overexpression of all the enzymes in the pathway should enable an increased flux (Niederberger *et al.*, 1992). In our study, the simultaneous

overproduction of a set of glycolytic enzymes did indeed lead, under specific conditions, to an enhanced glycolytic flux. However, in batch cultivation and continuous cultivation, the specific rate of glucose consumption in the recombinant strains was slightly lower than in CEN.PK.K45, and similarly, the specific ethanol production rate was lower in the recombinant strains than in CEN.PK.K45. The ethanol yield on glucose, i.e. the fraction of glucose directed towards ethanol, was the same in the recombinant and in the host strains.

The strategy taken to perform metabolic engineering in a given pathway might not lead to the expected results, since there may be unwanted effects due, for example, to protein burden and the regulatory network of the cell, which may counteract the intended changes of the metabolism. The recombinant strains characterized in the present study had reduced specific growth rates compared to their host strain, which is indicative of a protein burden effect. Protein burden may be ascribed to the dilution of essential enzymes necessary for protein production and to an increase in energy demand for the additional production of protein (Snoep *et al*., 1995), and consequently the effect of protein burden becomes important when the overproduced enzymes make up a substantial part of the total protein content. In *Zymomonas mobilis*, where 50% of the cytoplasmic proteins are glycolytic enzymes, simultaneous overexpression of glycolytic enzymes caused a decrease in glycolytic flux (An *et al*., 1991; Snoep *et al*., 1995). In *S. cerevisiae*, the glycolytic enzymes constitute 30–65% of the total cytoplasmic protein content (Hess *et al*., 1969; Fraenkel, 1982). The finding of Hauf *et al*. (2000), that the specific activities of most overexpressed enzymes decreased in the strains harbouring multiple integrated overexpression constructs when compared with strains containing only one or two overexpressed enzymes, is consistent with a possible protein burden effect. Furthermore, the presence of multiples of the *HXT7* promoter fragment in one genome could lead to a competition for important global transcription factors. This could cause a general alteration of transcriptional activities, with reduced expression of several housekeeping genes also leading to reduced growth rates.

Due to the rigidity of the regulatory network of the cell, an increase in enzyme activity expected from overexpressing an enzyme does not necessarily result in an increased flux through the manipulated pathway, as the enzyme activity *in vivo* depends both on the enzyme level and the allosteric regulation of that enzyme. This was observed when PFK was overproduced in *S. cerevisiae* (Davies and Brindle, 1992). The five-fold overproduction of PFK led to an increase in the number of binding sites for its activator, fructose-2,6-bisphosphate, which reduced the specific PFK activity and did not increase the flux through glycolysis. No difference in glycolytic flux could be observed in a *pfk26*/*pfk27* double deletion mutant, or in a fructose-2,6-bisphosphate overexpression mutant, compared to the corresponding wild-type strains (Boles *et al*., 1996; Müller *et al*., 1997). However, in this case it was indicated that fructose-2,6-bisphosphate had an effect on metabolite homeostasis and thus influenced the glycolytic flux indirectly, rather than exerting a direct control on glycolytic flux by regulating enzyme activities. In the present study, the intracellular concentrations of metabolites in upper glycolysis were shown not to be influenced by the overexpression of lower glycolysis enzymes, which indicates that the regulatory network is rigid enough to stabilize such an important pathway as glycolysis towards changes in enzyme levels.

In conclusion, although an entire sequence of glycolytic enzymes was overproduced in the present study, increased glycolytic flux was only found under conditions of enhanced ATP demand. Thus, the central carbon metabolism in *S. cerevisiae* is tightly regulated and resistant towards any changes, consistent with earlier observations of the limited effects of overproduction of enzymes on glycolytic fluxes (Heinisch, 1986; Brindle, 1988; Schaaff *et al*., 1989; Ruyter *et al*., 1991; Snoep *et al*., 1995). Although the implication here is that all enzymes of glycolysis need to be simultaneously overexpressed for a substantial enhancement of the glycolytic flux, it is likely that this strategy might fail because of an excessive protein burden. Therefore, a full recognition of the factors that hamper an increase in glycolytic flux is necessary for successful future metabolic engineering of the glycolytic pathway in *S. cerevisiae*.

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