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Chapter 7

Effects of the deletion of hexokinase II on the dynamics of glycolysis in continuous cultures of *Saccharomyces cerevisiae*

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7.1 Summary

In glucose-limited aerobic chemostat cultures of a wild-type *S. cerevisiae* CEN.PK113-7D and a derived *hxxk2* null strain, metabolic fluxes were identical at lower dilution rates. However, concentrations of intracellular metabolites, especially fructose-1,6-bisphosphate, were different in the wild-type and the *hxxk2* null strain at the various dilution rates. During oxidative growth at low dilution rates the maximal specific *in vitro* glucose-phosphorylating activity was higher in wild-type cells than in cells of the *hxxk2* mutant strain. The maximal hexose-phosphorylating velocity with fructose as a substrate compared to glucose as a substrate increased at higher dilution rates in the mutant cells yet decreased in the wild-type cells, suggesting an increase in hexokinase II activity in the wild-type cells and an increase in hexokinase I activity in the *hxxk2* mutant cells. Interestingly, the *hxxk2* null strain showed a higher maximal growth rate and ethanol production started to appear at a higher dilution rate, revealing a higher oxidative capacity for this strain.

After a pulse of glucose, aerobic glucose-limited cultures of wild-type *S. cerevisiae* displayed an overshoot in the intracellular concentration of glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate before a new steady-state was established. In the *hxxk2* null mutant glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate reached a new steady-state without overshoot. Furthermore, the steady-state level of fructose-1,6-bisphosphate after a glucose pulse was considerably lower in the *hxxk2* null strain. At low dilution rates the overshoot of intracellular metabolites in the wild-type strain coincided with the immediate production of ethanol after the glucose pulse. In contrast, in the *hxxk2* null strain the production of ethanol started gradually. In spite of the differences in intracellular properties, the steady-state fluxes after transition from glucose limitation to glucose excess were not significantly different in the wild-type strain and the *hxxk2* null strain at any dilution rate.
7.2 Introduction

Saccharomyces cerevisiae contains three genes that encode hexose-phosphorylating enzymes: hexokinase I encoded by HXK1, hexokinase II encoded by HXK2, and glucokinase encoded by GLK1. Hexokinase II has been known to play an important role in glucose repression (for reviews see (22, 23, 58)), however the exact mechanism of action still remains to be elucidated. In previous studies we found that the deletion of HXK2 from the yeast genome has profound effects on the physiology of S. cerevisiae in batch cultures with glucose as a carbon source (see Chapter 6 and (168)). An hxk2 null mutant displays fully oxidative growth at high concentrations of glucose, as evidenced by a redirection of carbon flux to the production of biomass. However, ethanol production was not completely abolished.

During batch cultivation of wild-type S. cerevisiae with glucose as a carbon source ethanol production seems inevitable, which makes S. cerevisiae a true Crabtree-positive yeast (32, 33). In the industrial production of yeast biomass ethanol is undesirable. Alcoholic fermentation is avoided by means of sugar-limited fed-batch cultivation. Incomplete mixing or insufficient air supply in bioreactors during the production of yeast biomass are potential causes for the production of ethanol (for instance see (253)) and limit the maximum biomass yield and productivity. Chemostat cultivation is an excellent method to mimic yeast at different stages of growth in the bioreactor at steady-state conditions.

Aerobic glucose-limited continuous cultures of S. cerevisiae show a behavior typical of Crabtree-positive yeasts. At low dilution rates glucose metabolism is strictly oxidative and has a relatively high biomass yield. Above a threshold or critical dilution rate metabolism switches to a respiro-fermentative mode which is accompanied with the production of ethanol and a decrease in biomass yield. The switch in metabolism after this Crabtree-threshold dilution rate coincides with changes in the metabolic machinery of the yeast cell; e.g. the kinetic parameters of glucose transport (see Chapter 2 and (249)) and levels of the glycolytic enzymes (196, 225). The Crabtree-effect has been ascribed to a limited respiratory capacity (178), and/or an overflow at the level of pyruvate (160, 163).

It was shown that rapid changes in the intracellular metabolites occur after injection of glucose to a glucose-limited culture (208). These rapid changes are associated with the production of ethanol or the short-term Crabtree-effect, which again is attributed to a limited respiratory capacity (160), or an overflow at the level of pyruvate (226). In a bioractor yeast responds rapidly to sugar gradients or oxygen shortages. To model the effect of an hxk2 null mutation on the physiology of S. cerevisiae in a bioreactor, transient responses of a glucose-limited culture to glucose excess were studied in a wild-type strain, CEN.PK113-7D, and a derived hxk2 null mutant.

7.3 Materials and Methods

**Strains** – Saccharomyces cerevisiae wild-type strain CEN.PK113-7D (MATa MAL2-8° SUC2) provided by Dr. P. Kötter (Frankfurt, Germany) was used (223) for a PCR-based gene disruption of HXK2. The HXK2 gene in CEN.PK113-7D was replaced by a kanMX-cassette
to create strain KY116 as follows: using primer AK53 (GTGGTAGGAATATAATTCTCCA CACATAAATAGTAGCTACGTCGAGTGCAGC; the underlined nucleotides correspond to the DNA immediately 5' of the HXK2 open reading frame) and primer AK54 (AAAAGCCACCTCTTGTGTTGCTCAATTTCTCAGAATCGCGCTCG; the underlined nucleotides correspond to the DNA 3' of the HXK2 open reading frame) the kanMX cassette of plasmid pFA6a-kanMX4 (235) was amplified using the Expand PCR kit as recommended by the manufacturer (Roche). The resulting PCR product was transformed into competent CEN.PK113-7D as described (61). G418-resistant isolates were tested for proper integration of the kanMX cassette at the HXK2 locus by analytical PCR using the TaqPlus Long PCR kit with the primers AK60 (GACGAAATACGCGATCGCTGT) and AK61 (GCCGAACATTTCAAAGTCAACC) as recommended by the manufacturer (Stratagene).

Chemostat cultivation — Chemostat cultures were run under aerobic glucose-limited conditions in laboratory fermenters (L.H. Engineering) at a stirrer speed of 1000 rpm and at 30°C in a defined mineral medium containing vitamins (232). The concentration of glucose in the reservoir media was 5 g litre⁻¹. The working volume in the culture vessel was kept at approximately 0.6 litre. The exact working volume was measured after each experiment. The pH was kept at 5.0 ± 0.1 via automatic addition of 1 M KOH. Silicone antifoam was added to prevent the development of foam. Chemostat cultures were flushed with air at a flow rate of approximately 1 volume of air per vessel volume per minute. Culture purity was routinely monitored by phase-contrast microscopy and by plating on YPD and YPD + G418 medium (see Chapter 6 Materials and Methods). O₂ and CO₂ concentrations were determined in the effluent gas with an oxygen analyser (paramagnetic O₂ transducer, Servomex) and an I.R. gas analyser (Servomex), respectively.

Pulse experiments — For the transition of the glucose-limited steady-state cultures to a condition of glucose excess, the medium feed to the culture vessel was switched off. Simultaneously, the cultures were pulsed with 20 mM of glucose (final concentration in the culture vessel) by means of a syringe. Samples for the determination of intracellular metabolites and extracellular metabolites were taken as frequently as possible during the first 1 min after the pulse, then samples were taken at regular time intervals until glucose depletion. During the pulse O₂ and CO₂ concentrations were measured from the effluent gas.

Sample extraction - Samples for the determination of intracellular metabolites were taken from the culture with a fast sampling device. Approximately 600 µl (accurately measured) of culture from the culture vessel was added within milliseconds to 100 µl 35% PCA (v/v) on ice. Samples were neutralized within an hour after extraction with 150 µl 2 M K₂CO₃ and stored at -20°C. Before analysis samples were centrifuged for 1 min at 16000 x g. Samples for the determination of extracellular metabolites were prepared by addition of 100 µl 35% perchloric acid (v/v) to 1 ml of culture supernatant, and stored at -20°C. Samples (on ice) were neutralized with 55 µl 7 M KOH, cooled on ice and centrifuged (1 min at 16000 x g) before analysis. For the determination of the protein concentration 1 ml of culture was centrifuged for 1 min at 16000 x g. The pellet was resuspended in 1 ml of 1 M NaOH, stored overnight at room temperature and centrifuged for 1 min at 16000 x g before analysis.
**Sample analysis** – Protein concentrations were determined by the method of Lowry *et al.* (115) using bovine serum albumin (fatty-acid free, Sigma) as a standard. Extracellular metabolites were determined by means of high performance liquid chromatography. Intracellular metabolites were determined by NAD(P)H coupled enzymatic reactions (11). Protein concentrations and intracellular metabolites were measured on a COBAS-FARA automatic analyser (Roche). Intracellular concentrations were calculated assuming that 1 mg of protein corresponds to 3.75 μl of intracellular volume (34, 177, 204). Furthermore, it was assumed that cells of the wild-type and the *hxk2* mutant have the same cell volume.

### 7.4 Results

**Effects of an hxx2 null mutation on the metabolism of yeast in continuous cultures** – The parental strain, CEN.PK113-7D and the derived *hxk2* null strain were grown in aerobic glucose-limited continuous cultures. Below the critical dilution rate of the wild-type strain (D = 0.28 h⁻¹) metabolism of both strains was indistinguishable (Figs. 7.1A and 7.1B); O₂ consumption, CO₂ production and other metabolic fluxes were virtually identical. In both strains metabolism was completely oxidative and the respiratory quotient (RQ: ratio of carbon dioxide production to oxygen consumption) was close to unity. Above D = 0.28 h⁻¹ (the critical dilution rate) the parental strain switched from oxidative metabolism to respiro-fermentative metabolism as was apparent from the production of ethanol and the increase in RQ (results not shown and Fig. 7.1A). In contrast, in the *hxk2* null strain glucose metabolism remained fully oxidative (Fig. 7.1B). In the *hxk2* null strain respiro-fermentative metabolism seemed to commence at D = 0.35 h⁻¹. At this dilution rate, results were erratic; i.e. production of ethanol and increase in RQ was sometimes present and sometimes absent. By rinsing out the culture, the maximal specific growth rate of the *hxk2* deletion strain was found to be 0.45 h⁻¹, compared to 0.39 h⁻¹ for the wild-type strain.

The biomass yield of the wild-type strain was approximately 0.5 g biomass · [g glucose]⁻¹ up to the critical dilution rate (0.28 h⁻¹), at higher dilution rates the biomass yield decreased (Fig. 7.1A). In the *hxk2* null mutant the yield remained 0.5 g biomass·[g glucose]⁻¹ up to a D of 0.35 h⁻¹ (Fig. 7.1).

**Figure 7.1** Physiology of *S. cerevisiae* in aerobic glucose-limited chemostat cultures. The wild-type strain CEN.PK113-7D (A) and the *hxk2* null strain KY116 (B) were grown in aerobic glucose-limited continuous cultures. Biomass yield (●; in g biomass·[g glucose]⁻¹) and respiratory quotient (○, RQ = specific carbon dioxide production divided by the specific oxygen consumption) were determined at the various dilution rates. Lines are drawn to indicate possible trends.
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Effects of an hxxk2 null mutation on the internal properties of yeast in continuous cultures – Despite the comparable overall metabolism below the critical dilution rate of the wild-type strain, some internal properties of the cell were clearly different in the hxxk2 null strain compared to the wild-type strain. The concentration of some key internal metabolites around hexokinase were determined (Fig. 7.2). The intracellular concentration of ATP was higher in the wild-type strain at the lower dilution rates, yet decreased and became comparable to the hxxk2 null strain approaching the critical dilution rate. The intracellular concentrations of glucose-6-phosphate and fructose-6-phosphate were somewhat lower in the hxxk2 null strain at the lower dilution rates. In the wild-type strain the concentration of these compounds seemed to increase approaching the critical dilution rate, yet decreased again at higher dilution rates. In the wild-type strain the concentration of fructose-1,6 bisphosphate increased above the critical dilution rate, but remained constant in the hxxk2 null mutant.

In the wild-type strain the maximal specific in vitro hexose-phosphorylating activity with either fructose or glucose as a substrate remained relatively constant from low to high dilution rates (results not shown). Consequently the ratio of the maximal fructose- and glucose-phosphorylating activity remained constant (at a value of approximately 1.5) at all dilution rates (Fig. 7.3). In the hxxk2 null strain the fructose-phosphorylating activity increased at higher dilution rate in contrast to the glucose-phosphorylating activity (results not shown). In the hxxk2 null strain the ratio of fructose- and glucose-phosphorylating activity increased at higher dilution rates from 1.5 to 3-4, and only at the highest sampled dilution rates the ratio decreased to approximately 2 (Fig. 7.3). However, at the higher dilution rates results where difficult to reproduce for the hxxk2 null strain; different steady-states were obtained at the same dilution rate.

Effects of an hxxk2 null mutation on the in vivo dynamics – The steady-state of aerobic glucose-limited continuous cultures of the wild-type strain CEN.PK113-7D and the hxxk2 null strain was disturbed with a pulse of 20 mM glucose (final concentration in the culture vessel) at dilution rates ranging from D = 0.1 h⁻¹ to 0.35 h⁻¹. After the injection of glucose the wild-

![Figure 7.2 Intracellular concentrations of glycolytic intermediates in aerobic glucose-limited chemostat cultures of S. cerevisiae. The wild-type strain (A) and the hxxk2 null strain (B) were grown in aerobic glucose-limited chemostats at different dilution rates. Samples were taken at each dilution rate for the determination of intracellular metabolites. The intracellular concentrations of glucose-6-phosphate (■), fructose-6-phosphate (▲), fructose-1,6-bisphosphate (△), and ATP (□) are expressed in mM in the cytosol.](image-url)
Dynamic s of glycolysis in an \textit{hxxk2} mutant
type culture responded instantaneously with the production of ethanol (short-term Crabtree
effect) at all dilution rates (Figs. 7.4A and 7.4B). The transition from completely oxidative to
respiro-fermentative metabolism was apparent from the increase in RQ from unity (results
not shown). In contrast to the wild-type strain, the \textit{hxxk2} mutant did not show any ethanol
production in the first minutes after a glucose pulse, at dilution rates lower than $D = 0.2 \text{ h}^{-1}$
(e.g. Fig. 7.4). After this initial lag the conversion rates of glucose to ethanol, acetate,
pyruvate, or glycerol were comparable in wild-type and \textit{hxxk2} null strain. Also the specific
oxygen consumption rates, specific carbon dioxide production rates and consequently the
respiratory quotient were comparable for both strains (results not shown). Since samples
were not always taken exactly at the moment of glucose depletion the exact distribution of
the metabolic fluxes during the glucose pulse was estimated. From the pattern of metabolite
production it was estimated that at all dilution rates glucose was converted (on a C-molar-
basis) to approximately 40% biomass, 25% ethanol, 7% acetate and small amounts of
pyruvate and glycerol. Above the critical dilution rate the wild-type culture already contained
ethanol. This did not influence the metabolite production pattern of the glucose pulse to a
great extent; although the production of acetate was decreased.

**Figure 7.3** Ratio between fructose- and
glucose-phosphorylating activities in aerobic
glucose-limited chemostat cultures of \textit{S. cerevisiae}. The wild-type strain (O) and the \textit{hxxk2}
null strain (■) were grown in aerobic glucose-
limited chemostats at different dilution rates.
Maximal specific glucose- and fructose-
phosphorylating activities were determined from cell
free extracts at the different dilution rates. Values
are means of duplicate samples from steady state
chemostat cultures.

In contrast to the comparable flux distribution in the wild-type strain and the \textit{hxxk2} null strain
after a glucose pulse, the transient response of intracellular metabolites of the two strains was
clearly different (Fig. 7.4). In the wild-type strain, the intracellular concentrations of glucose-
6-phosphate, fructose-6-phosphate and most markedly fructose-1,6-bisphosphate showed a
sharp increase in the first minute after the glucose pulse, concomitant with a decrease in the
intracellular concentration of ATP. After this initial overshoot the concentration of glucose-6-
phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate relaxed to a new steady state
level (Figs. 7.4C-F). Compared with the steady level during glucose limitation, the steady-
state level after the glucose pulse was higher for fructose-1,6-bisphosphate and
approximately equal for glucose-6-phosphate, fructose-6-phosphate, and ATP. Surprisingly,
in the \textit{hxxk2} null strain the intracellular metabolites responded with a gradual approach to a
new steady state level. The concentration of glucose-6-phosphate, fructose-6-phosphate, and
fructose-1,6-bisphosphate increased while the concentration of ATP remained constant. The
steady-state levels of glucose-6-phosphate, fructose-6-phosphate, and especially fructose-1,6-
bisphosphate were higher during the glucose pulse in the wild-type strain compared to the
\textit{hxxk2} null strain at all dilution rates.

At all dilution rates, both in the wild-type strain and the \textit{hxxk2} null strain, glycolytic
intermediates were drained as the cultures approached glucose depletion, as was evidenced
by the decrease in the concentration of glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate. Only the concentration of ATP remained around the steady state level.

**Figure 7.4** Transient responses of *S. cerevisiae* after a shift from glucose limitation to glucose excess at $D = 0.15 \text{ h}^{-1}$. The wild-type strain (A, C & E) and the *hxk2* null strain (B, D & F) were grown in aerobic glucose-limited chemostats at different dilution rates. Samples were taken at each dilution rate for the determination of extracellular (A and B) and intracellular metabolites (C and D). The initial responses of the internal metabolites are depicted in E an F (enlargement of C and D). Glucose (●), ethanol (○), and acetate (*) are expressed in mM in the culture vessel. The intracellular concentrations of glucose-6-phosphate (■), fructose-6-phosphate (▲), fructose-1,6-bisphosphate (Δ), and ATP (□) are expressed in mM in the cytosol. $D = 0.15 \text{ h}^{-1}$ is chosen as representative of all other dilution rates. Lines are drawn to indicate possible trends.

**Anaerobic growth** – To study whether the change towards oxidative metabolism in the *hxk2* mutant under aerobic glucose limited conditions implied impaired anaerobic growth and
Dynamics of glycolysis in an hxx2 mutant

metabolism, the wild-type and hxx2 mutant were cultivated in anaerobic glucose limited cultures. Both strains displayed identical metabolic fluxes (data not shown) at all dilution rates tested (0.1 to 0.25 h⁻¹). The similar fluxes suggest that the hxx2 mutant is capable of anaerobic glucose metabolism to the same extent as the wild-type.

7.5 Discussion

In previous investigations it was shown that some specific properties of glycolysis are affected by the deletion of HXXK2 during batch growth at excess glucose (e.g.: Chapter 5 and 6 and refs. (45, 168)). In particular, invertase, pyruvate decarboxylase, high-affinity glucose transport, mitochondrial activities and the concentration of fructose-1,6-bisphosphate are influenced by an hxx2 null mutation. It can be concluded that glucose repression is reduced in an hxx2 null mutant under repressing conditions. However, wild-type cells in a glucose-limited environment are non-repressed. The residual glucose concentrations in the wild-type culture and the culture of the hxx2 mutant were too low to be detected at any dilution rate, except for the wild strain above the critical dilution rate (results not shown, see Chapter 2); accordingly, glucose repression was absent under these conditions. During oxidative growth in glucose-limited cultures the wild-type strain and the hxx2 null strain showed equal biomass yields and RQ-values. In spite of the similar metabolic fluxes, different intracellular concentrations of metabolites in the wild-type strain and the hxx2 null strain at the different dilution rates were found. As has been shown before (e.g. (169)), comparable fluxes do not necessarily result in comparable intracellular metabolite concentrations. In fact, the different intracellular metabolite concentrations may reflect: i) the absence of hexokinase II via the different kinetic properties of the glucose-phosphorylating enzymes (59, 113), ii) the specific inhibitory characteristics of trehalose-6-phosphate towards hexokinase II activity (17), or iii) the inhibition of hexokinase II by high concentrations of ATP (91). Differences in the in vitro specificity of the hexose-phosphorylating activities between the glucose-limited cultures of the wild-type strain and the hxx2 null strain were clear from the ratio between the maximal fructose- and glucose-phosphorylating activities (Fig. 7.3), which reflect the differential expression of the genes encoding hexose-phosphorylating enzymes. Glucokinase does not phosphorylate fructose at all, a ratio of 3 is typical for hexokinase I and a ratio of 1.2 is typical for hexokinase II (72). In the hexokinase II deletion strain the ratio of the maximal specific fructose- and glucose-phosphorylating activity increased at higher dilution rates, suggesting an increased contribution of hexokinase I at the higher dilution rates. In contrast, in the wild-type strain the ratio of maximal specific fructose- and glucose-phosphorylating activity remained approximately constant at 1.5, which suggests an important contribution of hexokinase II at all dilution rates. Transcription of genes encoding the three hexose-phosphorylating enzymes was shown to be differentially regulated depending on the carbon source and growth condition (72, 196). In aerobic glucose-limited cultures of a wild-type S. cerevisiae strain it was shown that the transcription of HXX2 increased, while the transcription of HXX1 decreased with increasing dilution rate, yet the specific hexose-phosphorylating activity remained relatively constant (196).

We suggest that the difference in intracellular metabolites, in spite of the comparable metabolic fluxes, is due to the difference in specificity of the hexose-phosphorylating step in the wild-type and the hxx2 null strains. The different intracellular metabolite concentrations (especially intracellular glucose (see: (204))) might affect the
transcriptional regulation of enzymes further down glycolysis. In spite of the non-repressed conditions, glucose repression signals might still be present (to a lesser extent) and regulate gene expression.

Wild-type yeast cells respond rapidly to changes in the environment, as is apparent from the instant production of ethanol (short-term Crabtree-effect) after a transition from glucose limitation to glucose excess (this study, (160, 208, 226)). This fast response seems to be a consequence of a limited respiratory capacity (160) or, as suggested by others, the characteristics of hexose transport, the ability to synthesize reserve carbohydrates, or the pyruvate decarboxylase activity (226).

In the wild-type, the transition from glucose limitation to glucose excess resulted in a sharp drop in the intracellular ATP concentration immediately after the glucose pulse, at all dilution rates (Fig. 7.4E). As was suggested before, this is a result of an initial imbalance of the ATP consuming steps and the ATP producing steps (208), which is apparent from the sharp increase in glucose-6-phosphate and fructose-6-phosphate. Only after some minutes, when the lower part of glycolysis has been saturated, the internal metabolites relax to a new steady state not far from the glucose-limited steady-state (with the exception of the higher intracellular concentration of fructose-1,6-bisphosphate). In contrast, in the hxxk2 null mutant, the concentration of ATP remained at the glucose-limited steady-state level (Figs. 7.4D and 7.4F). The concentration of glucose-6-phosphate and fructose-6-phosphate showed a small initial overshoot before settling into a steady-state, which was again not far from the steady state under glucose limitation. Previous observations on limit-cycle oscillations in glycolysis already suggested an influence of the hexokinase reaction on the dynamic behavior of glycolysis (176). The results in the present study suggest that the decreased hexose-phosphorylating activity in the hxxk2 mutant results in a slower ATP consumption and consequently an absence of imbalance between the upper and lower parts of glycolysis. Instead, in the HXX2 null mutant glycolysis gradually fills up after a transition from glucose limitation to glucose excess.

In spite of the differences in intracellular metabolite concentrations between the strains (especially ATP and glucose-6-phosphate, a substrate and a product of the hexokinase reaction, respectively), alcoholic fermentation was not considerably influenced by the deletion of HXX2. In addition, overproduction of the separate enzymes of glycolysis does not significantly increase the glycolytic flux (188). This indicates that glycolysis is strongly regulated by allosteric interactions. The concentration of fructose-1,6-bisphosphate, which is clearly different in the wild-type strain and the hxxk2 null strain both under glucose-limited conditions and under conditions of glucose excess, is a good candidate as an important allosteric regulator of glycolysis under these conditions (e.g. as activator of pyruvate kinase). The differences in fructose-1,6-bisphosphate concentration might be an indirect consequence of the change in kinetic properties of the hexose-phosphorylating step. Homeostatic control of for instance the concentrations of adenine nucleotides, might result in changed intracellular metabolite pools further down glycolysis as response to the shifted demands.

In batch cultures under conditions of glucose excess the deletion of HXX2 results in a redirection of the metabolic flux to biomass and an initial absence of ethanol production (see Chapter 6). We concluded that this redirection was a consequence of a reduction in glucose repression. Under truly derepressed conditions (glucose-limited cultivation), both the
wild-type and *hxk2* mutant are capable of alcoholic fermentation. This contraction suggests that under conditions of glucose excess an active up-regulation of mitochondrial activities, hexose-transport properties (see Chapter 5), or other enzymatic activities (e.g. at the level of pyruvate) might be involved in the altered metabolism of the *hxk2* null mutant.

**Concluding remarks** – The *hxk2* mutant does not behave as a truly Crabtree-negative yeast. For Crabtree-negative yeast’s it was shown that the transition from glucose limitation to glucose excess results in increased biomass production rather than alcoholic fermentation. This was attributed to differences in the reserve carbohydrates, differences in the kinetics of glucose transport, and differences in pyruvate metabolism (226). From the present study it can be concluded that the flux through glycolysis after a transition from glucose limitation to glucose excess is not (additionally) limited in the *hxk2* null mutant, i.e. ethanol production is not considerably influenced. However, the instantaneous production of ethanol is delayed in the *hxk2* null mutant. This is very appealing for the industrial production of yeast biomass; the *hxk2* null mutant seems less vulnerable to sugar gradients (which occur in large-scale fermentation processes). Still, the mutant is able to produce ethanol (and carbon dioxide) to the full extent under anaerobic conditions, which is an important property for the application of yeast in dough. In addition, the higher critical dilution rate of the *hxk2* deletion strain reveals an enhanced oxidative capacity, which enables acceleration of the production process without the formation of the undesired ethanol. The unique phenotype of the *hxk2* mutant strain in chemostat cultures can contribute to a large extent in understanding the regulation of the distribution of fluxes in *S. cerevisiae*. If e.g., DNA arrays or 2D protein gels reveal what genes are affected in the *hxk2* mutant during growth rates higher than the critical dilution rate and maximal growth rate of the wild-type, we might understand why and how it manages to grow faster and has a higher oxidative capacity than its parent. Accordingly, a more focussed approach can be undertaken to construct strains with particular controlled and redirected fluxes by directed genetic changes.

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