Coordination of sucrose uptake and respiration in the yeast *Debaryomyces yamadae*

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Screening in batch cultures identified *Debaryomyces yamadae* as a yeast that exhibits the Kluyver effect for sucrose: this disaccharide can be respired but, even under oxygen-limited conditions, alcoholic fermentation of sucrose does not occur. Ethanol, glycerol and arabitol were the main fermentation products during oxygen-limited growth on glucose in chemostat cultures. None of these fermentation products were produced in oxygen-limited chemostat cultures grown on sucrose and the fraction of the sucrose that could not be respired remained unused in the culture medium. This absence of alcoholic fermentation was not due to repression of the key fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase. In contrast to some other yeasts that exhibit a Kluyver effect, *D. yamadae* did not exhibit a preference for ethanol in batch cultures grown on mixtures of ethanol and sucrose. Sucrose metabolism in *D. yamadae* involves intracellular hydrolysis by an α -glucosidase. Incubation of weakly buffered cell suspensions with sucrose led to a rapid transient alkalization, indicating the presence of a sucrose-proton symport system. The apparent substrate saturation constant of the sucrose-uptake system was 0.2 mmol l⁻¹. Sucrose-dependent alkalization rates were much lower in samples from oxygen-limited cultures than in samples from aerobic cultures. Transient responses of *D. yamadae* to oxygen limitation were investigated by applying a sudden decrease in the oxygen feed to aerobic sugar-limited chemostat cultures. In glucose-grown cultures, this led to alcoholic fermentation and no significant accumulation of sugar occurred after the switch. In sucrose-limited cultures, sugar accumulation occurred instantaneously after the switch, and ethanol formation was virtually absent. The results indicate that the Kluyver effect for sucrose in *D. yamadae*, i.e. the adjustment of the glycolytic flux to the cells' respiratory capacity, is effected by rapid down-regulation of the capacity of the sucrose carrier under oxygen-limited conditions.

Keywords: alcoholic fermentation, Kluyver effect, sucrose metabolism, invertase, transport

INTRODUCTION

The Kluyver effect is a widespread phenomenon among facultatively fermentative yeasts. Yeasts that exhibit the Kluyver effect are unable to ferment certain disaccharides to ethanol and CO₂, even though respiratory metabolism of these disaccharides, and alcoholic fermentation of the component hexose(s) are possible (Sims & Barnett, 1978; Weusthuis *et al.*, 1994a, b).

We have recently studied the Kluyver effect for maltose in the yeast *Candida utilis* in steady-state and transient-state chemostat cultures at various oxygen feeds (Weusthuis *et al.*, 1994a, b; Kaliterna *et al.*, 1995). In oxygen-limited, steady-state chemostat cultures grown on maltose, the rate of disaccharide metabolism was tightly adjusted to the cultures' respiratory activity which, under oxygen-limited conditions, was determined by electron-acceptor availability. As a consequence, alcoholic fermentation did not occur and the fraction of the disaccharide that could not be respired remained unconsumed (Weusthuis *et al.*, 1994a, b). However, when an aerobic, maltose-limited

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culture of *C. utilis* was suddenly exposed to oxygen limitation, a transient accumulation of ethanol occurred (Kaliterna *et al.*, 1995). The kinetics of this transient alcoholic fermentation were consistent with regulation of a key step in disaccharide metabolism, probably disaccharide transport, at the level of enzyme synthesis (Kaliterna *et al.*, 1995). In batch cultures of *C. utilis* grown on mixtures of maltose and ethanol, the latter compound was the preferred substrate, suggesting that ethanol or a related metabolite might be the effector molecule in this regulatory process (Weusthuis *et al.*, 1994b).

At present, it is unclear to what extent the observations made with *C. utilis* are representative for other 'Kluyver-positive' yeasts and disaccharides. A particularly interesting case in this respect is the fact that many yeasts exhibit a Kluyver effect for sucrose. According to the literature, the Kluyver effect for sucrose is widespread: of 137 facultatively fermentative sucrose-utilizing yeasts, 31 were reported to be unable to ferment this sugar (Barnett *et al.*, 1990). In many yeasts, including *Saccharomyces cerevisiae*, sucrose metabolism is initiated by extracellular hydrolysis, catalysed by invertase, followed by uptake of the monosaccharides. Clearly, the occurrence of such a system in a yeast that exhibits a Kluyver effect for sucrose would rule out a regulation of sucrose metabolism at the level of disaccharide transport. Both for this reason and in view of the observed substrate-specificities of yeasts with a Kluyver effect for sucrose, it has been postulated that occurrence of a Kluyver effect for this sugar should be accompanied by its intracellular hydrolysis (Sims & Barnett, 1978; Barnett, 1981). However, this possibility has not been studied experimentally.

The aim of this work was to test the hypothesis that sucrose hydrolysis in a yeast with a Kluyver effect for this disaccharide occurs intracellularly and to investigate to what extent regulation of disaccharide metabolism is comparable with that of maltose metabolism in *C. utilis*. *Debaryomyces yamadae* CBS 7035, a yeast that has previously been reported to exhibit a Kluyver effect for sucrose (Barnett *et al.*, 1990), was selected as the model organism.

METHODS

Yeast strains and maintenance. *Candida kruisii* CBS 6451, *Candida naeodendra* CBS 6032, *Candida peltata* CBS 5564, *Candida shehatae* CBS 5813, *Debaryomyces yamadae* CBS 7035, *Metschnikowia reukauffii* CBS 611, *Hansenula polymorpha* CBS 2575 and *Pichia stipitis* CBS 5773 were obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on malt agar slants at 4 °C. These strains have all been reported to grow on sucrose aerobically, but not to ferment this disaccharide (Barnett *et al.*, 1990).

Screening for the Kluyver effect in shake-flask cultures. The ability to ferment glucose and sucrose was investigated in 100 ml shake-flask cultures containing 50 ml mineral medium (Van Leeuwen *et al.*, 1992) supplemented with either 20 g glucose l⁻¹ or 20 g sucrose l⁻¹. After 24 h incubation at 30 °C on a rotatory shaker at 200 r.p.m., samples were withdrawn and analysed for biomass dry weight and ethanol.

Chemostat cultivation. Chemostat cultivation of *D. yamadae* CBS 7035 was performed in 2 l fermenters (Applikon) at a dilution rate of 0.10 h⁻¹, a temperature of 30 °C and a stirrer

speed of 1050 r.p.m. The culture pH was maintained at 5.0 by automatic addition of 2 M KOH via an Applikon ADI-1020 biocontroller. Aerobic cultivation (dissolved oxygen concentration above 70% of air saturation) was performed by maintaining an airflow through the culture of 0.53 l min⁻¹. This was administered to the culture by two means: 0.5 l min⁻¹ with a Brooks 5876 mass-flow controller and 30 ml min⁻¹ with a peristaltic pump. The working volume of the culture was kept at 1.0 l by removal of effluent from below the surface of the culture, via an electrical level controller. This set-up ensured that biomass concentrations in the effluent differed by less than 1% from those in samples taken directly from the culture (Noorman *et al.*, 1991). In order to obtain reproducible gas-transfer properties, positions of baffles, pipes, impellers and sensors were identical in the different fermenters used in this study. To avoid loss of volatile metabolites, the off-gas condenser was cooled to 2 °C. The mineral medium was prepared according to Van Leeuwen *et al.* (1992). Sucrose (10 g l⁻¹) or glucose (10 g l⁻¹) and vitamins were added to the cultures after separate sterilization. After sterilization, approximately 1% (w/w) of the sucrose was found to be hydrolysed to glucose and fructose. Culture purity was routinely checked by phase-contrast microscopy at 1000× magnification.

Transient-state experiments. At zero time, the gas flow through the mass-flow controller was switched from air to nitrogen gas, resulting in the addition of a mixture of ultra-pure nitrogen gas (0.5 l min⁻¹) and air (30 ml min⁻¹) to the culture. As a result, the dissolved-oxygen concentration in the culture, measured with an Ingold polarographic oxygen electrode, decreased to below 0.1% of air saturation within 2 min. At appropriate intervals, samples from the effluent line were collected on ice. The residence time in the effluent line was approximately 1 min. Samples were analysed for culture dry weight and, after centrifugation at 10000 g, for metabolites. Cell pellets for preparation of cell-free extracts were resuspended in 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, frozen and stored at -20 °C (Postma *et al.*, 1989).

Batch cultivation. Yeasts were pregrown in 100 ml shake-flasks on 20 ml of the mineral medium described above, with an initial pH of 6.0 and containing 5.0 g glucose or sucrose l⁻¹. Batch cultivation took place in the fermenters described above with an initial working volume of 1.0 l. The medium was the same as used for continuous cultivation with 5 g glucose or sucrose l⁻¹ and 4 g ethanol l⁻¹. The pH was controlled between 4.9 and 5.1 by the automatic addition of 2 M KOH or 1 M H₂SO₄. The airflow rate into the culture was 1.0 l min⁻¹. The dissolved oxygen concentration in the culture was kept above 50% air saturation by manual adjustment of the stirring speed between 750 and 1250 r.p.m. The temperature was 30 °C.

Metabolite analysis. Enzymic analysis of glucose concentrations and HPLC analysis of ethanol, glycerol, lactate and other low-molecular-mass metabolites was performed as described by Weusthuis *et al.* (1993). Ethanol was also determined with an enzymic assay (based on alcohol oxidase, EK 001, Leeds Biochemicals). This method gave the same results, but has a lower detection limit (approximately 10 µM) than the HPLC method (approximately 0.2 mM). Sucrose was determined via hydrolysis to glucose and fructose in 32 mM sodium citrate buffer (pH 4.6) with 50 U invertase ml⁻¹, followed by enzymic glucose analysis.

Biomass determination. Dry weights of culture samples were determined via filtration with 0.45 µm membrane filters and drying in a microwave oven (Postma *et al.*, 1989). Parallel samples varied by less than 1%. OD₆₆₀ was measured with a Vita-Lab 20 (Vita Scientific). When the OD₆₆₀ was above 0.3, samples were diluted with demineralized water.

Preparation of cell-free extracts. Cell-free extracts of chemostat-grown cells were prepared as described by Postma *et al.* (1989).

Pyruvate decarboxylase and alcohol dehydrogenase assays.

Assays of pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) activity in cell-free extracts were performed as described by Postma *et al.* (1989).

Sucrose-hydrolase assays. Sucrose-hydrolysing activity was measured in culture supernatants, cell-free extracts and washed cell suspensions. The latter were obtained by washing culture samples twice with 50 mM sodium acetate buffer (pH 5). Sucrose-hydrolysing activities of the various preparations were assayed at 30 °C by following the rate of glucose production in an incubation mixture containing 50 mM sodium acetate buffer (pH 5.0) and 56 mM sucrose. The reaction was stopped by the addition of Tris buffer (pH 10.5) to a final concentration of 60 mM. When assays were performed at pH 7.0, the sodium acetate buffer was replaced by 50 mM potassium phosphate buffer. For substrate-specificity assays, maltose or raffinose (56 mM) were used instead of sucrose. In the case of raffinose, production of fructose was determined with a commercial kit (Boehringer no. 139 106). One unit is defined as the hydrolysis of 1 µmol disaccharide min⁻¹.

Sucrose-proton symport assays Sucrose transport was determined by measuring initial rates of sucrose-dependent alkalization of weakly buffered cell suspensions according to the method of van Urk *et al.* (1989). To calculate proton-uptake rates, alkalization curves were calibrated with a standard NaOH solution. Kinetic parameters were estimated by non-linear regression, assuming Michaelis-Menten kinetics, using the program Fig.P (Fig.P Software).

RESULTS

Choice of model organism

In taxonomic studies, occurrence of alcoholic fermentation is generally tested with the Durham-tube method. Although this method allows rapid screening for vigorous

fermentation of substrates, low rates of alcoholic fermentation easily go unnoticed (van Dijken *et al.*, 1986; Barnett, 1992). For physiological studies on the Kluver effect, it is desirable to use strains with a clear-cut phenotype. Therefore, eight yeast strains previously reported to grow on sucrose aerobically, but not to ferment this disaccharide (Barnett *et al.*, 1990), were tested by enzymic analysis of ethanol formation in shake-flask cultures (Table 1). This method is based on the fact that shake-flask cultures of yeasts almost inevitably become oxygen limited (van Dijken *et al.*, 1986), which induces ethanol formation from fermentable sugars.

Surprisingly, four out of the eight yeast strains tested produced significant amounts of ethanol in sucrose-grown shake-flask cultures (Table 1). A fifth yeast, *M. reukaufii*, grew extremely slowly under the experimental conditions and was therefore rejected as a model organism. *C. peltata*, which did not exhibit alcoholic fermentation on sucrose, was not chosen because it produced large amounts of a viscous extracellular product. This left two strains which fermented glucose, but not sucrose: *C. naeodendra* and *D. yamadae*. The latter was chosen for further studies because it exhibited the highest ethanol concentration in glucose-grown cultures (Table 1).

Glucose and sucrose metabolism in steady-state chemostat cultures

To further investigate the Kluver effect for sucrose in *D. yamadae* CBS 7035, the organism was grown on glucose and sucrose in aerobic and oxygen-limited chemostat cultures ($D = 0.10 \text{ h}^{-1}$).

In aerobic, sugar-limited chemostat cultures grown on glucose or sucrose, all sugar entering the fermenter could be recovered as biomass and CO₂ and no fermentation

Table 1. Biomass and ethanol concentrations in 24 h oxygen-limited shake-flask cultures on glucose and sucrose of various facultatively fermentative yeast strains with reported Kluver effects for sucrose

Yeast strain	Glucose-grown cultures		Sucrose-grown cultures	
	Biomass (g l ⁻¹)	Ethanol (g l ⁻¹)	Biomass (g l ⁻¹)	Ethanol (g l ⁻¹)
<i>Candida kruisii</i> CBS 6451	2.7	5.8	2.7	1.3
<i>Candida naeodendra</i> CBS 6032	5.8	0.9	1.7	< 0.01
<i>Candida peltata</i> CBS 5564*	—	6.2	—	< 0.01
<i>Candida shehatae</i> CBS 5813	2.4	5.0	2.6	0.2
<i>Debaryomyces yamadae</i> CBS 7035	2.8	5.3	2.5	< 0.01
<i>Metschnikowia reukaufii</i> CBS 611	ND	ND	ND	0.02
<i>Hansenula polymorpha</i> CBS 2575	ND	ND	ND	1.6
<i>Pichia stipitis</i> CBS 5773	ND	ND	ND	1.5

ND, Not determined.

**C. peltata* CBS 5564 formed a viscous extracellular product, which prevented the measurement of culture dry weights by filtration.

Table 2. Concentrations of substrate, metabolites and biomass in carbon- and oxygen-limited chemostat cultures of *D. yamadae* grown on glucose or sucrose ($D = 0.10 \text{ h}^{-1}$, reservoir concentration $10 \text{ g sugar l}^{-1}$)

All metabolite concentrations are given in mmol l^{-1} . The experiments were performed in triplicate; data are from representative steady-state cultures.

	Glucose-grown cultures		Sucrose-grown cultures	
	Aerobic	O_2 limited	Aerobic	O_2 limited
Sucrose (mM)	< 0.1	< 0.1	< 0.1	23.3
Glucose (mM)	< 0.1	< 0.1	< 0.1	0.65
Ethanol (mM)	< 0.1	29.9	< 0.1	< 0.1
Glycerol (mM)	< 0.1	10.4	< 0.1	< 0.1
Arabitol (mM)	< 0.1	1.2	< 0.1	< 0.1
Biomass (g l^{-1})	4.9	2.2	5.1	1.4

products were detected in culture supernatants (Table 2). The biomass yield on the growth-limiting sugar was $0.5 \text{ g biomass (g sugar)}^{-1}$. In oxygen-limited chemostat cultures grown on glucose, a mixed fermentative metabolism was observed. In addition to ethanol, significant amounts of glycerol and arabitol were produced (Table 2; the latter compound was tentatively identified, based on its retention time on HPLC). No residual glucose was detected in the oxygen-limited steady-state cultures and carbon recoveries were 95–97%. The respiro-fermentative metabolism led to a decrease of the biomass yield to approximately $0.2 \text{ g biomass (g glucose)}^{-1}$.

In sucrose-grown oxygen-limited cultures of *D. yamadae*, the substrate was only partially consumed and the fermentation products observed in the glucose-grown oxygen-limited cultures were not detected (Table 2). Apparently, the mechanism responsible for the Kluver effect in this yeast not only prevents alcoholic fermentation, but also the formation of other fermentation products. The biomass yield on sucrose, corrected for the high residual sugar concentration, was $0.5 \text{ g biomass (g sucrose)}^{-1}$, consistent with a fully respiratory metabolism.

Localization of the sucrose-hydrolysing activity

Disaccharide uptake has been implicated as a key process in the regulation of sugar metabolism in yeasts which exhibit a Kluver effect (Sims & Barnett, 1978; Barnett, 1981, 1992; Kaliterna *et al.*, 1995). Since this can only hold when disaccharide hydrolysis occurs intracellularly, the localization of the sucrose-hydrolysing enzyme activity in *D. yamadae* was investigated.

Neither in aerobic, sucrose-limited nor in oxygen-limited chemostat cultures of *D. yamadae* could sucrose-hydrolysing activity be detected in culture supernatants. This observation does not necessarily rule out the possibility of extracytosolic sucrose hydrolysis since invertase can also be retained in the yeast cell wall (Barnett, 1981). However, washed cell suspensions also did not exhibit significant sucrose-hydrolysing activity. The same results were obtained when transport and

phosphorylation of sugars were inhibited by the addition of 0.1 mM 2,4-dinitrophenol. These observations argue against the presence of cell-wall-associated invertase and suggest that, instead, sucrose is hydrolysed intracellularly.

Indeed, cell-free extracts of aerobic, sucrose-limited chemostat cultures exhibited high sucrose-hydrolysing activities. Activity at $\text{pH } 7$ [$1.8 \text{ U (mg protein)}^{-1}$] was 2.4-fold higher than at $\text{pH } 5$, consistent with a cytosolic localization (Barnett, 1981). Sucrose-hydrolysing activities of extracts from oxygen-limited cultures were only approximately 30% lower than those in aerobic, sucrose-limited cultures [$1.2 \text{ U (mg protein)}^{-1}$], indicating that the inability to utilize all sucrose in the oxygen-limited cultures was not due to repression of the hydrolase.

In theory, intracellular hydrolysis of sucrose can be catalysed either by a β -fructosidase (invertase; EC 3.2.1.26) or by an α -glucosidase ('maltase', EC 3.2.1.20) (Barnett, 1981). One way to discriminate between these two possibilities is to investigate the substrate specificity of the sucrose-hydrolysing enzyme activities: β -fructosidase hydrolyses sucrose and raffinose, but not maltose; α -glucosidase hydrolyses maltose and sucrose, but not raffinose (Barnett, 1981). Rates of maltose and sucrose hydrolysis by cell-free extracts of *D. yamadae* grown under either aerobic or oxygen-limited conditions differed by less than 10% (data not shown). Raffinose was not hydrolysed, indicating that sucrose hydrolysis in this yeast is catalysed by an intracellular α -glucosidase.

Sucrose transport

Since sucrose cannot cross the yeast plasma membrane by passive diffusion, its metabolism via an intracellular α -glucosidase requires the presence of a sucrose carrier. Disaccharide transport in yeasts generally takes place via proton-symport (Serrano, 1977; Schulz & Höfer, 1986; Santos *et al.*, 1982; Dickson & Barr, 1983; Carvalho-Silva & Spencer-Martins, 1990).

Addition of sucrose to weakly buffered cell suspensions of *D. yamadae*, pregrown in aerobic, sucrose-limited chemo-

Fig. 1. (a) Sugar-dependent effects on the pH of weakly buffered cell suspensions of *D. yamadae* CBS 7035 grown on sucrose in aerobic (A) or oxygen-limited chemostat cultures (B). At the time indicated by the arrow, 20 mM sucrose was added. An identical biomass concentration ($2.0 \text{ g dry wt l}^{-1}$) was used in both experiments. (b) Kinetics of sucrose-dependent alkalization of weakly buffered cell suspensions of *D. yamadae* pregrown in aerobic, sucrose-limited chemostat culture. The line drawn through the points was calculated by non-linear regression, assuming Michaelis-Menten kinetics.

stat cultures, led to a rapid transient alkalization (Fig. 1a), which is indicative of the presence of a sucrose-proton symporter in this yeast. Uptake of sucrose by aerobically grown cells showed saturation kinetics (Fig. 1b) and non-linear regression analysis of the uptake activities revealed a K_m for sucrose uptake of $0.22 \pm 0.05 \text{ mmol l}^{-1}$ and a V_{max} of $7.9 \text{ mmol H}^+ \text{ g}^{-1} \text{ h}^{-1}$.

Assuming that, as has been observed for other yeast sugar-proton symporters (Williamson *et al.*, 1993; Weusthuis *et al.*, 1993), the proton-sugar stoichiometry is near unity, the capacity of this uptake system is 14-fold higher than the *in situ* rate of sucrose consumption in the aerobic, sucrose-limited chemostat cultures ($0.55 \text{ mmol g}^{-1} \text{ h}^{-1}$; calculated from Table 2).

If coordination of the glycolytic flux and the cells' respiratory capacity is indeed regulated at the level of sucrose uptake, this would require a drastic reduction in the capacity of the sucrose carrier under oxygen-limited conditions, where sucrose is present in excess (Table 2).

Fig. 2. Transient responses of *D. yamadae* CBS 7035 to oxygen limitation. At t_0 , an aerobic sucrose-limited (a) or glucose-limited (b) chemostat culture ($D = 0.10 \text{ h}^{-1}$) was switched to oxygen-limited growth conditions. Transient-state experiments were performed in three independent chemostat cultures with essentially the same results. The figure shows data from a single experiment.

Indeed, sucrose-dependent alkalization rates of washed cell suspensions harvested from oxygen-limited cultures were much lower than those of suspensions from aerobic cultures (Fig. 1a).

Although the above results clearly indicate the presence of a sucrose-proton symport mechanism in *D. yamadae*, the possibility that sucrose is also taken up by facilitated diffusion cannot be excluded.

Transient responses of *D. yamadae* to oxygen limitation

If, as suggested above, sucrose metabolism can be regulated at the level of disaccharide uptake, the high capacity of the sucrose-proton symporter should be reduced during a switch from aerobic to oxygen-limited conditions. In practice, this can be achieved in two ways: by a cessation of the synthesis of new carrier molecules or by inactivation of the carrier molecules already present in the cell. In the former case, the transport capacity of the cells will only decrease slowly due to turn-over of the carrier molecules and dilution by growth. This may lead to a transient accumulation of ethanol, as observed in the case of maltose utilization by *C. utilis* (Kaliterna *et al.*, 1995), even though ethanol is not detected in steady-state oxygen-limited cultures. In contrast, regulation of the

Fig. 3. Activities of alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) in *D. yamadae* CBS 7035 during a switch from aerobic to oxygen-limited conditions. At t_0 an aerobic, sucrose-limited (a) or glucose-limited (b) chemostat culture ($D = 0.10 \text{ h}^{-1}$) was switched to oxygen-limited conditions.

activity of existing enzymes can occur over a time-scale of minutes (Holzer, 1976).

When an aerobic, sucrose-limited culture of *D. yamadae* was switched to oxygen-limited conditions, very low concentrations of ethanol ($< 2 \text{ mM}$) were observed only during the first hours after the switch to oxygen-limited conditions (Fig. 2a) and sucrose started to accumulate in the culture within the first hour. From 5 h after the switch, neither ethanol nor other fermentation products were detected (Fig. 2a). When, as a control experiment, a glucose-limited chemostat culture was switched to oxygen limitation, this resulted in a rapid accumulation of ethanol and other fermentation products, without substantial accumulation of glucose (Fig. 2b).

Activities of key fermentative enzymes

Down-regulation of the key fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase has been suggested to contribute to the absence of alcoholic fermentation in yeasts that exhibit a Kluver effect (Sims *et al.*, 1991; Sims & Barnett, 1991; Barnett, 1992). To investigate whether this might also hold for *D. yamadae*, activities of these enzymes were followed during switch experiments from aerobic to oxygen-limited conditions.

Both pyruvate decarboxylase and alcohol dehydrogenase were clearly induced under oxygen-limited conditions,

Fig. 4. Growth and substrate consumption of *D. yamadae* CBS 7035 in aerobic, pH-controlled batch cultures on mixtures of (a) glucose and ethanol and (b) sucrose and ethanol. The media contained $5.0 \text{ g sugar l}^{-1}$ and $4.0 \text{ g ethanol l}^{-1}$.

activities being 10–20-fold higher than in the corresponding aerobic cultures (Fig. 3). In comparison with other facultatively fermentative yeasts, *D. yamadae* exhibited very low activities of alcohol dehydrogenase. Although even lower than in glucose-grown cultures, significant activities of alcohol dehydrogenase were detected in extracts from sucrose-grown, oxygen-limited cultures (Fig. 3).

Pyruvate decarboxylase was also present at significant activities in extracts from sucrose-grown, oxygen-limited cultures (Fig. 3). Therefore, the complete absence of alcoholic fermentation cannot be explained from a repression of the two fermentative key enzymes.

Growth on mixtures of sucrose and ethanol

It has recently been reported that in *C. utilis* and *Debaryomyces castellii*, two yeasts that exhibit the Kluver effect for maltose and lactose, respectively, ethanol is preferred over the disaccharide during growth on mixtures of the two substrates. In contrast, preferential utilization of ethanol was not observed during growth of these yeasts on mixtures of ethanol and fermentable sugars (Weusthuis *et al.*, 1994b).

To test whether preferential utilization of ethanol over sucrose occurred in *D. yamadae*, the yeast was cultivated in a pH-controlled, aerobic fermenter with ethanol (4 g l^{-1}) and sucrose (5 g l^{-1}) as carbon sources. As a control, growth on a mixture of ethanol and glucose was studied. In contrast to the diauxic growth patterns observed with

C. utilis and *D. castellii*, *D. yamadae* did not exhibit a preference for ethanol over the disaccharide. Instead, both glucose and sucrose were used simultaneously with ethanol (Fig. 4). The absence of extracellular glucose during batch cultivation on sucrose is consistent with the intracellular localization of the sucrose-hydrolysing enzyme.

DISCUSSION

A screening of eight yeast species that had previously been reported to exhibit a Kluver effect for sucrose (Table 1) confirmed earlier reports (van Dijken *et al.*, 1986; Barnett, 1992) about the insensitivity of the Durham-tube method, a procedure commonly used in yeast taxonomy to assess fermentation of carbohydrates. Therefore, the use of such tests may have led to an overestimation of the abundance of the Kluver effect among facultatively fermentative yeasts. Nevertheless, three of the eight strains tested exhibited a clear Kluver effect when ethanol formation was assayed by a sensitive enzymic method (Table 1).

The occurrence of a Kluver effect for sucrose in *D. yamadae* was confirmed by studies in oxygen-limited chemostat cultures. During oxygen-limited growth on sucrose, no fermentation products were detected. In contrast, glucose-grown, oxygen-limited chemostat cultures produced substantial amounts of ethanol, glycerol and arabinol (Table 2). In steady-state oxygen-limited cultures, the molar ratio of ethanol to glycerol was approximately 3. Thus, the relative amount of glycerol produced by *D. yamadae* is two- to three-fold higher than the maximum ratios observed in steady-state oxygen-limited chemostat cultures of *S. cerevisiae* and *C. utilis* (Weusthuis *et al.*, 1994a). This high glycerol production may be related to the low alcohol dehydrogenase activities in this yeast (Fig. 3), which are one to two orders of magnitude lower than those in *S. cerevisiae* and *C. utilis* grown under comparable conditions (Weusthuis *et al.*, 1994a; Kaliterna *et al.*, 1995). Increased formation of glycerol has also been observed in *S. cerevisiae* mutants with an impaired synthesis of alcohol dehydrogenase (Johansson & Sjöström, 1984).

The results presented in this paper demonstrate that sucrose metabolism in *D. yamadae* is initiated by its uptake across the plasma membrane, followed by intracellular hydrolysis, catalysed by an α -glucosidase. This observation is consistent with the prediction made by Sims & Barnett (1978) and Barnett (1981). It implies that, theoretically, the glycolytic flux during growth on sucrose can be controlled at the level of disaccharide uptake, as has been proposed for other yeasts that exhibit a Kluver effect (Barnett & Sims 1982; Kaliterna *et al.*, 1995). Several observations presented here indicate that occurrence of the Kluver effect, i.e. the adaptation of the glycolytic flux to the cells' respiratory activity, is indeed probably regulated at the level of sucrose uptake.

(i) The activity of the sucrose-hydrolysing enzyme is not repressed in oxygen-limited, sucrose-grown cultures.

Therefore, if down-regulation of the glycolytic flux were to occur at a reaction beyond sucrose uptake, accumulation of glucose and/or fructose would be expected after a switch to oxygen-limited conditions, rather than the observed accumulation of sucrose (Fig. 2).

(ii) Alkalinization studies suggest a decrease of the capacity of the sucrose-proton symporter after a switch to oxygen-limited conditions (Fig. 1). This observation should be interpreted with caution, since alkalinization is the net result of proton uptake by the sucrose carrier and acidification caused by the simultaneous action of the plasma membrane ATPase complex. In theory, the low alkalinization rates in oxygen-limited cultures could therefore also be due to an increased activity of the plasma-membrane ATPase under oxygen-limited conditions.

(iii) The Kluver effect for sucrose in *D. yamadae* not only encompasses the absence of alcoholic fermentation, but also absence of glycerol and arabinol, products that branch off from the main glycolytic pathway (Table 2).

It should be stressed that intracellular hydrolysis of sucrose in a yeast does not necessarily imply the occurrence of a Kluver effect. For example, sucrose metabolism in *H. polymorpha* is initiated by uptake via a proton-symport system and followed by intracellular hydrolysis by an α -glucosidase (J. Kaliterna, unpublished results). Nevertheless, this yeast ferments sucrose under oxygen-limited conditions (Table 1).

The rapid accumulation of sucrose after a switch to oxygen-limited conditions (Fig. 2) suggests that in *D. yamadae*, regulation of sucrose-uptake capacity occurs at the level of enzyme activity. This contrasts with recent observations on the Kluver effect for maltose in *C. utilis*, where a relatively slow decrease of the maltose-metabolizing activity appeared to be regulated at the level of enzyme synthesis (Kaliterna *et al.*, 1995).

In contrast to observations made with *C. utilis* and *D. castellii* (Weusthuis *et al.*, 1994b), the Kluver effect for sucrose in *D. yamadae* was not associated with a preferential use of ethanol during growth on mixtures with the disaccharide (Fig. 4). This makes it unlikely that ethanol is a key effector molecule involved in the regulation of sucrose uptake in this yeast. Further work is needed to identify the kinetics, molecular mechanism and signals involved in down-regulation of sucrose uptake in *D. yamadae* after a switch to oxygen limitation.

Sucrose-proton symport has been extensively studied in plant cells (for a recent review see Bush, 1993). Comparatively little is known about uptake of sucrose by yeasts, although proton-symport systems have been demonstrated in some, including *Candida albicans* (Williamson *et al.*, 1993) and *S. cerevisiae* (Santos *et al.*, 1982). In the latter yeast, a sucrose-proton symport mechanism is present in addition to the well-characterized extracytosolic invertase. The apparent K_m for sucrose of *D. yamadae* estimated from alkalinization studies (0.2 mmol l^{-1} ; Fig. 1b) is lower than the value reported for *S. cerevisiae* and plant sucrose transporters (apparent

K_m values of 6 and 1 mM, respectively; Santos *et al.*, 1982; Bush, 1993), but is much higher than that of the *C. albicans* sucrose-proton symporter ($K_m = 7 \mu\text{mol l}^{-1}$; Williamson *et al.*, 1993). A further characterization of sucrose-proton symport in yeasts and a detailed comparison with the proton symporters of plants requires the use of isolated plasma-membrane vesicles. In view of its very high sucrose-proton symport activities in aerobic, sucrose-limited chemostat cultures, *D. yamadae* seems an excellent model organism for such studies.

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