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Weusthuis, R. A., Visser, W., Pronk, J. T., Scheffers, W. A., & Van Dijken, J. P. (1994). Effects of oxygen limitation on sugar metabolism in yeasts: A continuous-culture study of the Kluver effect. *Microbiology*, 140(4), 703-715. <https://doi.org/10.1099/00221287-140-4-703>

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Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect

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Growth and metabolite formation were studied in oxygen-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 growing on glucose or maltose at a dilution rate of 0.1 h⁻¹. With either glucose or maltose *S. cerevisiae* could be grown under dual limitation of oxygen and sugar. Respiration and alcoholic fermentation occurred simultaneously and the catabolite fluxes through these processes were dependent on the magnitude of the oxygen feed. *C. utilis* could also be grown under dual limitation of glucose and oxygen. However, at very low oxygen feed rates (i.e. below 4 mmol l⁻¹ h⁻¹) growth was limited by oxygen only, as indicated by the high residual glucose concentration in the culture. In contrast to *S. cerevisiae*, *C. utilis* could not be grown anaerobically at a dilution rate of 0.1 h⁻¹. With *C. utilis* absence of oxygen resulted in wash-out, despite the presence of ergosterol and Tween-80 in the growth medium. The behaviour of *C. utilis* with respect to maltose utilization in oxygen-limited cultures was remarkable: alcoholic fermentation did not occur and the amount of maltose metabolized was dependent on the oxygen supply. Oxygen-limited cultures of *C. utilis* growing on maltose always contained high residual sugar concentrations. These observations throw new light on the so-called Kluyver effect. Apparently, maltose is a non-fermentable sugar for *C. utilis* CBS 621, despite the fact that it can serve as a substrate for growth of this facultatively fermentative yeast. This is not due to the absence of key enzymes of alcoholic fermentation. Pyruvate decarboxylase and alcohol dehydrogenase were present at high levels in maltose-utilizing cells of *C. utilis* grown under oxygen limitation. It is concluded that the Kluyver effect, in *C. utilis* growing on maltose, results from a regulatory mechanism that prevents the sugar from being fermented. Oxygen is not a key factor in this phenomenon since under oxygen limitation alcoholic fermentation of maltose was not triggered.

Keywords: *Saccharomyces cerevisiae*, *Candida utilis*, sugar metabolism, oxygen limitation, Kluyver effect

INTRODUCTION

Oxygen is a key factor in the regulation of sugar metabolism in yeasts. In the presence of oxygen, virtually all yeasts can respire sugars to CO₂ and water. The majority of the yeast species described so far are also

capable of fermenting sugars to ethanol and carbon dioxide (Barnett *et al.*, 1990; van Dijken *et al.*, 1986). The capacity to ferment sugars to ethanol does not imply the ability to grow under anaerobic conditions. In fact, most facultatively fermentative yeasts do not grow well in the complete absence of oxygen, not even in complex media (Visser *et al.*, 1990).

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Abbreviations: ADH, alcohol dehydrogenase; PDH, pyruvate dehydrogenase.

Oxygen-related physiological phenomena in yeasts have been categorized as four 'effects', the occurrence of which depends both on the yeast species and on the sugar

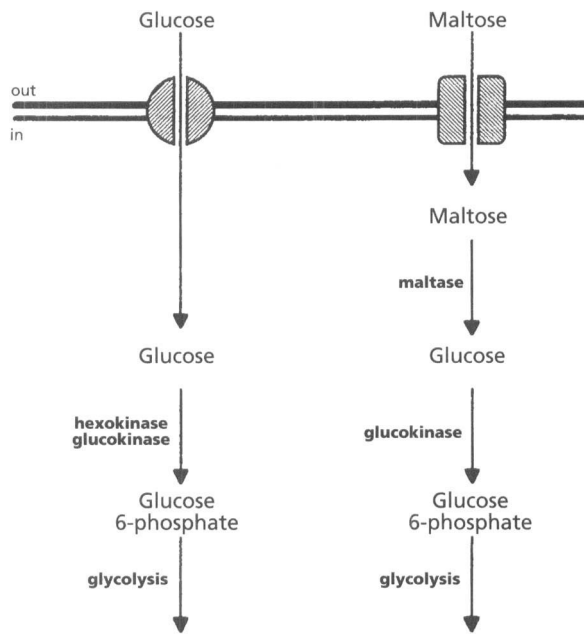


Fig. 1. Differences between glucose and maltose metabolism in yeasts. In this scheme it has been assumed that phosphorylation of glucose units derived from maltose occurs by glucokinase only, while glucose that is taken up from the medium can be phosphorylated by both glucokinase and hexokinase (Clifton *et al.*, 1993).

substrate. The Pasteur effect has been defined as an inhibition of fermentative sugar metabolism by oxygen (Lagunas *et al.*, 1982; Lagunas, 1986; Busturia & Lagunas, 1986; Lloyd & James, 1987). In contrast, the Custers effect describes the phenomenon that, in certain yeasts, fermentation is inhibited by the absence of oxygen (Custers, 1940; Scheffers, 1966; Carrascosa *et al.*, 1981; Wijsman *et al.*, 1984; Gaunt *et al.*, 1988). The Crabtree effect, defined as the occurrence of fermentative metabolism in the presence of oxygen occurs in some yeasts when these are exposed to excess sugar (Fiechter *et al.*, 1981; Petrik *et al.*, 1983; Käppeli *et al.*, 1985a, b; Käppeli, 1986; Käppeli & Sonnleitner, 1986; Postma *et al.*, 1988; 1989a, b; van Urk *et al.*, 1988, 1989).

A fourth phenomenon, the Kluver effect (Sims & Barnett, 1978), is probably the least understood of these oxygen-related metabolic responses. It has been defined as follows: '... certain yeasts can utilize particular disaccharides aerobically but not anaerobically, although these yeasts can use one or more of the component hexoses anaerobically' (Sims & Barnett, 1978). A screening for the occurrence of the Kluver effect among yeast species revealed that it is widespread among facultatively fermentative yeasts, the pattern of disaccharide fermentation being strongly strain-dependent (Sims & Barnett, 1978).

Clearly, the Kluver effect must be caused by differences between monosaccharide and disaccharide metabolism. As shown in Fig. 1, this leaves three possibilities: sugar transport, disaccharide hydrolysis or sugar-specific regulatory mechanisms.

A number of hypotheses have been proposed regarding the cause of the Kluver effect. Kluver & Custers (1940) postulated that the phenomenon might be caused by a reversible inactivation of the disaccharide hydrolase under anaerobic conditions. Later on, several authors proposed an effect of oxygen on disaccharide transport, either direct (e.g. by an effect on the conformation of the sugar carrier) or indirect [e.g. by an inadequate energy supply for active uptake of disaccharides during anaerobic fermentation (Barnett & Sims, 1982; Sims *et al.*, 1984; Schulz & Höfer, 1986; Höfer & Nassar, 1987)]. Alternatively, regulation of the key fermentative enzyme pyruvate decarboxylase by the sugar substrate has been suggested (Sims & Barnett, 1991). Furthermore, in recent papers it has been presumed that combinations of the factors shown in Fig. 1 may cause the Kluver effect (Sims *et al.*, 1991; Barnett, 1992).

The data presented in the original screening of yeast species by Sims & Barnett (1978) originated from two types of taxonomic tests. 'Aerobic' growth tests were performed in slowly shaking test-tubes; 'anaerobic' growth and fermentation tests were performed in static incubation of Durham tubes containing an inverted vial for monitoring gas production. It is clear that in the screening by Sims & Barnett (1978) the 'aerobic' cultures are likely to have been oxygen-limited. On the other hand, some oxygen must also have entered the static 'anaerobic' cultures.

Since none of the facultatively fermentative yeasts that exhibit the Kluver effect are capable of anaerobic growth (Visser *et al.*, 1990), occurrence of the Kluver effect must be confined to oxygen-limited growth conditions. Although oxygen has often been implicated as a key factor in its occurrence, the Kluver effect has not yet been studied under controlled oxygen feed regimes.

The aim of the present work was to investigate the function of oxygen in the Kluver effect. For this purpose, the effect of the oxygen feed rate on sugar metabolism was studied in sugar-limited chemostat cultures. *C. utilis* was chosen as a model organism that exhibits the Kluver effect for maltose (Sims & Barnett, 1978), whereas *S. cerevisiae* CBS 8066 is Kluver-negative for this disaccharide (Weusthuis *et al.*, 1993). Since both strains are capable of respiratory and fermentative glucose metabolism, cultures grown on glucose were included as references. The experimental results were compared to those predicted by a simple metabolic model.

METHODS

Organisms and maintenance. *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 were obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands) and maintained on malt agar slants at 4 °C.

Chemostat cultivation. Chemostat cultivation was performed in 2 litre fermenters (Applikon) at a dilution rate of 0.10 h⁻¹, a temperature of 30 °C and a stirrer speed of 750 r.p.m. The culture pH was maintained at 5.0 by automatic addition of 2 M KOH, via an Applikon ADI-1030 biocontroller. The working volume of the culture was kept at 1.1 litre by removal of effluent from the middle of the culture, via an Applikon

electrical level controller. This set-up ensured that biomass concentrations in the effluent line different by less than 1% from those in samples taken directly from the culture. To achieve an identical fermenter geometry, positions of baffles, pipes, impellers and sensors were kept the same in all experiments. To avoid loss of volatile metabolites, the condenser was cooled to 2 °C, using a cryostat. The mineral medium, supplemented with vitamins, trace elements and the anaerobic growth factors ergosterol (5,7,22-ergostatrien-3 β -ol; Sigma E-6510) and Tween-80 (polyoxyethylene-sorbitanmonooleate; Merck 822187) (see Andreasen & Stier, 1953, 1954) was prepared as described by Weusthuis *et al.* (1993). Glucose or maltose monohydrate, the growth factors and vitamins were added to the media after separate sterilization (Weusthuis *et al.*, 1993). Both the oxygen transfer properties of the cultures and the optimum Tween-80 concentration (Verduyn *et al.*, 1990) are functions of the biomass concentration in the reactor. Therefore, it was attempted to keep the biomass concentration in the cultures constant by increasing the reservoir concentration of the sugars with decreasing oxygen feed. In practice, biomass concentrations in the cultures typically varied between 2 and 3 g dry weight l⁻¹, with sugar concentrations in the reservoir medium ranging from 5 to 50 g l⁻¹. The purity of the chemostat cultures was routinely checked by phase contrast microscopy at 1000 \times magnification.

Oxygenation of the chemostat cultures. Oxygen was added to the cultures as air, using a peristaltic pump. The air flow rates ranged from 0 to 100 ml min⁻¹. The temperature of the ingoing air was kept constant at 20 °C. The overall gas flow into the cultures was maintained at 0.5 l min⁻¹ by the supplementary addition of nitrogen gas, using a Brooks 5876 mass flow controller. Addition of nitrogen gas assured good mixing of the air with the culture fluid and promoted anaerobiosis when air was not added. To minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation set-up (including medium reservoir and effluent vessel) was equipped with Norprene tubing and the reservoir vessel was flushed with nitrogen gas. The dissolved-oxygen concentration in the cultures was monitored with an Ingold polarographic oxygen electrode.

Gas analysis. Gas flows were measured with a self-constructed device, consisting of an inverted glass cylinder, filled with water. The cylinder was placed in a water-filled reservoir to prevent outflow of water, without touching the reservoir walls. A gas flow directed into the cylinder causes water to flow into the reservoir, which rested on an electronic balance. The weight of the water displaced per unit of time could, after the necessary corrections (e.g. ambient air pressure, temperature, pressure falls), be used to calculate the gas flow. Using this device, measurements were reproducible within 0.5%. Since the gas flows (nitrogen and air) into the cultures had to be interrupted before measurement, they were measured both before and immediately after steady-state analysis. Due to a slight loss of resilience of new tubing, usually a small difference between these measurements was found (on average 2%). The steady-state value was used in the calculations. The exhaust gas flow was determined on-line during the steady states. The oxygen content of the exhaust gas was determined with a Servomex oxygen analyser, CO₂ content with a Beckman infra-red CO₂ analyser. The exhaust gas entering both analysers was dried with a Perma Pure Dryer. Specific rates of CO₂ production and O₂ consumption were calculated according to van Urk *et al.* (1988). The amount of CO₂ leaving the culture with the effluent was negligible.

Metabolite analysis. Glucose and maltose concentrations were determined as described by Weusthuis *et al.* (1993). Ethanol,

glycerol and organic acids as pyruvate, succinate, fumarate and 2-oxoglutarate were determined by HPLC (Weusthuis *et al.*, 1993). Ethanol concentrations were also determined with an enzymic assay (based on alcohol oxidase; EK 001 Leeds Biochemicals). Both methods gave identical results.

Culture dry weights. Dry weights of culture samples were determined using a microwave oven and 0.45 μ m membrane filters as described by Postma *et al.* (1989b). Parallel samples varied by less than 1%.

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

Presentation of data. Several experimental approaches can be used to study the effects of oxygen on yeast metabolism. One possibility is to study the effect of dissolved-oxygen concentration (Brown & Rose, 1969; Moss *et al.*, 1969; Cysewski & Wilke, 1976; Nishizawa *et al.*, 1980; Furukawa *et al.*, 1983; Laplace *et al.*, 1991). However, at limiting oxygen supply rates, the dissolved-oxygen concentration falls below 1% air saturation and becomes difficult to measure accurately. Moreover, the anaerobic growth factors Tween-80 and ergosterol tend to foul oxygen electrode membranes, thereby further reducing the reliability of the measurements.

An alternative approach is to study the effect of oxygen feed rate (Oura, 1977; Grosz & Stephanopoulos, 1990; Kuriyama & Kobayashi, 1993). The effect of oxygen feed rate on growth and metabolism is strongly influenced by the gas transfer characteristics of the cultures, which are affected by biomass density and fermenter geometry. However, when oxygen feed rates are varied in identical fermentation set-ups with approximately equal biomass concentrations, this should allow a comparative study involving different carbon sources and yeast species. Nevertheless, data from this type of comparative studies cannot easily be extrapolated to alternative experimental set-ups.

In well-mixed systems, effects of gas transfer characteristics can be eliminated by using the specific oxygen uptake rate (q_{O_2}) as the experimental variable. This should allow extrapolation to other well-mixed fermenter set-ups, even if these exhibit different gas transfer properties. Unfortunately, since q_{O_2} is a derived parameter, its use will inevitably result in more scatter of the experimental data. In the present study, we have tried to relate experimental data to specific oxygen uptake rates whenever possible. However, at very low oxygen feeds, the off-gas oxygen analysis was not sufficiently sensitive to accurately calculate q_{O_2} (in some cases, this even resulted in negative apparent oxygen uptake rates). To enable comparison, even at low oxygen feed rates, of the four sets of experiments (two yeast species and two substrates), we attempted to keep fermenter geometry and biomass concentrations in all experiments constant (see above).

RESULTS

Relation between oxygen consumption rate and metabolic fluxes in *S. cerevisiae*: a simplified model

When the oxygen feed to sugar-limited cultures of the facultatively anaerobic yeast *S. cerevisiae* is varied, the biomass yield on sugar can be expected to vary between the growth yield observed under anaerobic conditions and the aerobic, 'respiratory' biomass yield. Because of the higher energetic efficiency of respiratory sugar metabolism, the biomass yield during respiratory growth

Table 1. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass and ethanol by *S. cerevisiae* CBS 8066 grown in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with glucose as a carbon source

Oxygen_{in}, oxygen feed rate. Oxygen_{out}, amount of oxygen leaving the culture. Glucose_{in}, reservoir concentration of sugar. Glucose_{out}, residual sugar concentration in culture.

Oxygen (mmol l ⁻¹ h ⁻¹)		Glucose (g l ⁻¹)		Dry weight (g l ⁻¹)	Ethanol (mM)
In	Out	In	Out		
0	0.32	24.6	< 0.1	2.52	242
0.70	0.85	22.8	< 0.1	2.64	201
0.93	0.73	23.7	< 0.1	2.70	240
4.7	2.6	15.5	< 0.1	2.56	172
7.3	6.0	11.4	< 0.1	1.99	99
13.5	8.9	7.6	< 0.1	2.07	58
17.5	13.7	6.5	< 0.1	2.15	41
24.2	18.2	5.1	< 0.1	2.67	14
33.1	25.6	5.2	< 0.1	3.18	< 1
46.6	39.3	4.6	< 0.1	2.79	< 1

Table 2. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass and ethanol by *S. cerevisiae* CBS 8066 grown in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with maltose as a carbon source

Oxygen (mmol l ⁻¹ h ⁻¹)		Maltose (g l ⁻¹)		Dry weight (g l ⁻¹)	Ethanol (mM)
In	Out	In	Out		
0	0	30.0	< 0.1	2.16	259
0	0	30.3	0.1	2.13	259
5.4	4.2	24.0	0.2	2.40	211
10.8	8.3	20.5	< 0.1	2.55	121
18.7	15.4	14.1	< 0.1	2.36	80
22.3	17.3	9.9	< 0.1	2.51	41
24.8	19.1	7.7	< 0.1	2.68	26
30.8	24.8	5.3	< 0.1	2.41	35
35.3	29.6	4.1	< 0.1	2.52	< 1
40.2	34.0	4.1	< 0.1	2.51	< 1
45.8	39.8	4.6	< 0.1	2.71	< 1

is ca. 5-fold higher than that under strictly anaerobic conditions (Verduyn *et al.*, 1990, 1991). Over this range of oxygen feeds, the specific oxygen uptake rate can be expected to vary between zero (under anaerobic conditions) and the rate corresponding to sugar-limited, fully respiratory growth.

At submaximal oxygen consumption rates, both respiration and fermentation can contribute to glucose metabolism. An increase of the oxygen consumption rate implies that more sugar is respired, thereby increasing the biomass yield. Alternatively, an increase of the biomass

yield can be achieved by increasing the concentration of sugar in the feed rate, which allows more sugar to be fermented. Growth under these conditions can therefore be described as dually limited by oxygen and glucose.

A simplified model describing biomass yield and metabolic fluxes in *S. cerevisiae* cultures growing under this dual limitation can be constructed by assuming that the energetic efficiency of fermentative and respiratory sugar metabolism is not affected by the simultaneous occurrence of both processes. If this assumption holds, and products other than CO₂, ethanol, biomass and water are neglected, the biomass yield in the oxygen- and sugar-limited cultures will be a simple function of the fraction of glucose that is metabolized by respiration. This fraction is equal to the ratio of the actual specific oxygen uptake rate (q_{O_2}) and the specific oxygen uptake rate during fully respiratory growth ($q_{O_2,R}$). The actual biomass yield on glucose is given by equation 1, in which Y is the actual biomass yield, Y_F is the anaerobic, fermentative biomass yield and Y_R is the aerobic, respiratory biomass yield on sugar.

$$\frac{1}{Y} = \frac{1}{Y_F} \left(1 - \frac{q_{O_2}}{q_{O_2,R}} \right) + \frac{1}{Y_R} \frac{q_{O_2}}{q_{O_2,R}} \quad (1)$$

Equation 1 is based on the assumption that the fraction of sugar that is fermented ($1/Y_F$) decreases linearly with increasing q_{O_2} . Correspondingly, with increasing q_{O_2} , the specific ethanol production rate (q_{ethanol}) will decrease linearly from the rate that is observed during anaerobic growth ($q_{\text{ethanol},F}$) to zero, according to equation 2.

$$q_{\text{ethanol}} = q_{\text{ethanol},F} \left(1 - \frac{q_{O_2}}{q_{O_2,R}} \right) \quad (2)$$

Dissimilatory production of CO₂ occurs both during respiration and fermentation. During complete respiratory dissimilation of glucose, the rate of CO₂ production is equal to the oxygen consumption rate [the respiratory quotient (RQ) being 1]. The rate of fermentative CO₂ production during alcoholic fermentation should be equal to the specific ethanol production rate given by equation 2.

Because yeast biomass is more oxidized than the substrate sugars, production of CO₂ also occurs as a result of assimilatory processes (see e.g. Bruinenberg *et al.*, 1984; Gommers *et al.*, 1988). If it is assumed that the biomass composition does not vary substantially, the specific rate of assimilatory CO₂ production should not be influenced by the oxygen feed rate. For *S. cerevisiae* cultures growing at a dilution rate of 0.10 h⁻¹, an assimilatory $q_{CO_2,A}$ of 0.58 mmol g⁻¹ h⁻¹ has been reported by Verduyn *et al.* (1990). When the three sources of CO₂ production are combined, this results in equation 3.

$$q_{CO_2} = q_{CO_2,A} + q_{O_2} + q_{\text{ethanol}} \quad (3)$$

Substitution of equation 2 in equation 3 gives equation 4, which indicates also that the specific CO₂ production rate of the cultures is a linear function of q_{O_2} .

$$q_{CO_2} = q_{CO_2,A} + q_{O_2} + q_{\text{ethanol},F} \left(1 - \frac{q_{O_2}}{q_{O_2,R}} \right) \quad (4)$$

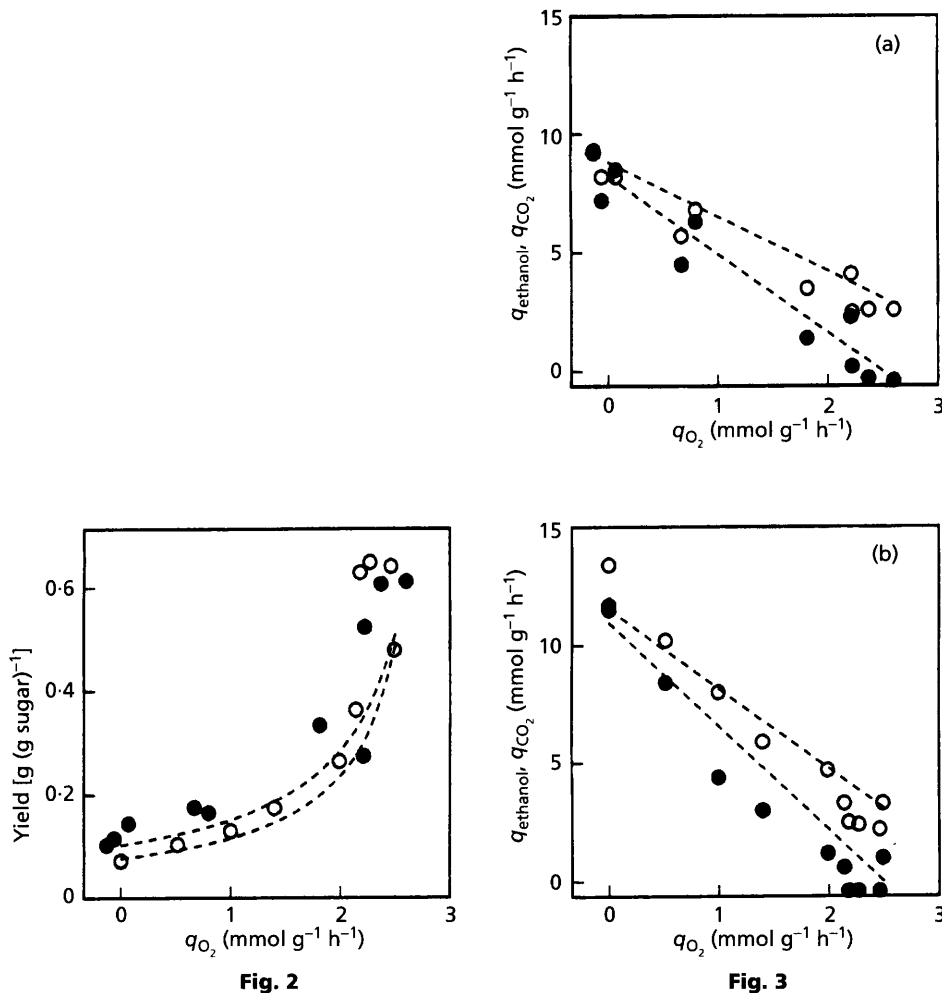


Fig. 2. Relation between the specific oxygen uptake rate (q_{O_2}) and biomass yield of *S. cerevisiae* CBS 8066, grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$), with glucose (●) or maltose (○) as a carbon and energy source. The data were fitted with equation 1 (dashed lines). Note that in this and the following figures, some negative q_{O_2} values are presented. This is due to the inaccuracy of the gas analysis at very low oxygen feed rates (see Methods).

Fig. 3. Relation between the specific oxygen uptake rate (q_{O_2}) and the specific production rates of ethanol ($q_{ethanol}$, ●) and CO₂ (q_{CO_2} , ○) of *S. cerevisiae* CBS 8066, grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$), with glucose (a) or maltose (b) as a carbon and energy source. The ethanol data were fitted with equation 2, the CO₂ data with equation 4 (dashed lines).

Effect of oxygen feed on growth of *S. cerevisiae* on glucose and maltose

The effects of oxygen on the physiology of *S. cerevisiae* were studied by varying the oxygen feed rate to glucose- and maltose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1} . At oxygen feed rates above $30 \text{ mmol l}^{-1} \text{ h}^{-1}$ and sugar concentrations below 5.0 g l^{-1} , growth of *S. cerevisiae* was not oxygen-limited: a further increase in air supply did not result in higher biomass yields (Tables 1 and 2). Growth was fully respiratory, as was evident from the absence of ethanol in the culture supernatants (Tables 1 and 2) and an RQ of approximately 1 (Fig. 3). The q_{O_2} of these oxygen-sufficient, glucose-limited chemostat cultures was ca. $2.5 \text{ mmol g}^{-1} \text{ h}^{-1}$ (Fig. 2). When q_{O_2} was lowered by decreasing the oxygen feed

rate, alcoholic fermentation set in (Tables 1 and 2; Fig. 3). This coincided with a decrease of the biomass yield (Fig. 2).

Over a range of oxygen consumption rates, respiratory and fermentative glucose metabolism occurred simultaneously. In these cultures, the biomass concentration could be increased either by increasing the oxygen feed rate (Fig. 2), or by increasing the sugar concentration in the reservoir medium (which led to increased alcoholic fermentation; data not shown). Therefore, such cultures grew under a dual limitation of sugar and oxygen.

Qualitatively, equation 1 gave a good description of the observed biomass yields at non-saturating oxygen feeds (Fig. 2). However, the apparent biomass yield of $0.63 \pm 0.02 \text{ g (g sugar)}^{-1}$ (Fig. 2) in the oxygen-sufficient cultures

Table 3. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass, ethanol and pyruvate by *C. utilis* CBS 621 grown in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with glucose as a carbon source

Oxygen ($\text{mmol h}^{-1} \text{ l}^{-1}$)		Glucose (g l^{-1})		Dry weight (g l^{-1})	Ethanol (mM)	Pyruvate (mM)
In	Out	In	Out			
0.17	0.51	45.5	28.3	1.50	170	5.8
0.33	0.61	45.5	17.3	1.99	265	4.1
0.77	0.69	45.5	17.8	2.00	262	3.8
1.8	1.3	45.5	0.9	2.22	340	8.7
2.9	2.3	45.5	16.2	2.50	242	8.0
4.3	3.0	28.4	< 0.1	3.20	252	2.4
9.3	5.8	14.2	< 0.1	2.70	90	0.2
11.4	8.9	10.5	< 0.1	2.21	82	< 0.1
16.7	13.1	7.7	< 0.1	2.38	42	< 0.1
18.6	14.9	7.7	< 0.1	2.57	54	< 0.1
24.4	18.8	6.4	< 0.1	2.99	24	< 0.1
22.3	15.9	5.9	< 0.1	3.14	17	< 0.1
32.6	25.3	4.6	< 0.1	3.03	< 1	< 0.1
47.8	40.1	4.6	< 0.1	2.96	< 1	< 0.1

was substantially higher than the 0.51 g g^{-1} reported by Verduyn *et al.* (1991) for aerobic growth of *S. cerevisiae*. An explanation for the high biomass yield is that all cultures were supplied with the anaerobic growth factors Tween-80 and ergosterol. Both were added as a stock solution in ethanol, resulting in an ethanol concentration of ca. 12 mM in the reservoir media. Under oxygen-sufficient growth conditions, ethanol was co-metabolized by the cultures, as evident from the apparent 'negative' q_{ethanol} at high oxygen feed rates (Fig. 3). Also oleic acid, added as Tween-80, may have a slight carbon-sparing effect and thus contribute to the biomass yield. When a correction is made for co-metabolism of ethanol (by assuming a growth yield of 0.61 g g^{-1} on ethanol; Verduyn *et al.*, 1991), the corrected growth yield on glucose under oxygen-sufficient conditions would be 0.53 g g^{-1} .

Both with glucose and maltose, q_{ethanol} and q_{CO_2} increased with decreasing q_{O_2} to reach a maximum in the anaerobic cultures (Fig. 3). Equations 2 and 4 gave a good fit of the experimental data.

The main difference between growth of *S. cerevisiae* on glucose and maltose was the consistently lower biomass yield (Fig. 2) and higher q_{ethanol} (Fig. 3) during oxygen-limited growth on maltose. The lower growth efficiency with maltose as the energy source can be fully explained by the fact that, unlike glucose, maltose is transported via a proton symport mechanism in *S. cerevisiae*. The net energy requirement for maltose transport is 1 mol ATP per mol maltose. Therefore, when maltose metabolism is fully fermentative, 1 out of the 4 ATP produced in glycolysis is used for maltose uptake, leading to a 25% lower biomass yield and a 43% higher q_{ethanol} (Weusthuis *et al.*, 1993). This tendency was indeed observed during anaerobic growth on maltose and glucose (Figs 2 and 3).

When sugar metabolism is respiratory, the ATP yield on maltose is much higher, and it is no longer possible to detect significant differences between the biomass yields on glucose and maltose (Fig. 2).

Effects of oxygen feed on growth of *Candida utilis* on glucose

As in *S. cerevisiae*, glucose metabolism of *C. utilis* was fully respiratory at oxygen feed rates above $30 \text{ mmol l}^{-1} \text{ h}^{-1}$ (Table 3). When the oxygen feed rate was reduced below this value, corresponding to an oxygen consumption rate of $2.5 \text{ mmol g}^{-1} \text{ h}^{-1}$, the biomass yield decreased (Fig. 4) and alcoholic fermentation set in (Table 3, Fig. 5).

At oxygen feed rates between 4.3 and $30 \text{ mmol l}^{-1} \text{ h}^{-1}$, virtually all the glucose was consumed (Table 3), with respiration and fermentation occurring simultaneously (Fig. 5). Qualitatively, the behaviour of the *C. utilis* cultures over this range of oxygen feeds was very similar to that of *S. cerevisiae* and growth could be considered as dually limited by glucose and oxygen (Table 3, Figs 4 and 5). However, when the oxygen feed rate was decreased below $4.3 \text{ mmol l}^{-1} \text{ h}^{-1}$, the residual glucose concentration in the cultures increased from below 0.1 g l^{-1} to 28.3 g l^{-1} , resulting in a decrease of the culture dry weights (Table 3). At these low oxygen feed rates, the biomass concentration in the cultures could no longer be increased by adding more glucose to the reservoir media. Apparently, at these low oxygen feed rates, growth of *C. utilis* was only limited by oxygen.

Attempts to grow *C. utilis* on glucose at oxygen consumption rates below $0.17 \text{ mmol g}^{-1} \text{ h}^{-1}$ resulted in wash-out. Apparently, the maximum anaerobic growth rate of *C. utilis* is lower than the dilution rate (0.10 h^{-1}). This is in agreement with the reported maximum growth rate of *C. utilis* in anaerobic batch cultures (0.01 h^{-1} ; Visser *et al.*, 1990).

As mentioned above for *S. cerevisiae*, the biomass yield of *C. utilis* under fully aerobic, respiratory conditions was higher than the value of 0.51 g g^{-1} reported by Verduyn *et al.* (1991), probably due to the presence of ethanol and oleic acid in the media. In view of the inability of *C. utilis* to grow anaerobically, the experimental data could not be fitted with equations 1 to 4. However, it is clear from Fig. 5 that during dual-substrate-limited growth, q_{ethanol} and q_{CO_2} did not increase linearly with decreasing q_{O_2} as predicted by equations 2 and 4. The observed non-linearity suggests that either fermentative metabolism or respiratory metabolism changes under dual substrate limitation. Factors that may be involved are changes in biomass composition or composition of the respiratory chain.

Compared to *S. cerevisiae*, the maximum q_{ethanol} of *C. utilis* at very low oxygen feeds was substantially higher and the biomass yield correspondingly lower (Figs 4 and 5). This lower efficiency of fermentative growth may be due to the involvement of a proton symport carrier in glucose transport by *C. utilis*. Also increased maintenance requirements, for example caused by the production of uncoupling weak acids, may contribute to the high q_{ethanol} at

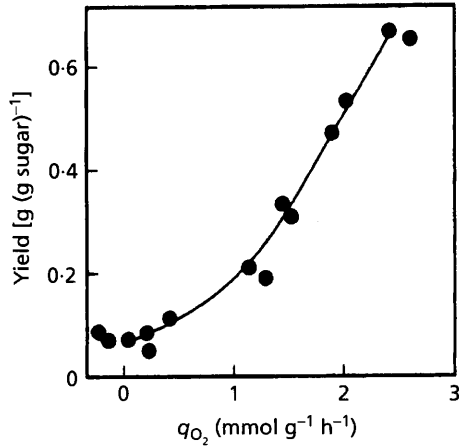


Fig. 4

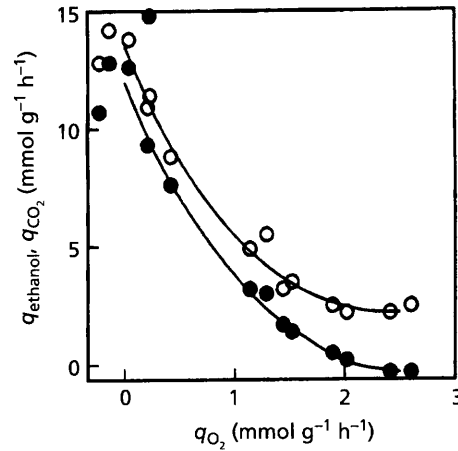


Fig. 5

Fig. 4. Relation between the specific oxygen uptake rate (q_{O_2}) and the biomass yield of *C. utilis* CBS 612, grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$), with glucose as a carbon and energy source.

Fig. 5. Relation between the specific oxygen uptake rate (q_{O_2}) and the specific production rates of ethanol (q_{ethanol} , ●) and CO_2 (q_{CO_2} , ○) of *C. utilis* CBS 612, grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$), with glucose as a carbon and energy source.

low oxygen feeds. Indeed, organic acid concentrations increased with decreasing oxygen feed (data not shown), with pyruvate concentrations up to 8 mM occurring in oxygen-limited *C. utilis* cultures (Table 3).

Table 4. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass, ethanol and pyruvate by *C. utilis* CBS 621 grown in chemostat ($D = 0.10 \text{ h}^{-1}$) with maltose as the carbon source

The presence of small amounts of ethanol in cultures growing at low oxygen feed results from the presence of this compound in the reservoir medium.

Oxygen ($\text{mmol h}^{-1} \text{ l}^{-1}$)		Maltose (g l^{-1})		Dry weight (g l^{-1})	Ethanol (mM)	Pyruvate (mM)
In	Out	In	Out			
2.5	2.0	4.6	ND*	0.28	10.3	< 0.1
4.7	4.0	45.5	48.4	0.43	10.2	< 0.1
4.7	3.8	4.6	4.6	0.38	4.7	< 0.1
10.9	7.0	12.3	11.0	1.88	< 1	< 0.1
14.5	11.1	10.5	9.2	1.88	< 1	< 0.1
14.8	11.2	4.6	2.6	1.76	< 1	< 0.1
15.6	11.3	4.6	ND	1.97	< 1	< 0.1
18.0	12.7	6.8	1.8	3.46	< 1	< 0.1
18.1	12.8	4.6	ND	3.07	< 1	< 0.1
21.1	17.2	6.8	0.8	3.21	< 1	< 0.1
22.8	19.2	5.5	4.0	2.45	< 1	< 0.1
32.5	26.2	4.6	0.7	2.87	< 1	< 0.1
47.6	40.4	4.6	ND	2.90	< 1	< 0.1

ND, Not determined.

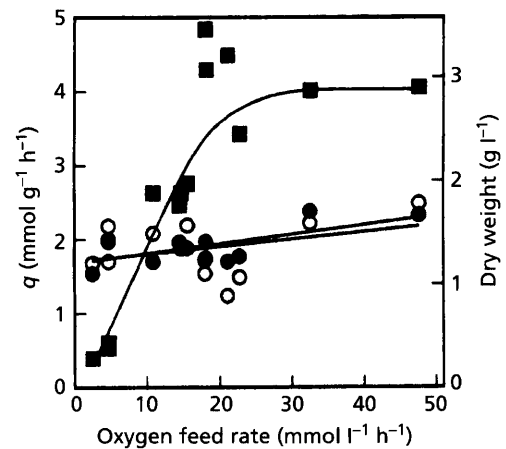


Fig. 6. Relation between oxygen feed rate and the dry weight (■), specific production rate of CO_2 (q_{CO_2} , ○) and specific consumption rate of oxygen (q_{O_2} , ●) of *C. utilis* CBS 612, grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$), with maltose as a carbon and energy source. Note that on the x-axis the oxygen feed rate is plotted and not, as in the other graphs, q_{O_2} .

Effects of oxygen feed on growth of *C. utilis* on maltose

The effect of the oxygen feed rate on growth of *C. utilis* on maltose was markedly different from the three situations described above (Fig. 6). In all cultures, the ethanol concentration was lower than in the reservoir medium (Table 4). Apparently, alcoholic fermentation did not occur at any of the oxygen feed rates tested. Instead, maltose metabolism was respiratory over the whole range

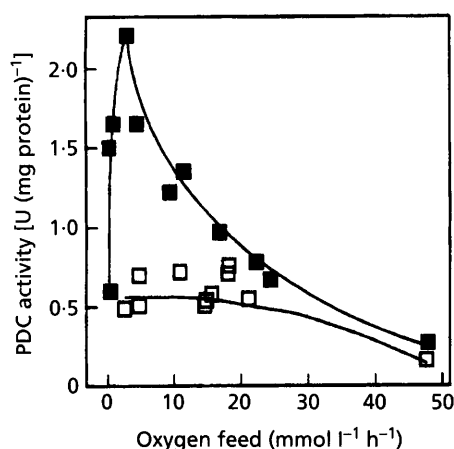


Fig. 7

Fig. 7. Relation between oxygen feed rate and PDC activity of *C. utilis* CBS 612, grown in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with glucose (■) or maltose (□) as a carbon and energy source.

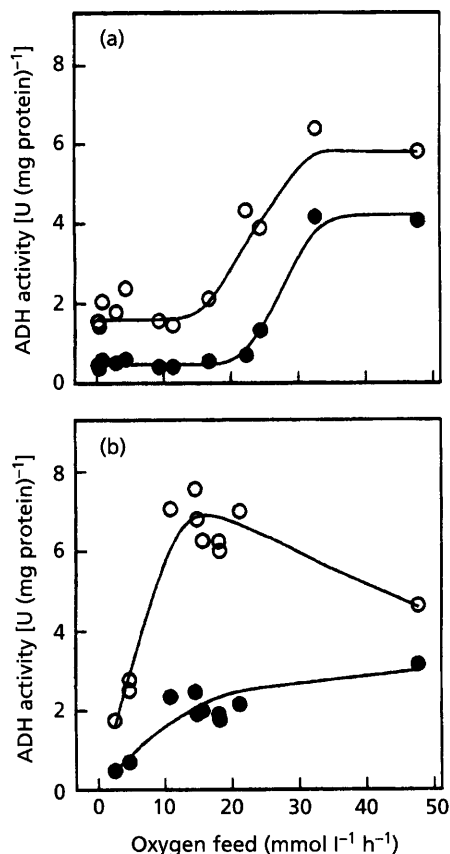


Fig. 8

Fig. 8. Relation between oxygen feed rate and ethanol-(○) and pentanol-(●) dependent ADH activities of *C. utilis* CBS 612, grown in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with glucose (a) or maltose (b) as a carbon and energy source.

of oxygen feed rates tested, as indicated by the constant q_{CO_2} and q_{O_2} (Fig. 6). Since the q_{O_2} hardly changed, culture parameters were plotted against the oxygen feed rate. In contrast to the results on glucose, organic acids could not be detected in any of the cultures (only shown for pyruvate, Table 4).

At oxygen feed rates below $30 \text{ mmol l}^{-1} \text{ h}^{-1}$, residual maltose concentrations in the cultures increased and the amount of biomass decreased (Table 4, Fig. 6). At these suboptimal oxygen feed rates, the culture dry weight could not be increased by adding maltose to the reservoir media (Table 4). A dual limitation of sugar and oxygen, as observed during growth of *C. utilis* on glucose, could not be attained with maltose, and growth was only limited by oxygen. The high residual maltose concentrations resulted in small differences between culture and reservoir sugar concentrations. As a consequence, a large variation occurred in the calculated biomass yields and maltose fluxes (results not shown).

Due to the low culture dry weights at low oxygen feed rates, it was not possible to test at which oxygen feed rate

maltose-grown cultures of *C. utilis* washed-out. The ethanol added to the medium reservoir accumulated in the culture at oxygen feed rates below $10.9 \text{ mmol l}^{-1} \text{ h}^{-1}$. With a biomass yield of *C. utilis* on ethanol of 0.69 g g^{-1} (Verduyn *et al.*, 1991), ethanol present in the media (12 mM) could only account for $0.38 \text{ g biomass l}^{-1}$. The observed biomass concentrations and ethanol concentrations in the culture (Table 4) hence indicate that respiratory growth of *C. utilis* on maltose occurred at all oxygen feed rates.

C. utilis exhibited a clear Kluyver effect for maltose: alcoholic fermentation of the disaccharide was not observed, while both respiratory metabolism of maltose and fermentation of its hydrolysis product glucose were possible (Kluyver & Custers, 1940; Sims & Barnett, 1978). An explanation for this apparent intrinsic inability to ferment maltose might be the absence of the fermentative key enzymes pyruvate decarboxylase and/or alcohol dehydrogenase during growth on the disaccharide. Therefore, the activities of these enzymes were assayed in cell-free extracts of maltose-grown *C. utilis*.

Effect of oxygen on pyruvate decarboxylase and alcohol dehydrogenase activities of *C. utilis*

Pyruvate decarboxylase (PDC) activities in glucose-grown *C. utilis* increased from 0.3 to 2.2 U (mg protein)⁻¹ with decreasing oxygen feed rates, parallel to the q_{ethanol} . An increase of PDC activities was also observed in the maltose-grown cultures: with decreasing oxygen feed, PDC activities increased from 0.2 to 0.6 U (mg protein)⁻¹ (Fig. 7). The maximum fermentation rates that can be sustained by the PDC activities measured in cell-free extracts can be calculated by assuming a soluble protein content of yeast biomass of 30%. Thus, the maximum observed PDC activity of 0.6 U (mg protein)⁻¹ would be sufficient to account for a maximum rate of ethanol formation of as high as 11 mmol (g dry wt)⁻¹ h⁻¹.

Various forms of the second key enzyme in alcoholic fermentation, alcohol dehydrogenase (ADH), occur in yeasts (Verduyn *et al.*, 1988). *S. cerevisiae*, for example, contains three isoenzymes: a constitutive cytoplasmic ADH functioning in alcoholic fermentation, an inducible cytoplasmic ADH active during growth on ethanol, and a mitochondrial ADH of which the function is unknown. The 'fermentative' ADH activity can be differentiated from the other two ADH activities by its inability to use pentanol as a substrate (Lutstorf & Megnet, 1968). A similar situation probably exists in *C. utilis*, although the role of the several isoenzymes has been less well studied. During growth of *C. utilis* on glucose, the pentanol-dependent ADH activity was approximately 4 U (mg protein)⁻¹ at high oxygen feed rates and decreased sharply to a basal level of 0.2 U (mg protein)⁻¹ when the oxygen feed rate was reduced. The high pentanol-dependent ADH activities coincided with the consumption of ethanol present in the reservoir media. Ethanol-dependent ADH activity showed a similar pattern, but was approximately 2 U (mg protein)⁻¹ higher in all cultures, suggesting a constitutive expression of the 'fermentative' ADH. During growth on maltose, pentanol-dependent ADH activity remained high over a broader oxygen feed range than during growth on glucose. Also in this case a good correlation existed with the occurrence of ethanol cometabolism. Under oxygen-limited conditions, the ADH activities of maltose-grown cells were equal to or higher than those of glucose-grown cells (Fig. 8).

Production of glycerol

In equations 1 to 4, fermentative sugar metabolism in yeasts has been simplified by assuming that ethanol is the sole fermentation product. However, it is well-known that in addition to ethanol, other metabolites may be excreted by yeasts. Quantitatively, glycerol is one of the major byproducts of alcoholic fermentation under anaerobic conditions.

Glycerol formation by yeasts may have various functions, some involved in osmoregulation. Under anaerobic conditions, however, the main physiological role of glycerol formation is related to redox metabolism. Since, as mentioned above, yeast biomass is more oxidized than

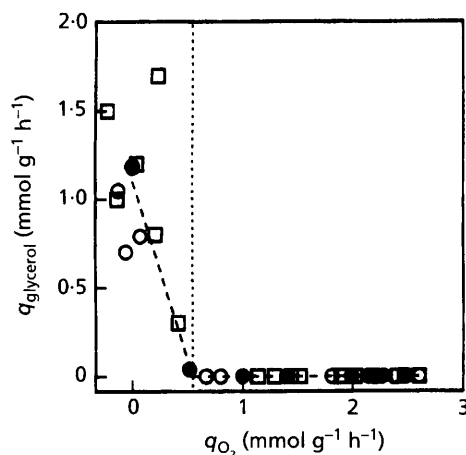


Fig. 9. Relation between specific oxygen uptake rate (q_{O_2}) and specific glycerol production rate (q_{glycerol}) of *S. cerevisiae* CBS 8066 (circles) and *C. utilis* CBS 612 (squares), grown at different oxygen feed rates in chemostat cultures ($D = 0.10 \text{ h}^{-1}$) with glucose (open symbols) or maltose (filled symbols) as a carbon and energy source. The dashed line indicates the q_{O_2} value below which glycerol formation must occur to obtain a closed redox balance.

the carbohydrate substrate, NADH is produced during assimilation of sugars. In the presence of oxygen, reduced cofactors can be reoxidized by respiration. When respiration is not possible, yeasts can reduce dihydroxyacetone phosphate to glycerol to close the redox balance (Holzer *et al.*, 1963; Gancedo *et al.*, 1968; Oura, 1977).

Glycerol formation branches off from the glycolytic pathway before the reactions that involve substrate-level phosphorylation. Therefore, formation of 1 mol glycerol from glucose requires the net hydrolysis of 1 mol ATP. In contrast, respiratory reoxidation of reduced cofactors yields metabolic energy by oxidative phosphorylation. To investigate the regulation of redox metabolism in yeasts, we studied the formation of glycerol at limiting oxygen feed rates.

Verduyn *et al.* (1990) reported that formation of 1 g *S. cerevisiae* biomass results in the formation of 11 mmol NADH. Under respiratory conditions, this NADH can be oxidized with 5.5 mmol oxygen. If redox metabolism in yeasts is regulated to minimize ATP expenditure for glycerol formation, glycerol should not be produced at specific oxygen consumption rates above $5.5 \times 0.1 = 0.55 \text{ mmol g}^{-1} \text{ h}^{-1}$ (product of oxygen requirement per unit yeast biomass and dilution rate). This could indeed be confirmed experimentally for growth of *S. cerevisiae* on either maltose or glucose, and for *C. utilis* grown on glucose (Fig. 9). Cultures in which q_{O_2} was decreased below this threshold invariably produced glycerol (Fig. 9). The glycerol production rates in anaerobic cultures of *S. cerevisiae* were in good agreement with the data of Verduyn *et al.* (1990). No glycerol formation was observed in the cultures of *C. utilis* grown on maltose (data not shown), consistent with their respiratory mode of metabolism.

The physiological necessity of glycerol production at very low oxygen feeds implies that the energetic efficiency of sugar metabolism is dependent on the oxygen feed. When the reducing equivalents produced during anabolic processes are oxidized by respiration, ATP is produced. However, when reoxidation occurs via glycerol formation, ATP is consumed. Consequently, equations 1 to 4 that were used to model growth and alcoholic fermentation in oxygen-limited cultures are an oversimplification.

DISCUSSION

Regulation of alcoholic fermentation in yeasts

Crabtree-positive yeasts, including *S. cerevisiae*, have a very strong tendency towards alcoholic fermentation. In practice, ethanol formation by these yeasts can only be avoided by growth under fully aerobic conditions with a limited supply of sugar. In contrast, fermentative metabolism in Crabtree-negative yeasts, including *C. utilis*, cannot be induced in the presence of excess oxygen. However, under oxygen-limited growth conditions, fermentation rates in Crabtree-positive and Crabtree-negative yeasts are comparable.

In the Crabtree-positive yeast *S. cerevisiae*, high levels of the fermentative key enzyme PDC are present under aerobic, glucose-limited growth conditions. These activities increase only approximately twofold upon a switch to respiro-fermentative growth. In contrast, only low PDC activities could be detected in aerobic, glucose-limited cultures of the Crabtree-negative yeast *C. utilis*. These activities increased sharply when *C. utilis* was grown under oxygen limitation, suggesting that oxygen may be a key factor in the regulation of PDC activity in this yeast (Franzblau & Sinclair, 1983). In fact, in cultures in which growth is limited only by glucose (i.e. at dissolved-oxygen concentrations above ca. 1% air saturation) the actual dissolved-oxygen concentration had a significant effect on PDC activities (data not shown). These data suggest that regulation of sugar metabolism at the level of pyruvate may well be responsible for the different behaviour of Crabtree-positive and Crabtree-negative yeasts.

Oxygen requirements for growth

Oxygen plays a dual role in yeast physiology: it is used as the terminal electron acceptor for mitochondrial respiration and for assimilatory oxygenation reactions. Respiration has two major functions: oxidation of reduced cofactors and generation of metabolic energy in the form of ATP. Under oxygen-limited conditions, energy transduction can be taken over by alcoholic fermentation. However, the conversion of glucose into ethanol is redox-neutral, and can therefore not be used to reoxidize the 'excess' reducing equivalents generated in assimilation. Glycerol production can serve as an alternative redox sink but requires a net input of ATP (van Dijken & Scheffers, 1986). Theoretically, therefore, yeasts can optimize their sugar metabolism under oxygen-limited growth con-

ditions by preferentially using oxygen for the regeneration of 'excess' NADH. The data presented in Fig. 9 indicate that in both *S. cerevisiae* and *C. utilis*, sugar metabolism is regulated in this way. At present, it is unclear at which level the preferential use of oxygen as an electron acceptor for NADH regeneration is regulated. At the kinetic level, a higher affinity of the mitochondrial external NADH dehydrogenase complex for NADH as compared to the cytosolic dihydroxyacetone phosphate reductase could be significant.

In addition to its role in respiration, oxygen has an essential role in assimilatory oxygenation reactions. Cellular constituents, the synthesis of which require molecular oxygen, must be added to growth media to allow anaerobic growth. In the case of *S. cerevisiae*, these 'anaerobic growth factors' are well-defined, and the organism grows well anaerobically in defined media supplemented with ergosterol, unsaturated fatty acids and nicotinic acid (Andreasen & Sier, 1953, 1954) (Table 1, Figs 2 and 3). In the same medium, however, *C. utilis* was not able to grow at a rate of 0.10 h^{-1} at zero oxygen feed (Table 4, Fig. 6). This confirmed earlier reports that *C. utilis* is unable to grow anaerobically at rates above 0.01 h^{-1} (Visser *et al.*, 1990). *C. utilis* is not an exception in this respect: apart from *S. cerevisiae*, none of the type species of the 75 yeast genera studied were able to grow under strictly anaerobic conditions with specific growth rates higher than 0.10 h^{-1} (Visser *et al.*, 1990). It is as yet unclear if unidentified assimilatory oxygen requirements are involved, or that these yeasts require oxygen for other cellular processes. This hitherto unexplained inability of non-*S. cerevisiae* yeasts to grow anaerobically is of great importance for some industrial applications of these organisms. The range of sugar substrates is much larger for these yeasts than for *S. cerevisiae*. Numerous processes have been suggested in which non-*Saccharomyces* yeasts could be used for the production of ethanol from waste streams and complex raw materials. A fact, overlooked in many of these publications, is that oxygen is required by these yeasts, even during fermentative growth. At very low oxygen feeds, growth (and eventually fermentation) becomes inhibited due to the intrinsic inability of these yeasts to grow anaerobically. At higher oxygen feeds, the glycolytic flux is preferentially directed towards respiration, thereby lowering the ethanol yield. This makes it rather difficult to optimize ethanol yields in large-scale industrial fermentations using non-*Saccharomyces* yeasts. However, in small-scale laboratory experiments, the oxygen requirements of these yeasts may easily go unnoticed. For example, if in the present study, oxygen-permeable silicone tubing (instead of Norprene) was used on the fermenters, *C. utilis* could be grown at a dilution rate of 0.10 h^{-1} in cultures sparged with pure nitrogen gas.

The Kluyver effect

The observation of Sims & Barnett (1978) that *C. utilis* exhibits a Kluyver effect during growth on maltose is clearly supported by the results presented in this study.

Over a range of oxygen feed rates, *C. utilis* grew strictly respiratorily on maltose, without the occurrence of alcoholic fermentation (Fig. 6, Table 4). Our results imply that, even in a situation where the enzymes for maltose uptake and hydrolysis were present, *C. utilis* did not ferment this disaccharide. In contrast, during oxygen-limited growth of *C. utilis* on glucose, respiration and fermentation occurred simultaneously (Fig. 5). The fermentation rates in these cultures (Fig. 5) were equal to or even exceeded those of the Kluver-negative yeast *S. cerevisiae*, which exhibited a respiro-fermentative metabolism during oxygen-limited growth on both glucose and maltose (Fig. 3).

The intrinsic inability of *C. utilis* to ferment maltose is important for the interpretation of the results of Sims & Barnett (1978). From experiments with a CO₂ electrode, these authors concluded that the fermentative activity of this yeast with maltose responded rapidly and reversibly to changes in the oxygen concentration. The results presented in this paper indicate that the CO₂ production measured in the experiments of Sims & Barnett (1978) was due to respiratory rather than fermentative maltose metabolism.

In earlier reports, low PDC activities have been reported for Kluver-positive yeasts grown on disaccharides (Sims & Barnett, 1991; Sims *et al.*, 1991). In the present study, the PDC and ADH activities in *C. utilis* grown on maltose in oxygen-limited chemostat cultures (Figs 7 and 8) were theoretically sufficient to sustain a q_{ethanol} of 11 mmol g⁻¹ h⁻¹. This theoretical flux is even higher than that observed in *S. cerevisiae* grown under the same conditions. Apparently, in *C. utilis* the Kluver effect for maltose is not caused by an insufficient capacity of PDC and ADH. Also, the absence of significant concentrations of organic acids in the maltose-grown *C. utilis* cultures (Table 4) indicates that a limited capacity of these fermentative enzymes is unlikely to cause the Kluver effect. Instead, maltose metabolism appears to be regulated before the level of pyruvate, i.e. at the level of disaccharide uptake, hydrolysis or glycolysis.

An inhibition of disaccharide transport activity by the absence of oxygen has been proposed as one of the possible causes of the Kluver effect (Sims & Barnett, 1978; Schulz & Höfer, 1986). However, low, but significant disaccharide uptake rates have been reported for Kluver-positive yeasts under anaerobic conditions (Sims & Barnett, 1978; Schulz & Höfer, 1986). Barnett & Sims (1982) therefore concluded that the Kluver effect cannot be solely caused by absence of transport activity. This conclusion is supported by the present study: under all oxygen feed regimes studied, *C. utilis* continued to take up and respire maltose. The total absence of alcoholic fermentation in maltose-grown, oxygen-limited cultures of *C. utilis* indicates that uptake and hydrolysis of maltose were stoichiometrically balanced with the amount of this disaccharide that could be respired. A mechanism which tunes disaccharide uptake and hydrolysis in response to oxygen concentration or redox potential may indeed be responsible for this phenomenon.

The original description of the Kluver effect (Sims & Barnett, 1978) discriminated between aerobic and anaerobic utilization of sugars. However, *C. utilis* and other yeasts exhibiting the Kluver effect are not capable of anaerobic growth (Visser *et al.*, 1990). Furthermore, the data presented here indicate that occurrence of the Kluver effect does not depend on the oxygen concentration, but reflects an intrinsic inability to perform fermentative metabolism with the disaccharide. We therefore propose a new definition of the Kluver effect: 'The inability to ferment certain disaccharides to ethanol and CO₂, even though respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexose(s) can occur'.

ACKNOWLEDGEMENTS

We are indebted to Dr Cornel Verduyn (BIRD Engineering, Schiedam, The Netherlands) for valuable discussions and Kjeld Bangma and Yvonne van Klei for their skilful technical assistance.

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Received 8 October 1993; revised 29 November 1993; accepted 7 December 1993