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Research Article

Co-consumption of sugars or ethanol and glucose in a *Saccharomyces cerevisiae* strain deleted in the *HXK2* gene

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Abstract

In previous studies it was shown that deletion of the *HXK2* gene in *Saccharomyces cerevisiae* yields a strain that hardly produces ethanol and grows almost exclusively oxidatively in the presence of abundant glucose. This paper reports on physiological studies on the *hxk2* deletion strain on mixtures of glucose/sucrose, glucose/galactose, glucose/maltose and glucose/ethanol in aerobic batch cultures. The *hxk2* deletion strain co-consumed galactose and sucrose, together with glucose. In addition, co-consumption of glucose and ethanol was observed during the early exponential growth phase. In *S. cerevisiae*, co-consumption of ethanol and glucose (in the presence of abundant glucose) has never been reported before. The specific respiration rate of the *hxk2* deletion strain growing on the glucose/ethanol mixture was 900 $\mu mol \cdot min^{-1} \cdot (g\ protein)^{-1}$, which is four to five times higher than that of the *hxk2* deletion strain growing oxidatively on glucose, three times higher than its parent growing on ethanol (when respiration is fully derepressed) and is almost 10 times higher than its parent growing on glucose (when respiration is repressed). This indicates that the *hxk2* deletion strain has a strongly enhanced oxidative capacity when grown on a mixture of glucose and ethanol.

Keywords: yeast (*Saccharomyces cerevisiae*); hexokinase II; glucose repression; mixed sugar metabolism

Introduction

Saccharomyces cerevisiae or baker's yeast ferments under aerobic conditions and is therefore a Crabtree-positive yeast. Aerobic fermentation is a consequence of a phenomenon called glucose repression. In the presence of high concentrations of glucose the expression of genes involved in the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, glyoxylate cycle, gluconeogenesis and metabolism of sugars other than glucose is repressed (for recent reviews, see Carlson, 1998; Gancedo, 1998). Additionally, the expression of genes involved in alcoholic fermentation is induced. This regulatory mechanism in *S. cerevisiae* results in the preferential consumption of glucose over other carbon sources. Thus, when *S. cerevisiae* grows on a mixture of glucose and another carbon source such as sucrose, maltose, galactose or ethanol, growth is diauxic, i.e. glucose is metabolized first, whereas the other carbon sources are not metabolized until glucose is exhausted. Only after all sugars have been depleted is the ethanol produced during the fermentation of the sugars consumed.

S. cerevisiae contains three distinct hexose-phosphorylating enzymes that catalyse the first step of glycolysis: hexokinase I (encoded by *HXK1*), hexokinase II (encoded by *HXK2*) and glucokinase (encoded by *GLK1*). Hexokinase I and II phosphorylate fructose as well as mannose and glucose, whereas glucokinase is only able to phosphorylate glucose and mannose. Each isozyme

has a different affinity for glucose and ATP and is subject to a different transcriptional regulation mechanism, depending, among other things, on the concentration and the kind of carbon source available (Herrero *et al.*, 1995). Unlike most other species, the activity of the hexokinases in *S. cerevisiae* is not inhibited by their metabolic product, glucose-6-phosphate, but appears to be regulated by trehalose-6-phosphate, which is assumed to regulate the flux through the upper part of glycolysis (Teusink *et al.*, 1998; Thevelein and Hohmann, 1995).

In many previous studies, it has been shown that hexokinase II is involved in glucose repression (for reviews, see Carlson, 1998; Gancedo, 1998). Nevertheless, the exact role of hexokinase II in glucose repression is still a matter of debate. On the one hand, it is thought that deletion of *HXK2* causes relief of glucose repression as a consequence of the reduced phosphorylation capacity (Ma *et al.*, 1989; Rose *et al.*, 1991); on the other hand, a direct regulatory role has been ascribed to hexokinase II (Entian and Frohlich, 1984; Entian *et al.*, 1985).

In favour of the last proposal, hexokinase II is a phosphoprotein *in vivo* (Vojtek and Fraenkel, 1990) which can exist in either a dimeric or monomeric form. The oligomerization state of hexokinase II shifts to the monomeric state by phosphorylation which is initiated at low glucose (Behlke *et al.*, 1998). The phosphorylated form of hexokinase II was recently shown to enter the nucleus (Randez-Gil *et al.*, 1998); the nuclear protein participates in a DNA–protein complex, which regulates repression of at least the *SUC2* gene by glucose (Herrero *et al.*, 1998). The phosphorylation of hexokinase II at low glucose (Behlke *et al.*, 1998) contradicts the results that show that phosphorylation of this enzyme is necessary to enter the nucleus and initiate glucose repression (Herrero *et al.*, 1998; Kraakman *et al.*, 1999). At this moment, the exact mechanism by which hexokinase II enters the nucleus and causes repression of at least *SUC2* is unclear.

Early characterization of a *S. cerevisiae* strain deleted in the *HXK2* gene showed that the absence of hexokinase II relieved glucose repression of the indicator gene *SUC2*, encoding invertase (Entian, 1980; Ma *et al.*, 1989; Michels *et al.*, 1983). Further, the expression of the genes encoding the maltose transporter and maltase (Zimmermann and Scheel, 1977) and the genes encoding the high-affinity glucose transporters Hxt2p and Hxt7p were shown to be derepressed (Wendell and Bisson, 1994; Petit *et al.*, 2000). DNA arrays performed on an *hxk2* deletion strain grown with excess glucose showed clearly that the expression of many genes involved in glucose-repressible routes, such as the TCA cycle, glyoxylate cycle, oxidative phosphorylation (e.g. *SDH4*, a subunit in the succinate dehydrogenase complex, 2.7; *ICL1*, isocitrate lyase, 2.2; *ATP3*, F_1-γ ATP synthase, 2.1 times overexpressed) and the consumption of sugars other than glucose were upregulated (e.g. *MAL32*, maltase, 2.3 times), whereas the expression of those gene products involved in glycolysis were downregulated (e.g. *GPM1*, phosphoglycerate mutase, 2.1; *ENO2*, enolase 2, 1.9 times). Accordingly, the *hxk2* deletion strain displayed an increased flux through the TCA cycle and oxidative phosphorylation in the presence of excess glucose. The flux through glycolysis and the fluxes to ethanol and glycerol were decreased, resulting in almost exclusively oxidative growth and thus diminished ethanol production (Diderich *et al.*, 2001). As a consequence, the growth yield of the *hxk2* deletion strain during aerobic growth on glucose is much higher than that of its parent strain, since fermentation of glucose to ethanol yields only two ATPs per glucose consumed, whereas complete oxidation of glucose to carbon dioxide and water yields roughly 20 ATPs.

The derepression of the genes encoding enzymes involved in maltose metabolism (*MAL* genes) and sucrose metabolism (*SUC2*) triggered the question of whether the *hxk2* deletion strain would be capable of consuming sucrose and maltose in the presence of abundant glucose. Therefore, in continuation of the physiological characterization of the *hxk2* deletion strain, the performance of this strain was studied on mixed carbon sources, i.e. mixtures of glucose with either sucrose, galactose, maltose or ethanol, both on plates and in batch cultures.

Materials and methods

Strains

The wild-type *S. cerevisiae* strain, X2180-1A (*MAT***a** *SUC2 mal mel*), was obtained from the Yeast Genetic Stock Center (Berkeley, CA). The prototrophic *S. cerevisiae* wild-type strain, CEN.PK113-7D (*MAT***a** *MAL2-8^c SUC2*), was obtained from P. Kötter, Johann Wolfgang Goethe Universität

(Frankfurt, Germany). The *HXK2* gene was deleted in CEN.PK113-7D to create strain KY116 as follows: using primers AK53 (GTTGTAGGAATA TAATTCTCCACACATAATAAG TACGCTAA TTCGTACGCTGCAGGTCGAC) and AK54 (AA AAGGGCACCT TCTTGTTGTTCAAACTTAA TTTACAAATTAAGTATCGATGAATTCGAGC TCG), the kanMX cassette of plasmid pFA6a–kanMX4 (Wach *et al*., 1994) 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 (Gietz and Schiestl, 1995). After 2 h cultivation in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose), the transformed cells were plated on solid YPD medium (2% w/v agar) containing G418 (200 μg/ml) and incubated at 30°C. 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 (GACGAAA TACGCGATCGCTGT) and AK61 (GCCGAA CATTTCAAAGTCAACC), as recommended by the manufacturer (Stratagene).

Plate assays

Solid medium contained 2% w/v of each carbon source and 1 mM 5-thio-D-glucose in YNB medium, which consisted of 0.17% w/v Yeast Nitrogen Base without amino acids and ammonium sulphate (Difco) and 0.5% $(NH_4)_2SO_4$. The strain X2180 was supplemented with casamino acids.

Co-consumption batch experiments

The yeast strains were cultivated in batch fermentors at 30°C. The cells were pregrown in 20 ml medium, which contained 1% w/v glucose, 0.17 % w/v Yeast Nitrogen Base without ammonium sulphate and amino acids (Difco), 0.5% $(NH_4)_2SO_4$ and 100 mM potassium phthalate, pH 5.0. The next day, the fermentors were inoculated containing 1 l 1% w/v fresh medium with glucose, plus 0.4% w/v sucrose, 0.4% w/v galactose, 0.4% w/v maltose or 100 mM ethanol. The *hxk2* mutant and the isogenic parent strain were cultivated on each of these carbon mixtures. To exclude the possibility that the onset of fermentation is caused by oxygen limitation, the fermentors were aerated at 1 vessel volume/min and stirred at 1000 rpm. The cells were grown overnight and samples were taken the next day during and beyond the exponential phase. The specific growth rate and the growth phase were determined by measuring the optical density at 600 nm.

Extracellular metabolites were measured by spinning down 1 ml culture and injecting the supernatant into 100 μl 35% PCA (at 0°C). After 15 min, part of the PCA was precipitated by adding 55 μl 7 N KOH. After centrifugation, the supernatant was filtered and analysed for glucose, ethanol, glycerol, acetate and pyruvate by HPLC (column, Phenomenex type Rezex Organic Acid; eluent, 7.2 mM H_2SO_4 at 40°C).

Oxygen consumption and carbon dioxide production were determined by passing the gas from the fermentor through an oxygen analyser (Taylor Servomex Type OA 272) and a carbon dioxide analyser (Servomex IR Gas Analyser PA 404). The protein content of the culture was measured according to Lowry *et al*. (1951) using bovine serum albumin (fatty acid-free) as a standard and measured on a COBAS-FARA automatic analyser (Roche).

The RQ equals the quotient of CO_2 production to O_2 consumption and is calculated to give an indication of the distribution of the carbon fluxes over the fermentative and oxidative pathways. When *S. cerevisiae* completely oxidizes a fermentable carbon source to CO_2 and water, $RQ=1$ (for each molecule of CO_2 produced, one molecule of O_2 is consumed). When a fermentable carbon source is partially fermented and partly oxidized, the RQ will reach values (much) larger than 1. When *S. cerevisiae* grows on ethanol the RQ has the specific value of 0.67 (Locher *et al*., 1993).

Results

Glucose repression through *HXK2*, revisited

The glucose analogue 5-thioglucose is known to induce glucose repression in *S. cerevisiae,* yet cannot be metabolized after it has been taken up by the cell (Egilsson *et al*., 1986). Consequently, a wild-type *S. cerevisiae* strain will not be able to grow on medium containing 5-thioglucose plus another carbon source, such as sucrose, galactose, maltose or ethanol, since the required pathways and enzymes are repressed. In Figure 1A, it can clearly be seen that the isogenic wild-type strain (*HXK2*), in the presence of 5-thioglucose, has a glucose-repressed metabolism because it cannot consume

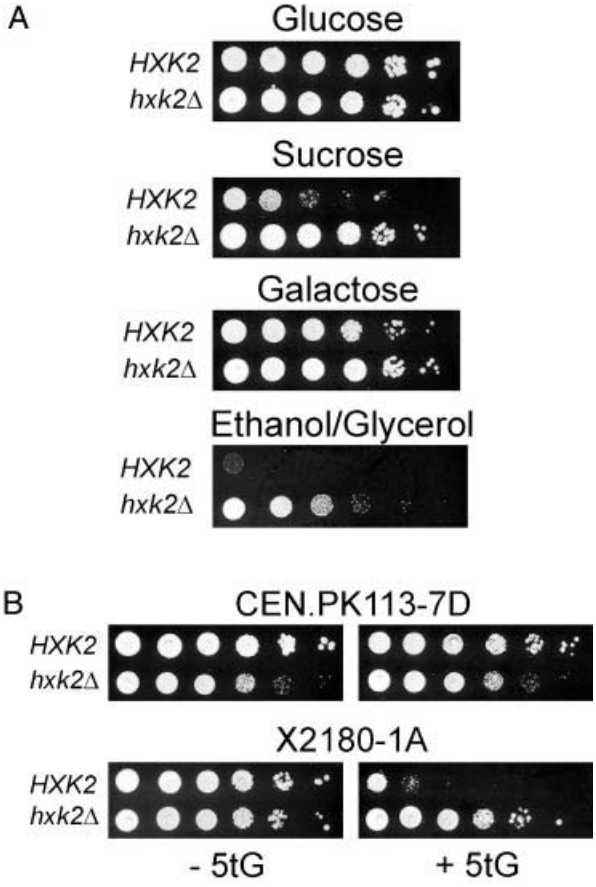

Figure 1. Growth on plates containing various carbon sources in the presence or absence of 5-thioglucose. Growth on glucose, sucrose, galactose or ethanol/glycerol of the wild-type strain, CEN.PK113-7D (*HXK2*) and the *hxk2* deletion strain (*hxk2*Δ) derived from it, in the presence of 5-thioglucose (A). Growth on maltose of the wild-type strain CEN.PK113-7D (*HXK2*) and its *hxk2* deletion strain (*hxk2*Δ) compared to the wild-type strain, X2180-1A (*HXK2*), and the *hxk2* deletion strain (*hxk2*Δ) in the presence (+5tG) or absence (−5tG) of 5-thioglucose (B)

either sucrose or ethanol/glycerol in the medium, whereas glucose can still be consumed. Galactose consumption does not seem to be strongly glucose repressed in the parent strain, since quite some growth appeared on galactose in the presence of 5-thioglucose. The expression of genes whose products are involved in galactose metabolism are not only regulated by glucose repression but are also induced by galactose (Oshima, 1982). Possibly, the induction of the galactose metabolism by high galactose overrules the induced glucose repression by the 5-thioglucose. Due to the constitutive expression of the *MAL* genes in the CEN.PK113-7D strain, growth of the parent strain on maltose in the presence of 5-thioglucose was not inhibited (Figure 1B). Further, growth on maltose in the presence or absence of 5-thioglucose was reduced in the *hxk2* deletion strain compared to its parent (Figure 1B). In another background strain, X2180-1A, the *MAL* genes seems glucose-repressible and, accordingly, growth on maltose is impaired in the presence of 5-thioglucose, whereas in the absence of 5-thioglucose growth in both the mutant strain and its parent is restored. These results show that this phenotype is strain-dependent (Figure 1B).

The *hxk2* deletion strain is capable of growth on all carbon sources tested in the presence of 5-thioglucose (Figures 1A, B). Thus, the enzymes involved in the metabolism of sucrose, galactose, maltose and ethanol/glycerol were expressed in the *hxk2* deletion strain in the presence of 5-thioglucose. This is confirmed by the derepressed levels of invertase, galactose permease, maltase and maltose permease in the *hxk2* deletion strain (Entian, 1980; Entian *et al.*, 1977; Michels *et al.*, 1983). However, these data show that genes involved in ethanol/glycerol metabolism are expressed as well. Additionally, DNA arrays performed on the *hxk2* deletion cells grown on glucose showed that *ALD7* (aldehyde dehydrogenase) and *ACSI* (acetyl-coA synthetase), both involved in ethanol metabolism, are upregulated 2.6 and 1.7, respectively, in the mutant. Surprisingly, *ADH2* (alcohol dehydrogenase), whose product catalyses the conversion of alcohol to acetaldehyde, was downregulated by a factor of 1.3 compared to the wild-type strain, suggesting that ethanol needs to be present for the upregulation of *ADH2*. The same is true for the gluconeogenic enzyme fructose-1, 6-bisphosphatase, which is not expressed in the *hxk2* deletion strain growing on glucose but which must be expressed on ethanol/glycerol plus 5-thioglucose to form sugar phosphates from either glycerol or ethanol for anabolism.

Deletion of *HXK2* enhances oxidative metabolism; co-consumption of glucose and sucrose

The physiology of the *hxk2* deletion strain co-consuming glucose with either sucrose, galactose, maltose or ethanol was studied in greater detail and more quantitatively in the CEN.PK

background in aerated batch fermentors. To obtain a better insight in the distribution of the carbon fluxes over the fermentative and the oxidative routes, *RQ* was calculated during the entire batch growth.

The CO_2 production, O_2 consumption, and *RQ* of the parent strain grown in an aerated batch fermentor on a mixture of glucose and sucrose were measured in time where $t=0$ is the time of inoculation (Figure 2A). Growth on glucose yielded an *RQ* of approximately 9, confirming repressed growth. After 20.5 h, glucose had been completely consumed and the shift to growth on sucrose was made, as can be observed from the small decrease in CO_2 production and O_2 consumption rates. Subsequently, the value of *RQ* was about 6, indicating fermentative growth and thus repressed metabolism, but more derepressed compared to growth on glucose. Growth on sucrose was rather brief (1–2 h), since the biomass concentration was high and the original sucrose concentration added to the medium was relatively low. After approximately 22 h, the sucrose was exhausted and the shift to growth on ethanol set in. Accordingly, the CO_2 production, and to a lesser extent the O_2 consumption rates, collapsed. Within a few hours metabolic fluxes were re-established with an *RQ* of 0.6, which is characteristic for growth on ethanol. In the last phase of growth, at approximately 40 h, the *RQ* rose from 0.6 to 1, which is indicative for growth on acetate after the ethanol has been exhausted. After 42 h the *RQ* values scattered as growth had ended

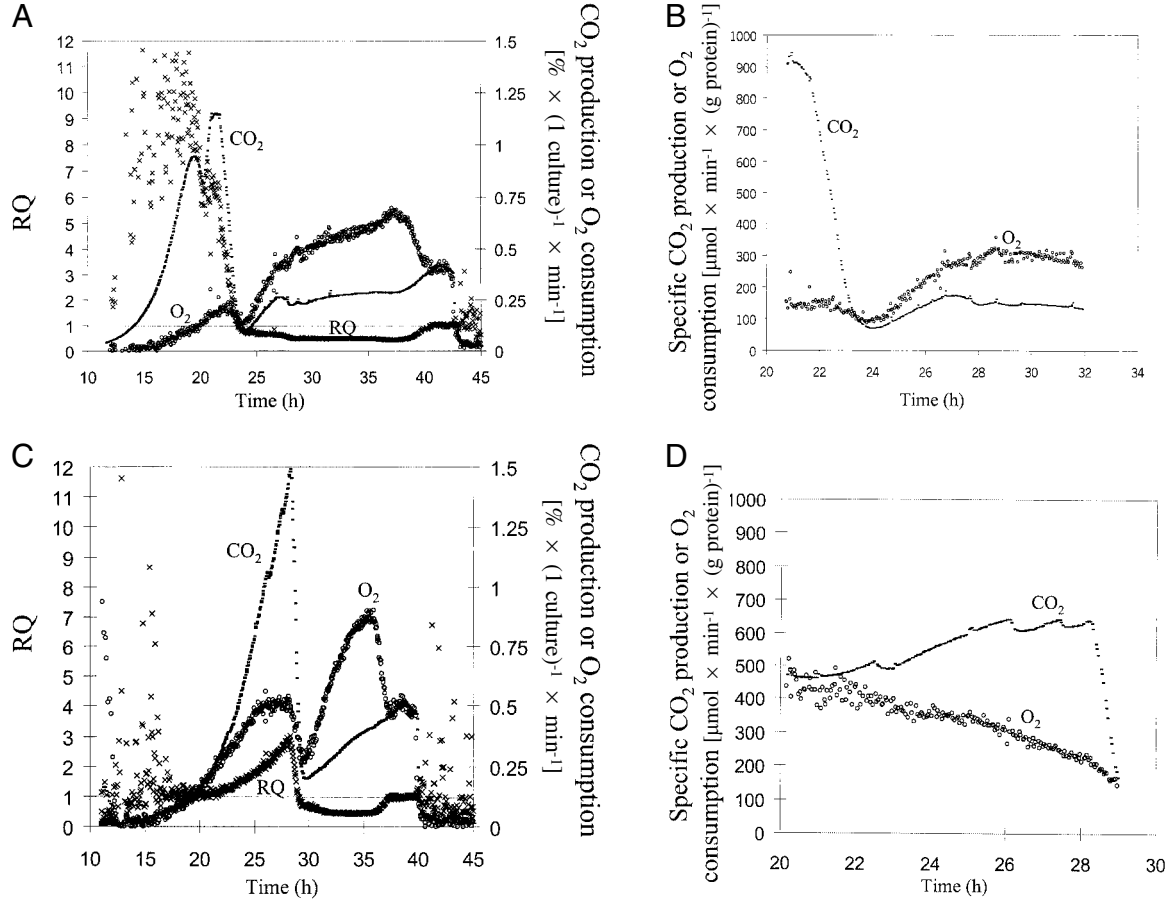

Figure 2. The gas-profiles of the wild-type strain and hxk2 deletion strain growing in batch culture on a mixture of glucose and sucrose. The respiratory quotient (*x*) ($RQ=CO_2/O_2$), the CO_2 production rate (—) and the O_2 consumption rate (*o*) [%gas × min^{-1} × (l culture)$^{-1}$] in the wild-type strain (A) and the *hxk2* deletion strain (C). The specific CO_2 production (—) and O_2 consumption (*o*) [µmol × min^{-1} × (g protein)$^{-1}$] of a part of the growth curve of the wild-type strain (B) and the *hxk2*deletion strain (D)

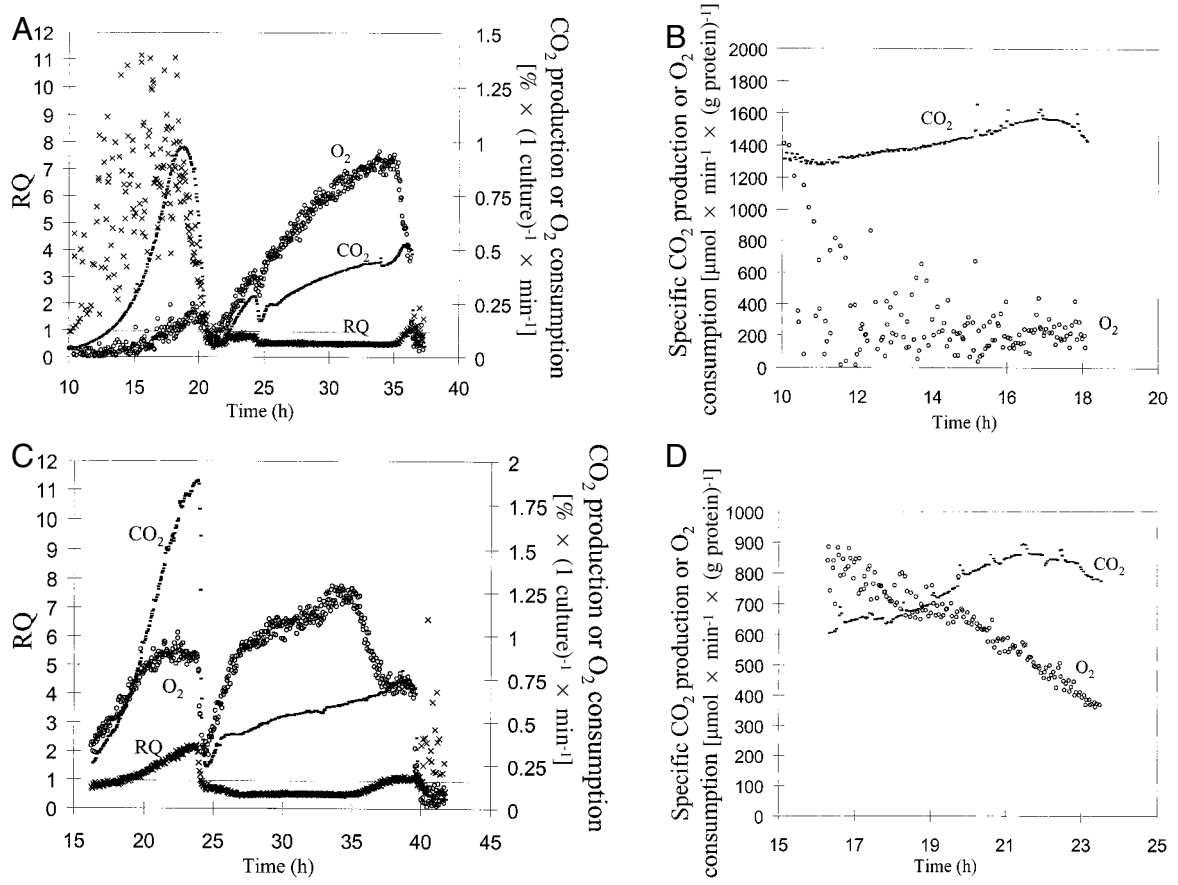

Figure 3. The gas-profiles of the wild-type strain and hxk2 deletion strain growing in batch on a mixture of glucose and ethanol. The respiratory quotient (*x*) ($RQ = CO_2/O_2$), the CO_2 production rate (−) and the O_2 consumption rate (*o*) [%gas × min^{-1} × (l culture)$^{-1}$] in the wild-type strain (A) and the *hxk2* deletion strain (C). The specific CO_2 production (−) and the specific O_2 consumption (*o*) [µmol × min^{-1} × (g protein)$^{-1}$] of a part of the growth curve of the wild-type strain (B) and the *hxk2*deletion strain (D)

and the O_2 consumption was almost zero. The scatter of the *RQ* within the first 20 h was also caused by the low oxygen consumption rate, due to glucose repression in combination with a low biomass concentration.

The *RQ* profile, the CO_2 production rate and O_2 consumption rate in the *hxk2* deletion strain are very different from the wild-type (cf. Figures 2A, C). First, the gas data reveal no discernible shift from glucose to sucrose metabolism, suggesting that the sucrose is simultaneously metabolized with the glucose. Figure 4B indeed shows that sucrose is already hydrolysed by derepressed invertase activity to glucose and fructose. The fructose formed was not metabolized until practically all the glucose was consumed. This is not caused by glucose repression but is a consequence of the lower affinity of the hexose transporters for fructose than for glucose (Reifenberger *et al.*, 1995). The parent strain, however, does not hydrolyse the sucrose until glucose levels have dropped strongly and derepressed invertase expression (Figure 4A).

Second, the growth of the *hxk2* deletion strain on ethanol (approximately 30–37 h) takes only half the time of that of the parent strain (24–38 h) (cf. Figures 2A, 2C). This is caused by the much lower ethanol production during the predominantly oxidative growth on glucose and sucrose by the *hxk2* deletion strain, whereas the wild-type strain converts glucose mainly to ethanol (see also Diderich *et al.*, 2001). In the first 22 h, the *RQ* of the *hxk2* deletion strain was close to 1, which is characteristic

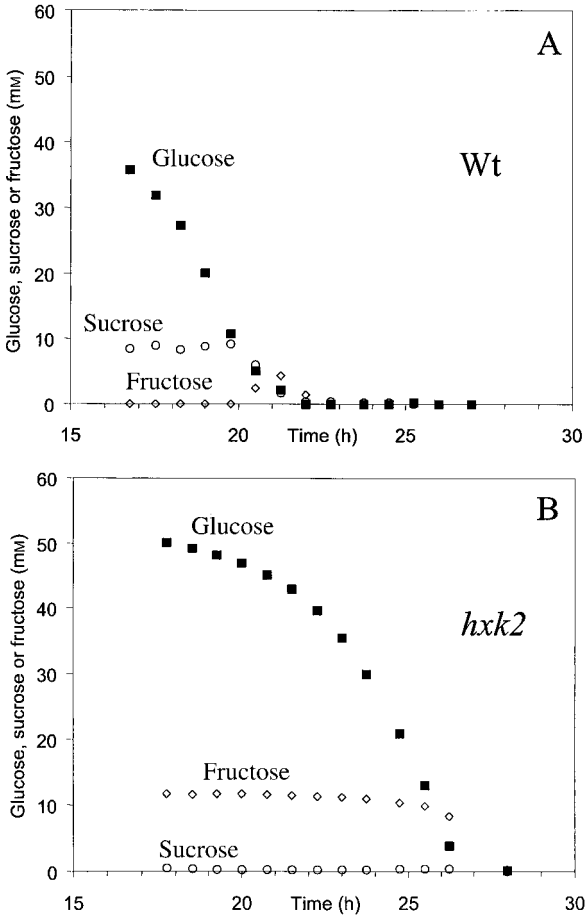

Figure 4. Growth on a mixture of glucose and sucrose. The extracellular glucose (■), sucrose (o) and fructose (◇) concentrations (mM) during growth of the wild-type strain (A) and the *hxk2* deletion strain (B)

of fully respiratory growth (Figure 2C). Then, when the *RQ* rose above 1, fermentation set in at around 23 h and the *RQ* rose above 1 and gradually increased further to almost 3 before glucose and sucrose were exhausted at 28 h.

The specific respiration rates of the parent strain (Figure 2B) and *hxk2* deletion strain (Figure 2D) during exponential growth on glucose (and sucrose) as carbon source, show that the highest specific CO_2 production rate is much higher in the parent than in the mutant, 900 μmol $CO_2 \times min^{-1} \times (g\ protein)^{-1}$ and 600 μmol $CO_2 \times min^{-1} \times (g\ protein)^{-1}$ respectively. The specific oxygen consumption is approximately 150 μmol $O_2 \times min^{-1} \times (g\ protein)^{-1}$ for the parent and maximally 450 μmol $O_2 \times min^{-1} \times (g\ protein)^{-1}$ for the *hxk2* deletion strain.

Co-consumption of glucose and galactose or maltose

Growth of both the parent and the *hxk2* deletion strain growing batch-wise on glucose plus galactose essentially showed the same *RQ*, CO_2 and O_2 profile as Figures 2A and 2C (results not shown). In the *hxk2* deletion strain the co-consumption of glucose and galactose was obvious: the extracellular galactose concentration was already decreasing when glucose was still abundant (Figure 5B). In the parent first the glucose was exhausted (at 18 h), after which the galactose was consumed (Figure 5A). At 23 h the galactose was exhausted and the shift to growth on ethanol set in.

The parent strain grew without any difficulty on a mixture of glucose and maltose; first the glucose was consumed and then the maltose was taken up and hydrolysed to glucose (Figure 6A). The *hxk2* deletion strain, however, grew poorly on a mixture of maltose and glucose (or maltose alone), with a μ_{max} of $0.12\ h^{-1}$ instead of $0.32\ h^{-1}$ on other fermentable carbon sources (data not shown). Nevertheless, maltose concentrations decreased, indicating uptake and intracellular hydrolysis of maltose (Figure 6B). Simultaneously, the extracellular glucose levels increased slightly at around 16–18 h, indicating that the maltose was hydrolysed faster than it was metabolized, resulting in the export of intracellular glucose into the medium by the available hexose transporters. After all the maltose had been hydrolysed, the glucose was consumed next and growth was restored. The poor growth in the presence of maltose was not due to ATP depletion as a consequence of uncontrolled active transport of maltose over the membrane, since the intracellular ATP concentration was approximately 8 mM and the ATP : ADP ratio approximately 5.

Co-consumption of glucose and ethanol

On a mixture of glucose and ethanol the parent consumed the glucose first and converted the glucose mainly to ethanol; only after glucose exhaustion was ethanol consumed (Figures 3A, 7A). Interestingly, in the early to mid-exponential stages of growth, the ethanol concentration in the medium of the *hxk2* deletion strain decreased (Figure 7B). This suggests consumption of ethanol, but might be an artefact caused by evaporation of ethanol in the vigorously aerated fermentor.

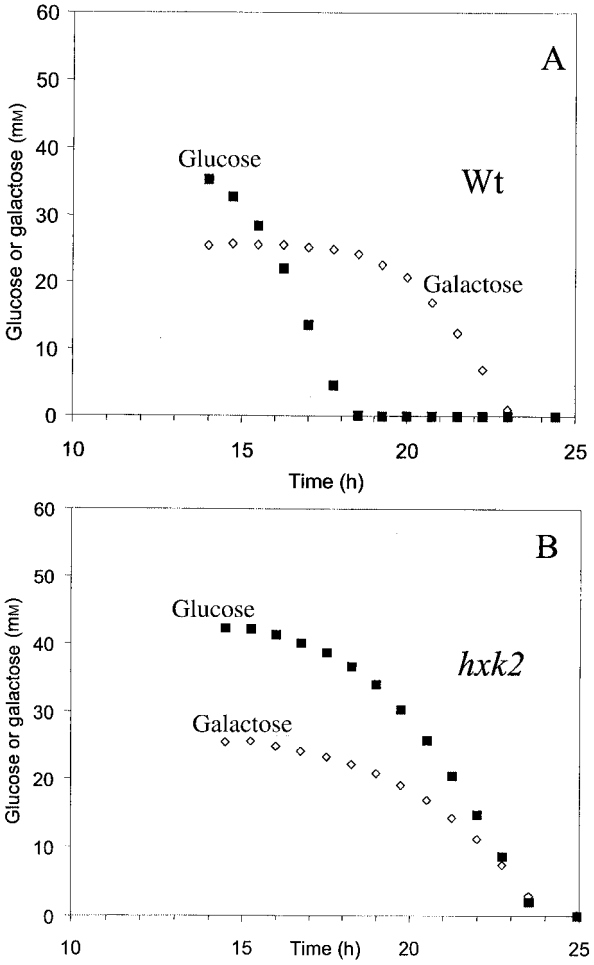

Figure 5. Growth on a mixture of glucose and galactose. The extracellular glucose(■) and galactose (◇) concentrations (mM) during growth of the wild-type strain (A) and the *hxk2* deletion strain (B)

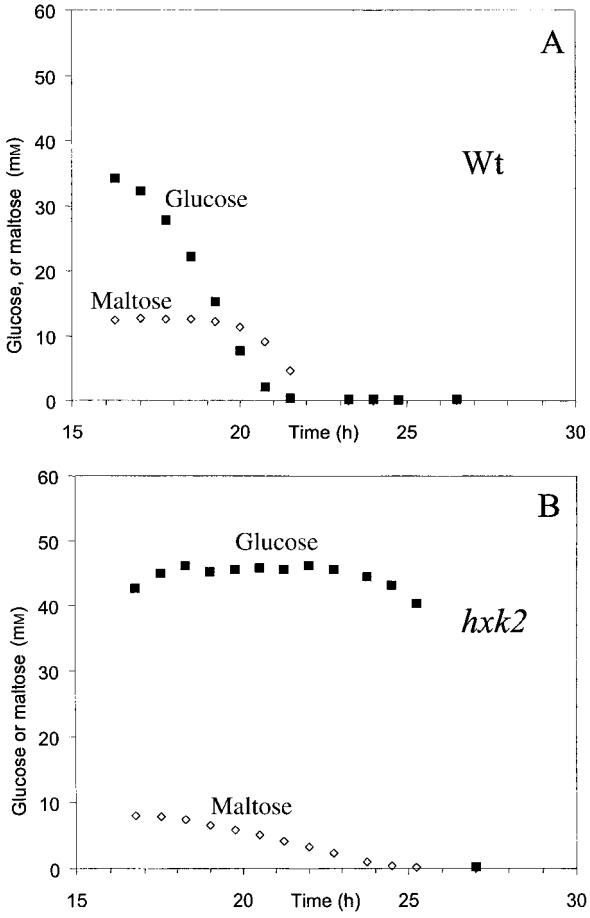

Figure 6. Growth on a mixture of glucose and maltose. The extracellular glucose (■) and maltose (◇) concentrations (mM) during growth of the wild-type strain (A) and the *hxk2* deletion strain (B)

However, the gas data revealed that the *RQ* value was lower than 1, which irrefutably shows that ethanol was actually consumed simultaneously with glucose (Figure 3C). The *RQ* gradually increased and at 18 h the value became larger than 1 and net fermentation set in. Thus, the *hxk2* deletion strain consumed ethanol in the presence of abundant glucose in early exponential growth, then switched to ethanol production during late exponential growth, and then switched back to ethanol consumption after the glucose was exhausted (Figures 3C, 7B). The parent strain produced ethanol due to fermentation of glucose (Figure 7A), which agrees with the high *RQ* values of 8 until glucose was exhausted (Figure 3A). The specific oxygen consumption by the *hxk2* deletion strain was extremely high and reached values of almost $900\ \mu mol \times min^{-1} \times (g\ protein)^{-1}$ (Figure 3D) compared to 200 for the parent (Figure 3B). Even during fully derepressed or oxidative growth by the parent on ethanol, this value did not exceed $300\ \mu mol \times min^{-1} \times (g\ protein)^{-1}$ (Figure 2B).

Discussion

This paper reports on the physiological behaviour of *S. cerevisiae* strains deleted in the *HXK2* gene during growth on mixtures of carbon sources. The *hxk2* deletion strain was investigated for co-consumption properties both on plates and, in

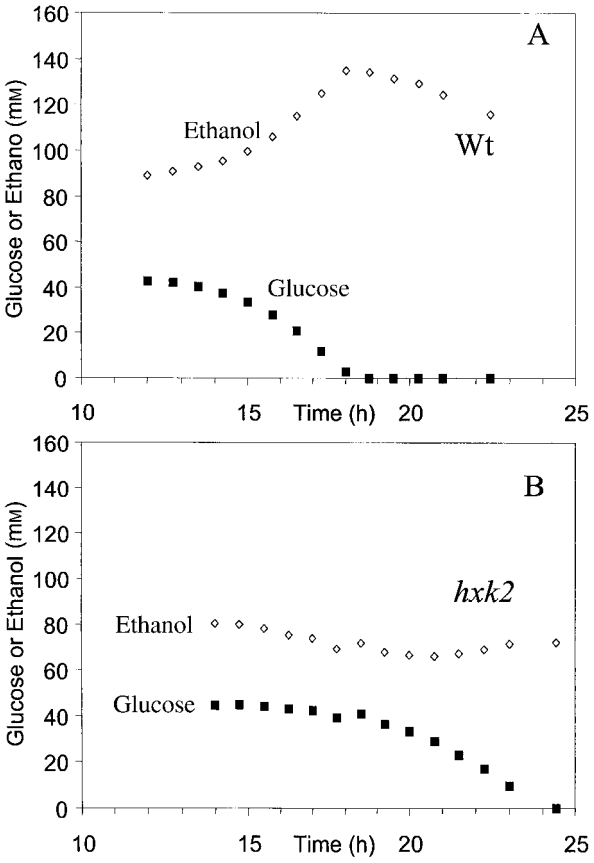

Figure 7. Growth on a mixture of glucose and ethanol. The extracellular glucose (■) and ethanol (◇) concentration (mM) during growth of the wild-type strain (A) and the *hxk2* deletion strain (B)

more detail, in batch fermentors. The plate assays revealed that the *hxk2* deletion strain could grow on sucrose and even on ethanol/glycerol in the presence of the glucose analogue 5-thioglucose, whereas the parent could not (Figure 1A). Comparably, more quantitative results were found in the batch fermentors. In batch cultures, the *hxk2* deletion strain co-consumed sucrose with glucose (Figure 4B), galactose with glucose (Figure 5B) and ethanol together with glucose (Figure 7B), of which the latter could be confirmed by the *RQ* value, which was smaller than 1 (Figure 3C).

It was observed consistently that the specific oxygen consumption in the *hxk2* deletion mutant decreased in time (Figures 2D, 3D). Recently, we found that the *hxk2* deletion strain suffers increasing biotin deficiency during growth (Raamsdonk *et al.*; paper in preparation). The gradual decrease in specific respiration rate can be (partly) prevented by the addition of extra biotin to the standard growth medium.

In liquid medium and on plate, the *hxk2* deletion strain consumed galactose in the presence of glucose and 5-thioglucose, respectively. The parent strain, however, grew in the presence of 5-thioglucose on plates as well, thus galactose metabolism was not fully repressed. In the batch fermentor, the parent strain could not consume glucose together with galactose (Figures 1A, 5A). This discrepancy might be caused by the different concentration of galactose in the medium or the different molar ratio of glucose (or 5-thioglucose) to galactose. On plates a higher concentration of galactose (2%) was present than in the fermentors (0.4%). The higher galactose concentration in the solid medium may be sufficient to induce galactose metabolism and overrule the glucose repression in the parent strain, whereas the lower initial galactose concentration in the batch fermentor may not be sufficient to overrule glucose repression.

Remarkably, the X2180-1A strain was capable of growth on maltose (Figure 1B), which is in contrast to earlier observations. The X2180-1A is characterized as *mal* minus; however it is not clear whether this characterization is based on phenotypological tests or relates to its genotype. The absence of maltose uptake and growth on maltose in liquid medium that was found for the diploid strain, in contrast to the growth on plates of the haploid, might be related to differences in preceding growth conditions. We presume that the X2180 strains are able to grow on maltose, but induction of the *MAL* genes is dependent on the preceding growth conditions. However, this hypothesis has to be tested further.

The CEN.PK strain deleted for *HXK2*, grew poorly on a mixture of glucose and maltose in liquid medium. First, all maltose was taken up and hydrolysed before the glucose was consumed (Figure 6B). The hydrolysed maltose was probably accumulated in the cytoplasm and subsequently transported out of the cell, since the extracellular glucose concentration actually rose. On plates it could be observed as well that the *hxk2* deletion strain in the CEN.PK background grew worse on maltose in the presence of 5-thioglucose than its parent, CEN.PK113-7D (Figure 1B), probably as a result of the constitutive expression of the *MAL* genes in this strain. The uncontrolled uptake of

maltose by the *hxk2* deletion strain costs ATP, since maltose permease is an active transporter. This may suggest that the strain cannot grow well due to exhaustion of intracellular ATP. However, the ATP concentration was 8 mM and the ATP : ADP ratio was approximately 5, values which are even higher than the average on fermentable carbon sources. The *hxk2* deletion strain may show impaired growth as a result of osmotic damage and/or unspecific O-glycosylation of proteins due to high intracellular glucose levels.

A completely novel observation was the ability of a *S. cerevisiae* (with a *HXK2* deletion) strain to simultaneously metabolize glucose and ethanol in the presence of excess glucose. Co-consumption of glucose and ethanol in *S. cerevisiae* has been reported earlier but could only be accomplished by using dual-substrate limited chemostat cultures (Geurts *et al.*, 1980). In such cultures, the glucose and ethanol consumption could be manipulated by changing the relative concentrations of glucose and ethanol in the medium feed.

The specific respiration rates measured in the batch fermentors varied strongly, depending on the carbon sources available and the type of strain used. The parent strain displayed respiration rates that were maximally 200 μmol $O_2 \times min^{-1} \times (g\ protein)^{-1}$ in the presence of glucose, sucrose or galactose and 300 μmol $O_2 \times min^{-1} \times (g\ protein)^{-1}$ during growth on ethanol (fully derepressed conditions) (Figures 2B, 3B). The *hxk2* deletion strain had an increased specific respiration rate on galactose and sucrose of approximately 450 μmol $O_2 \times min^{-1} \times (g\ protein)^{-1}$ and on a mixture of glucose and ethanol even 900 μmol $O_2 \times min^{-1} \times (g\ protein)^{-1}$ (Figures 2D, 3D). Especially under these conditions, where high biomass concentrations are present in combination with high specific growth rates, the specific respiration rates decline in time (Figures 2D, 3D). This is most likely due to the earlier-mentioned biotin requirement (Raamsdonk *et al.*; paper in preparation).

During industrial processes in complex medium, *S. cerevisiae* will first consume the most favoured carbon source and then switch to the next, and so on. A *S. cerevisiae* strain, such as the *hxk2* deletion strain, that can co-consume different substrates would exhaust the available carbon sources faster and is therefore appealing for use in processes such as the production of baker's yeast or heterologous proteins. Additionally, if most of the available sugars can be consumed in industrial media, the environmental burden of squandering used media will clearly be relieved. The *hxk2* deletion strain therefore appears to meet several of the properties desired for a *S. cerevisiae* strain to be used for commercial yeast biomass production or heterologous protein production.

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