Stellingen

Behorende bij het proefschrift van Wiebe Visser: Oxygen requirements of fermentative yeasts

1. Bij studies naar de invloed van verschillende suikers op de vorming van esters bij brouwersgist wordt onvoldoende rekening gehouden met een verminderde biomassavorming bij groei op maltose als gevolg van het actief transport van deze suiker.
   Bijvoorbeeld: Yoshioka, K. & N. Hashimoto. (1984). *Agric. Biol. Chem.* **48**:333-340.

2. van der Aar *et al.* gaan in "possible misconceptions about oxygen consumption and carbon dioxide production measurements in stirred microbial cultures" ten onrechte voorbij aan de effecten van temperatuur en druk op het ingaande gasdebiet.
   van der Aar, P.C, A.H. Stouthamer & H.W. van Verseveld. (1989). *J. Microbiol. Meth.* **9** (4): 281-286.

3. Bij de ontwikkelingen in de Europese wetgeving met betrekking tot de toelaatbare concentraties nitraat in babyvoeding wordt onvoldoende rekening gehouden met eventuele positieve effecten van kleine concentraties nitraat op de preventie van schadelijke darminfecties.
   Benjamin, N., F. O'Driscoll, H. Dougall, C. Duncan, M. Golden & H. McKenzie. (1994). *Nature* **368**:502.

4. *Photobacterium phosphoreum*, een organisme dat licht uitstraalt bij voldoend hoge zuurstofconcentraties, brengt op gevoelige wijze aan het licht dat laboratoriumfermentoren vaak ten onrechte beschouwd worden als homogeen gemengd.
   J.P. van Dijken & and W. Visser, niet gepubliceerde resultaten.

5. Gezien het hoge aantal magnetosoom deficiënte mutanten in het relatief geringe aantal gescreende conjuganten, is het, in tegenstelling tot hetgeen in de titel wordt gesuggereerd, onwaarschijnlijk dat de geïsoleerde genen direct betrokken zijn bij de synthese van magnetosomen.
   Matsunaga, T., C. Nakamura, J.G. Burgess & K. Sode. (1992). *J. Bact.* **174**:2748-2753.

6. Ter verklaring van de vorming van glycerol in anaërobe cultures van *Saccharomyces cerevisiae* wordt in hoofdstuk 5 van dit proefschrift ten onrechte aangegeven dat de biomassa meer is geoxideerd dan de substraatsuikers.

7. De door Verduyn *et al.* gepresenteerde reactievergelijkingen zijn niet stoichiometrisch, en kunnen derhalve niet dienen als compleet model voor de metabole fluxen in dergelijke fermentaties.
   Verduyn, C., Postma, E., Scheffers, W.A. & J.P. van Dijken. (1990). *J. Gen. Microbiol.* **136**:395-403.

8. Het twee keer publiceren van dezelfde resultaten door dezelfde auteurs, met vrijwel letterlijk dezelfde teksten en dezelfde figuren in hetzelfde tijdschrift in hetzelfde jaar is een onaanvaardbare vervuiling van de wetenschappelijke literatuur.
   Garuti, G., M. Donahanyos and A. Tilch. 1992. *Wat. Sci. Tech.* **12** No 7 :383-394;
   Garuti, G., M. Donahanyos and A. Tilch. 1992. *Wat. Sci. Tech.* **25** No 12:185-195.

9. In verklaringen dat een lage contaminatie van pindakaas met *Salmonella* geen risico voor de volksgezondheid vormt, wordt ten onrechte voorbij gegaan aan de beschermende effecten van vet-encapsulatie.

10. De term non-respiring mitochondria verdient de voorkeur boven non-functioning mitochondria (dit proefschrift).

11. De positieve resultaten van "air ionisers" op het micro-klimaat die in de betreffende reclame-uitingen worden aangehaald berusten grotendeels op onzorgvuldig wetenschappelijk onderzoek.
   F.W.J. Schreij (1990). Kunstmatige ionisatie van de lucht; zin of onzin. Gezondheidsdienst (GGD) Stadsgewest 's Hertogenbosch.

12. Het is onaanvaardbaar dat veel koelkasten worden afgeleverd zonder deugdelijke thermometers.
   L. de Cezanne (1994). Koelkast temperaturen thuis. Keuringsdienst van Waren Leeuwarden.

13. De stelling dat de norm voor externe kwaliteitsborging ISO 9001 zich niet richt op de kwaliteit van de produkten of diensten is onjuist.

14. Bij de analyse van rampen met veerschepen als de Estonia wordt ten onrechte onvoldoende aandacht gegeven aan de invloed van de strakke dienstregelingen.

15. In de "snijtijdenwet" voor medici die na de "rijtijdenwet" voor chauffeurs is ingevoerd ontbreekt ten onrechte een analogon voor de tachograaf.

16. Van het oormerk voor runderen is alleen het oogmerk goed.

17. De rekenregels van de Vedische mathematica verdienen een grotere bekendheid.

18. Teneinde begripsverwarring te voorkomen moet ook de Nederlandse wetgever op officiële stukken de termen massa en gewicht niet onjuist gebruiken.
   zie b.v. de tekst op het rijbewijs.

19. Organisaties zijn altijd volop in beweging. Het is alleen jammer dat de analogie met de Brownse beweging in een gas vaak pijnlijk duidelijk is: alles beweegt, de som der bewegingen is nul, een richting valt niet te ontdekken, alleen de druk blijft.

Oxygen requirements of fermentative yeasts

Cover: Astrid van de Graaf, "*Discussie*" (1992)
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Foto: Fred Hammers, fotografische dienst STM (TUD)

Oxygen requirements of fermentative yeasts

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof. ir. K.F. Wakker,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College van Dekanen aangewezen,
op dinsdag 2 mei 1995 te 16.00 uur

door

Wiebe VISSER

doctorandus scheikunde

geboren te Rotterdam

Dit proefschrift is goedgekeurd door de promotor: Prof. dr. J.G. Kuenen

Dr. J.P. van Dijken heeft als begeleider in belangrijke mate aan het totstandkomen van het proefschrift bijgedragen.

Samenstelling promotiecommissie:

Rector Magnificus	Technische Universiteit Delft
Prof. dr. J.G. Kuenen	Technische Universiteit Delft
Prof. dr. N. Nanninga	Universiteit van Amsterdam
Prof. dr. ir. J.J. Heijnen	Technische Universiteit Delft
Prof. dr. K. Hellingwerf	Universiteit van Amsterdam
Dr. J.P. van Dijken	Technische Universiteit Delft
Ir. W.A. Scheffers	Technische Universiteit Delft
Dr. P.C. van der Aar	Heineken Technical Services B.V.

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Aan mijn vader

Contents

CHAPTER 1

General introduction

The role of oxygen in nature

Oxygen is an important element in the earth's biosphere. The earth contains approximately 410×10^3 Emole (1 examole = 10^{18} mole) of which roughly 10 % is present as water. Molecular oxygen (O_2), also roughly 10% of the total content, is for 99% found in the atmosphere, contributing 21% of all gases. For 1%, molecular oxygen is found in the hydrosphere (Elstner, 1987).

Oxygen is involved in many biological reactions, leading to a continuous turnover of approximately 15×10^3 Emole/Myear (M= mega= 10^6) according to Gilbert (1981). An overview of the oxygen cycle is presented in Fig. 1.

Respiration.

Although molecular oxygen is not a very reactive gas chemically, after first an activating reaction (at the expense of some energy), the reduction of activated oxygen ($O_2^{\cdot-}$, superoxide radical anion) eventually yields a lot of energy (ΔG_0= 120 kcal). This is due to the high redox potential between O_2 and H_2O.

Although O_2 is utilised by organisms to produce energy via the respiratory chain, oxygen is not always available to them. Apart from a total lack of oxygen, as found under anaerobic conditions, low solubility in water (approximately 240 μmole·l^{-1} at 30 °C) readily leads to a shortage of this electron acceptor.

Since the aquatic environment plays a pivotal part in the earth's life cycles, it is not

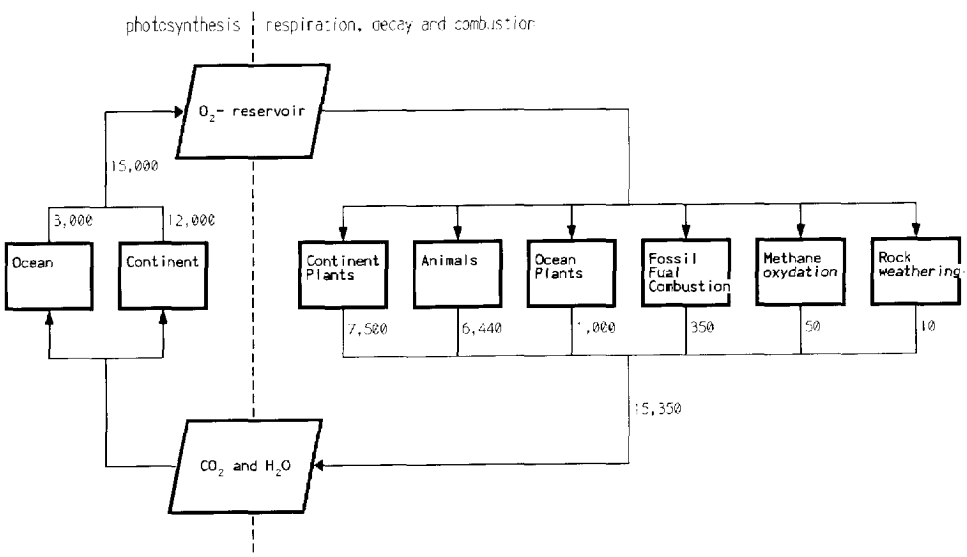


Fig. 1. Overview of the oxygen cycle, indicating the importance of individual processes with respect to the total turnover. Note the importance of biological processes and the disturbance introduced by fossil fual combustion. Figures, in Emoles/Myears, are from Gilbert (1981).

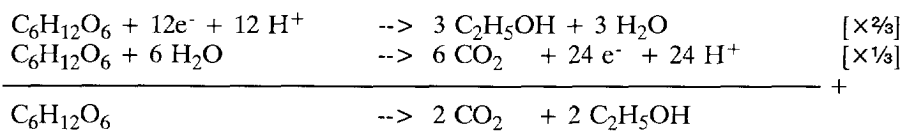
surprising that other mechanisms exist for biological energy supply, circumventing this shortage of oxygen. Other electron acceptors may be used for respiration instead of oxygen, e.g. nitrate (reacting to nitrite, nitrous oxide or even molecular nitrogen), sulphate, and bicarbonate, yielding sulphide and methane respectively. However, many organisms are unable to use these alternative electron acceptors. For a long time nitrate respiration was thought to be an exclusive capacity of prokaryotic organisms, until nitrogen respiration of a protozoa (*Loxodes*) was reported (Finlay, 1983). This finding prompted the question: "Will yeast never learn?" (Cole, 1988). So far, however, no yeast has been reported with respiration mechanisms using other electron acceptors than oxygen.

Fermentation

Yeasts, as well as many other organisms, pro- and eukaryotes, can solve this shortage of electron acceptor under oxygen-limited conditions by a completely different type of metabolism, known as fermentation.

In fermentation reactions the energy source, e.g. glucose, is also used as a source of electron acceptors. Part of the glucose is oxidized (as is the case in respiration), but another part is metabolized to reduced products and subsequently released as waste product by the organism.

The most important fermentation for yeasts is alcoholic fermentation, in which part of the glucose is reduced to ethanol. In principle this is a simple redox reaction, clearly shown by adding the two half-reactions for alcoholic fermentation:



$C_6H_{12}O_6 + 12e^- + 12\ H^+ \quad \text{--> } 3\ C_2H_5OH + 3\ H_2O \quad [\times 2/3]$

$C_6H_{12}O_6 + 6\ H_2O \quad \text{--> } 6\ CO_2 + 24\ e^- + 24\ H^+ \quad [\times 1/3]$

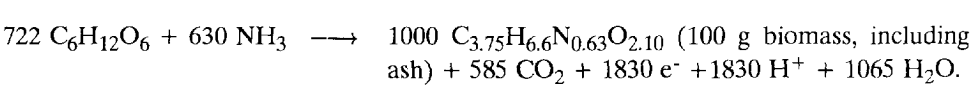
$+$

$C_6H_{12}O_6 \quad \text{--> } 2\ CO_2 + 2\ C_2H_5OH$

Redox balance

Whenever an external electron acceptor is not available, the maintenance of a redox balance as presented above for alcoholic fermentation, is of vital importance for an organism (van Dijken and Scheffers, 1986). This maintenance of a closed redox balance during fermentation processes is a complex interplay of oxidation and reduction processes. Biomass itself is more reduced than glucose and thus may serve as electron acceptor for some of the excess reducing equivalents produced, but during biomass production biochemical restrictions leading to the formation of CO_2 force the organism to produce much more reducing equivalents from glucose than can be absorbed in the biomass (585 mmol CO_2 and 1170 mmol NADH per 100 g biomass, Verduyn *et al.*, 1990).

Using the biomass composition as determined by Verduyn *et al.* (1990) the following reaction equation for biomass synthesis from glucose and NH_3 can be calculated:



$722\ C_6H_{12}O_6 + 630\ NH_3 \longrightarrow 1000\ C_{3.75}H_{6.6}N_{0.63}O_{2.10}$ (100 g biomass, including ash) $+ 585\ CO_2 + 1830\ e^- + 1830\ H^+ + 1065\ H_2O.$

The total redox balance of biomass formation therefore yields a surplus of electrons which must be disposed of. In absence of oxygen, yeast may produce the more reduced metabolite glycerol from glucose. Although this requires extra glucose consumption and additional input of energy, it may restore the redox balance, according to:

$$C_6H_{12}O_6 + 4\ e^- + 4\ H^+ \longrightarrow 2\ C_3H_8O_3$$

By multiplying the last reaction by 1830/4 and subsequently adding both equations, a redox neutral reaction equation is found for biosynthesis:

$$1180\ C_6H_{12}O_6 + 630\ NH_3 \longrightarrow 1000\ C_{3.75}H_{6.6}N_{0.63}O_{2.10} + 585\ CO_2 + 915\ C_3H_8O_3 + 1065\ H_2O.$$

The energy required for the production of biomass and glycerol is obtained from alcoholic fermentation as described above. With a cell yield of 0.103 g (g glucose)$^{-1}$ (Verduyn *et al.*, 1990), under anaerobic conditions 5394 mmol glucose is needed for the formation of 100 gram biomass. Since 1180 mmol is used in anabolic reactions, 4214 mmol glucose is fermented to ethanol and CO_2.
The complete fermentation reaction therefore is:

$$5394\ \text{glucose} + 630\ NH_3 \longrightarrow 100\ \text{g biomass} + 8428\ \text{ethanol} + 915\ \text{glycerol} + 9013\ CO_2 + 1065\ H_2O.$$

During anaerobic growth of yeast some organic acids may be produced: succinate, pyruvate and acetate. Succinate and pyruvate are more oxidized than glucose, and during the production of succinate and acetate, carbon dioxide is an inevitable by- product. This leads again to the generation of excess reducing power and hence the organism must produce even more glycerol to remove the extra electrons. Using the concentrations found by Verduyn (1990) for these acids, the fermentation reaction is adapted as follows:

$$5394\ \text{glucose} + 630\ NH_3 \longrightarrow 100\ \text{g biomass} + 8308\ \text{ethanol} + 962\ \text{glycerol} + 8\ \text{pyruvate} + 38\ \text{succinate} + 21\ \text{acetate} + 8893\ CO_2 + 1065\ H_2O.$$

The calculated concentrations of ethanol and glycerol fit with the measurements of Verduyn *et al.* (1990): 8263 mmol ethanol and 898 mmol glycerol per 100 g biomass.
An overview of the fermentative metabolism in yeast is presented in Fig. 2.

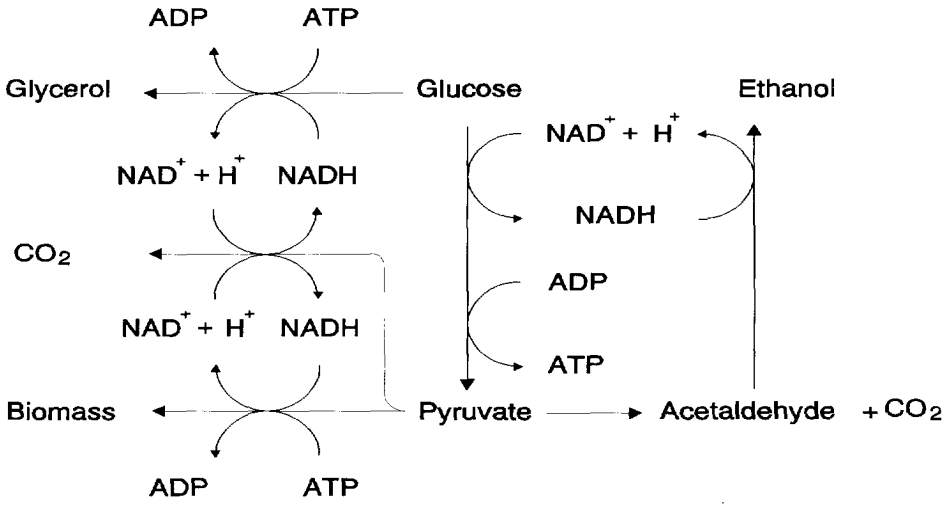


Fig 2. Schematic presentation of redox balances in anaerobically grown yeasts. Note that it is the excess CO_2 production concomittant to biosynthesis causing a surplus of NADH. Glycerol production is carried out to restore the redox balance.

Industrial applications of fermentation

Mankind has exploited fermentation reactions of yeast species for the production of alcoholic beverages and for the leavening of dough for many centuries. The production of beer and wine nowadays still is of significant economical importance. Breweries belong to the biggest companies in the world.

Knowledge of the microorganisms and, subsequently, of the fermentation mechanisms involved, initiated by the findings of Louis Pasteur at the end of the nineteenth century, broadened the industrial use of fermenting microorganisms. For example, during the first world war, glycerol needed for the production of explosives in Germany was produced by the addition of sodium bisulphite to fermenting yeast, a process called after its inventor, Carl Neuberg (Neuberg's second fermentation). The bisulphite reacts with acetaldehyde wh2alsoich thus is no longer available as electron acceptor for the production of ethanol. As a result, the yeast is overproducing NADH and is forced to produce glycerol as shown above in order to restore its disturbed redox balance.

For the leavening of dough, a huge amount of biomass (approximately 2 million tons in 1990) has to be produced on a daily base. Since this bulk product has little added value, efficiency of production (optimizing biomass yield and fermentation capacity) is of economical importance. For this purpose detailed knowledge of the physiology is necessary. The most important property of baker's yeast, its capability to ferment immediately when mixed with dough, easily leads to fermentation during biomass production, thereby lowering the yield.

During the last two decades the production of ethanol by fermentation (bioethanol, biofuel) has become more important, since it is evident that fossil fuel reserves are

rapidly being consumed. Bioethanol therefore is a logic alternative, since it is a renewable resource and does not contribute to a net increase in atmospheric carbon dioxide.
The yeast *Saccharomyces cerevisiae* (baker's yeast and brewer's yeast) is the most extensively used industrial yeast at present. However, although this yeast ferments hexoses very efficiently, it is not able to use pentoses as a substrate. But, when bioethanol is to be produced from lignocellulosic material like agricultural crop residues and wood, hydrolysis of its hemicellulose component will yield a mixture of pentoses and hexoses. In order to achieve an optimal ethanol production from these substrates, during the past decade yeasts other than *S. cerevisiae* capable of pentose fermentation were studied more extensively. From this research it became evident that oxygen plays a very important role in the fermentation of pentoses (e.g. Bruinenberg *et al.*, 1984; Skoog and Hahn-Hägerdahl, 1988; Grootjen *et al.*, 1990).

Not only yeasts, but also bacteria are used in industrial fermentations. For example, *Zymomonas mobilis* is capable of a highly efficient conversion of glucose to ethanol (low growth yield of the organism) under strictly anaerobic conditions (Sahm and Bringer-Meyer, 1987). However, the high substrate specificity (Swings and De Ley, 1977) and its sensitivity towards high osmotic pressure (Bajpai and Margaritis, 1984) limits its industrial use.

Other bacteria may have broader substrate spectra (*Clostridium saccharolyticum*, *Thermoanaerobacter ethanolicus*), but since these bacteria show a heterofermentative metabolism, the forming of secondary fermentation products as acetic acid will inhibit their growth. Furthermore, this formation by bacteria of by-products like acetate and formate, is complicating the downstream processing of ethanol (Esser and Karsch, 1984). Apart from ethanol production, a new technical use for yeasts became apparent during the last decade: expression of heterologous DNA. Large-scale production of heterologous proteins requires a thorough knowledge of the genetics and physiology of the yeasts involved. For example, the high cell densities required for economical production of such proteins may easily lead to gradients in the oxygen and/or substrate concentrations as a result of imperfect mixing. These gradients will definitely influence the metabolism of the organism, thus disturbing the production.

A better understanding of the biochemical background of the regulation of metabolic fluxes in yeasts therefore is of great importance for biotechnological applications of these organisms.

Alcoholic fermentation by yeasts.

For a long time, yeasts have been divided into three different groups with respect to their ability to ferment sugars, namely:

1. ***Obligatory fermentative yeasts.***
   These organisms will dissimilate glucose exclusively by fermentation. Mostly these organisms are indicated as respiratory deficient. Example: *Arxiozyma telluris* (formerly: *Torulopsis pintolopesii*; *Candida slooffii*).

2. ***Facultatively fermentative yeasts.***
   Organisms from this group are able to alter their metabolism between respiratory and fermentative according to the environment, e.g. *Saccharomyces cerevisiae* and

Candida utilis.

3. ***Non-fermentative yeasts.***
Non-fermentative yeasts are defined as not being capable of alcoholic fermentation at all, e.g. some *Rhodotorula* species.

Approximately 40% of the yeast species described so far (590) belong to the last group. However, this classification is based upon a test in which visible gas formation in Durham tubes is used as the criterion for alcoholic fermentation. Although this test may be useful for taxonomic purposes, it has been shown (Van Dijken *et al.*, 1986) that the criterion, as such, is not valid, since small amounts of ethanol were detected even under the standard conditions used and, more strikingly, that for a number of strains (e.g. *Pichia nonfermentans*) high fermentation rates could be achieved by adaptation of the culture conditions, e.g. in to shake-flask cultures. This again confirmed that the occurrence and intensity of alcoholic fermentation strongly depend on the culture conditions.

Regulation of alcoholic fermentation in yeasts

The metabolism of yeasts is drastically influenced by the growth conditions, especially the occurrence of alcoholic fermentation which has been categorized into the Pasteur-, the Crabtree-, the Custers-, and the Kluyver-effect.

Influence of the oxygen concentration.

Many organisms are facultative anaerobes, respiring whenever oxygen is available and using other compounds as electron acceptors when transferred to anaerobic conditions. During respiration the energy yield obtained from the energy source, e.g. sugars, is much higher, i.e. more ATP is formed per mole of sugar, then during fermentation. This large difference in ATP yield may lead to a smaller rate of sugar consumption in aerobiosis than in anaerobiosis. This slowing down, or inhibition of sugar consumption is called the "Pasteur effect". It is not restricted to yeasts; also many cell types of higher organisms show this behaviour (Krebs, 1972; Malaise,, 1988). In fact, *S. cerevisiae*, the most well-known facultatively anaerobic yeast, generally does not show the Pasteur effect when transferred from anaerobic to aerobic conditions. In growing cells of this organism, the rate of ethanol production is hardly influenced by the oxygen concentration. In this yeast the Pasteur effect only occurs under special conditions, like in sugar-limited chemostat cultures (Lagunas, 1981). Other yeasts, like *Candida utilis*, do not perform alcoholic fermentation under aerobic conditions.

Despite the fact that *S. cerevisiae* has frequently been used as a model organism for studying the Pasteur effect, in some cases the effect of aerobiosis is just the opposite, as already was shown by Pasteur himself: fermentation is stimulated when the anaerobic conditions are alleviated. This can be explained by the inability of *S. cerevisiae* to grow anaerobically for more than a few generations (see also below: anabolic oxygen demand). Also in other cases it was reported that the absence of oxygen could lead to an inhibition of alcoholic fermentation. Custers (1940) demonstrated that oxygen increased the fermentation rate of cells of *Brettanomyces spp.*, a phenomenon he called "negative Pasteur effect". It was shown by Scheffers (1966) that this effect was due to a disturbed redox balance, since the addition of an H-acceptor like acetoin could eliminate this

negative Pasteur effect. Scheffers introduced the term Custers effect for the phenomenon. Thus, availability of oxygen plays a complex role in the fermentative behaviour of yeasts. When available in excess, it blocks alcoholic fermentation in many yeasts (e.g *C. utilis*, Van Dijken and Scheffers, 1984), but on the other hand fermentation may be enhanced by limited oxygen supply in many species (Scheffers, 1966; van Dijken *et al.*, 1986; Moss *et al.*, 1969).

Even when glucose fermentation does occur under anaerobic conditions, fermentation of other sugars like xylose still may require oxygen.

For *C. utilis* it has been shown that this oxygen requirement for xylose fermentation finds it origin in a disturbed redox balance. Xylose metabolism in this yeast is started by an NADPH-dependent xylose reductase, whereas the subsequent step is carried out by an NAD^+-linked xylitol dehydrogenase. The reduced co-enzyme in the second reaction (NADH) is not reoxidized and the fermentation cannot proceed (Bruinenberg *et al.*, 1983 and 1984).

Influence of the substrate concentration.

Although oxygen limitation may trigger alcoholic fermentation in many yeasts, it is not an exclusive prerequisite. Yeasts like *S. cerevisiae*, as already indicated above, will ferment glucose despite the presence of sufficient oxygen. This phenomenon is called the "glucose"- or Crabtree effect (De Deken, 1966; Fiechter *et al.*, 1981) and was initially found in tumour cells (Warburg, 1926; Crabtree, 1929). However, aerobic fermentation is dependent on the glucose concentration in the medium. At low concentrations, not exceeding 100-200 $mg \cdot l^{-1}$, the metabolism in *S. cerevisiae* is fully respiratory (Verduyn *et al.*, 1984; Van Dijken and Scheffers, 1984).

Facultatively fermenting yeasts are categorized according to their ability to ferment under aerobic conditions into Crabtree-positive (showing the Crabtree effect, e.g. *S. cerevisiae*) and Crabtree-negative yeasts (e.g. *C. utilis*).

The Crabtree effect clearly complicates the production of baker's yeast, since alcoholic fermentation reduces the biomass yield significantly. The sudden fermentative response to high sugar concentrations under fully aerobic conditions is called the "short-term Crabtree effect", as opposed to the occurrence of alcoholic fermentation under steady-state conditions at high growth rates, the long-term Crabtree effect.

The short-term Crabtree effect has been explained by a limited capacity of the respiratory pathways (Petrik *et al.*, 1983; Rieger *et al.*, 1983) and by a limited capacity of assimilatory pathway (Van Urk *et al.*, 1988).

The long-term Crabtree effect has been ascribed to a limited respiratory capacity of the yeasts involved (Käppeli, 1986; Petrik *et al.*, 1983). However, chemostat studies on the effects of weak acids have shown that *S. cerevisiae* can respire glucose at very high rates (20 mmol $O_2 \cdot g^{-1} \cdot h^{-1}$) and that its respiratory capacity therefore is not intrinsically lower than that of Crabtree-negative yeasts.

At high growth rates in continuous cultures acetate is formed also. It has been shown (Postma *et al.*, 1989) that the uncoupling effect of weak acids on respiratory energy production triggers alcoholic fermentation.

Influence of the nature of the sugar.

In yeast physiology, an old rule of thumb, the Kluyver rule, tells that a yeast that does not ferment glucose will not ferment any sugar (Kluyver, 1914). However, when glucose is fermented by a yeast this does not imply that other sugars will be fermented as well. As already explained above, the specific biochemistry of *C. utilis* involved in the metabolism of xylose prevents its fermentation.

In fact, facultatively fermentative yeasts growing aerobically on a particular sugar may differ widely in their capacities to ferment that sugar under anaerobic conditions. For disaccharides this effect has been designated the Kluyver effect (Sims and Barnett, 1978). The mechanism of the Kluyver effect is still unclear; for a recent review the reader is referred to Barnett (1992) and Weusthuis (1994).

Although oxygen has often been implicated as a key factor for the occurrence of the Kluyver effect, its role has not yet been studied under controlled conditions of oxygen limitation.

Anabolic oxygen demand.

Although, as explained above, fermentation can provide energy for growth, this is not the only requirement for strictly anaerobic growth. The influence of oxygen is not only exerted on catabolic processes as respiration and fermentation, but also on anabolic processes. It was already demonstrated by Cochin, a pupil of Pasteur, that prolonged anaerobic growth is dependent on the composition of the medium. In a specially developed apparatus (Fig. 3) he showed that malt extract could not sustain anaerobic growth after three serial transfers, whereas in yeast-extract containing media anaerobic growth continued. Clearly in yeast extract growth-promoting factors are available that were missing in the malt extract medium.

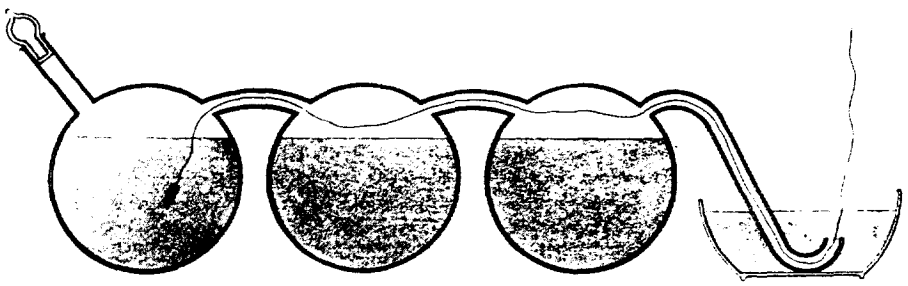


Fig. 3. The Cochin apparatus used in educational experiments to demonstrate that under strict anaerobiosis S. cerevisiae does not grow after three serial transfers in malt extract, in contrast to a medium with glucose and yeast extract. By pulling the wire through a bowl with mercury the yeast is transferred from one compartment to the next.

Some decades later these factors were identified to be sterols and unsaturated fatty acids (Andreasen and Stier, 1953 and 1954) and nicotinic acid (Suomalainen *et al.*, 1965). The synthesis of these compounds requires traces of molecular oxygen, due to the involvement of oxygenase-catalyzed reactions. It has been shown by these authors that for the yeast *S. cerevisiae* the addition of ergosterol, Tween 80 and nicotinic acid may successfully supplement the medium, thereby circumventing this anabolic oxygen requirement.

This clearly shows, that for *S. cerevisiae* (electron transport coupled) mitochondrial ATP-synthesis is not a necessity for growth. For other yeasts, especially Crabtree-negative yeasts, anaerobic growth appears impossible, despite the addition of the above-mentioned growth factors. This may be an indication that for these organisms mitochondrial ATP-synthesis is required. Support for this view is found in the inability of Crabtree-negative yeasts to form respiratory- deficient mutants. These mutants, often called "petites" in view of their smaller colonies on aerobic plates (Ephrussi *et al.*, 1949), have lost their ability to respire glucose and hence all ATP-production must result from fermentation as is the case in anaerobic cultures. Most probably such petite-mutations are lethal to Crabtree-negative yeasts (De Deken, 1966), indicating their dependence on mitochondrial ATP-synthesis.

Anaerobic continuous cultures.

Although many reports exist on the anaerobic cultivation of yeasts, continuous cultures under these conditions have rarely been described (e.g. Schatzmann, 1975). A possible reason is that strictly anaerobic conditions are not easy to achieve, not even in batch cultures.

This is nicely illustrated by the problem of growing strictly anaerobic bacteria, like sulphate reducers, as already discussed by Beijerinck (1895):

> "Hat man *Spirillum desulfuricans* einmal in Reinkultur gebracht, so muß man nicht glauben, daß dann die Schwierigkeiten für die weitere Untersuchung verschwunden sind. Ganz im Gegenteil, dieselben werden dann noch viel größer wie beim Rohmaterial. Dieses resultiert aus der Notwendigkeit der Durchführung von Kautelen, wodurch der Sauerstoff vollständig entfernt wird, und Jeder, welcher sich mit solchen Versuchen beschäftigt hat, welche sehr oft wiederholt werden müssen, weiß, wie aufreibend es ist, in dieser Beziehung radical zu handeln, wenn man dabei nicht aërobische Microbiën zu Hilfe nehmen kann."

When continuous cultures are used, the difficulties are rapidly increasing, since the fermenter used cannot be closed completely. Not only is it difficult to prevent leakages in the more complex fermenter system, but also medium will be pumped in and out continuously.

Control of the anaerobic conditions achieved also has its difficulties. The actual concentrations of oxygen or the redox potential of the culture medium can be measured, but, due to rapid consumption, this hardly gives information on the amount of oxygen actually entering the fermenter.

It can be calculated that during anaerobic cultures the oxygen does not contribute significantly to the overall metabolic fluxes in the organism. For example, glycerol production is indicating that the redox balance is not restored by the traces of oxygen entering the culture.

However, only traces of oxygen are needed for the synthesis of sterols and fatty acids.

Leakage or diffusion of traces of oxygen into the culture system is illustrated by reports on the anaerobic growth in the absence of these growth requirements (Macy and Miller, 1983; Verduyn *et al.*, 1990).

The small scale of laboratory fermenters also complicates anaerobic growth, since a larger surface/volume ratio increases the effect of diffusion. Therefore, despite all precautions taken, "strictly anaerobic conditions" have probably not yet been achieved in anaerobic chemostat cultures of yeasts.

Mitochondrial functions

In eukaryotic cells many processes are carried out in different compartments or organelles. Yeast cells are no exception in this respect and therefore they have been used frequently as model organisms to study specific organelles (Scheffers and Van Dijken, 1993). Its facultatively anaerobic character has made *S. cerevisiae* an attractive experimental system for studying the biogenesis of mitochondria.

Mitochondria are well known for containing the oxidative phosphorylation system and TCA cycle enzymes, but they also contribute to the biosynthesis of haem, pyrimidines, amino acids, phospholipids, nucleotides, folate coenzymes, urea and many other metabolites (Attardi and Schatz, 1988).

Under anaerobic conditions mitochondria loose their function in the generation of ATP. It is now more generally accepted that compartments surrounded by biological membranes can only be propagated and transferred to new cells by division of pre-existing compartments (Palade, 1983). Although early reports claimed that mitochondria were absent in anaerobically-grown cells (Wallace and Linnane, 1964; Linnane, 1965), later on membranous structures resembling mitochondria were found and *de novo* synthesis of yeast mitochondria was finally ruled out (Damsky *et al.*, 1969). Since such membranous structures exhibit many properties typical of mitochondria but yet are very different from mitochondria in aerobically-grown cells, the term "promitochondria" was proposed to express this relation (Schatz, 1965).

Complete absence of mitochondria under anaerobic conditions would also conflict with the above-mentioned functions apart from ATP-synthesis, unless, under anaerobiosis, the enzymes involved are localized elsewhere under anaerobiosis.

However, the fragile structure of promitochondria (Criddle and Schatz, 1969; Damsky *et al.*, 1969) is a complicating factor in the localization of these enzymatic activities. A gentle method for the isolation of promitochondria therefore will be necessary for this purpose.

Recently more evidence for the indispensability of mitochondria has been put forward. Only five mitochondrial proteins have been found to be essential for viability of *S. cerevisiae*; all of them are key components of the mitochondrial import system (Baker and Schatz, 1991).

When indeed promitochondria fulfil an important function in anaerobically-grown cells, an intriguing problem is the way in which the cell accomplishes energy-requiring transport across the mitochondrial membranes under those conditions. Under aerobic conditions the required ATP is synthesized within the mitochondria by respiration and the majority is exported to the cytoplasm. This transport is catalyzed by a special carrier, the ADP/ATP-translocator, which is the most abundant protein in the inner mitochondrial membrane (Klingenberg, 1976). Under anaerobic conditions, however, the ATP produced

by glycolysis is cytoplasmatic. Import of ATP and subsequent hydrolysis in the mitochondria therefore seems inevitable for strictly anaerobic growth. Evidence for such ATP import is given by the effect of a specific inhibitor of the ADP/ATP-translocator, bongkrekic acid, on the growth of a respiratory-deficient yeast (Šubík *et al.*, 1972; Gbelská *et al.*,1983) and, more recently, by genetic studies in which the genes coding for the translocator have been deleted (Drgoň *et al.*, 1991).

Mitochondria of aerobically-grown yeasts are easily detected by thin-section electron microscopy, and are regularly distributed in the periphery of the cytoplasm (Fig. 4). However, serial sections have revealed the existence of a single, highly branched mitochondrion and it was postulated that the idea of numerous mitochondria resulted from misinterpretation of random thin sections (Hoffman and Avers, 1973). Subsequent studies with serial section microscopy have revealed that both views may be correct: the life cycle and physiological state determines the number and volume of mitochondria (Grimes *et al.*, 1974; Stevens, 1977). These findings suggest that mitochondria are not rigid structures, but are highly flexible. Large mitochondria may break up into smaller ones and these may fuse again to a larger organelle. So far, very little information is available on this dynamic behaviour and on its importance for the mitochondrial function.

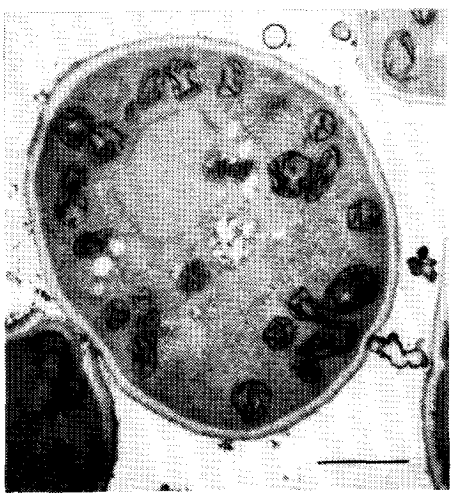


Fig. 4. Electron micrographs of aerobically grown S. cerevisiae cells (bar, 1 μm), showing the small mitochondria distributed in the periphery of the cell.

Outline of this thesis.

Most yeast species are able to perform alcoholic fermentation, yet few can grow in the complete absence of oxygen. The aim of this study is to acquire more insight in the oxygen requirements of yeasts and in the role of the respiring organelles under conditions of oxygen limitation.

In chapter 2, a screening among the type species of the yeast genera is described for the ability of strictly anaerobic growth. Furthermore a comparative study on anaerobic growth is carried out with a number of selected strains.

In chapter 3, the physiological role of mitochondria is investigated via enzyme localization studies. Since physiological activity of the mitochondrion is likely to require energy, also the import of ATP into the mitochondrion is studied using a specific inhibitor.

Changes in the physiology of yeasts are reflected in the morphology of the mitochondria. In chapter 4 the effects of environmental conditions on the structure of mitochondria of living *S. cerevisiae* cells are described, clearly showing a relationship between mitochondrial morphology and metabolic activity.

Many yeasts need a small amount of oxygen, also when they are able to ferment the sugar supplied. Studies on the Kluyver effect therefore should include conditions of oxygen limitation, in order to reveal the physiological explanation for this phenomenon. So far all studies towards the Kluyver effect have been performed in batch cultures, complicating the interpretation of the results. In chapter 5 a comparison is made between oxygen-limited chemostat cultures of *S. cerevisiae* and *C. utilis*, growing on glucose and maltose. Of the two yeasts only *C. utilis* is known to show the Kluyver effect for maltose.

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CHAPTER 2

Oxygen Requirements of Yeasts

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Abstract

Type species of 75 yeast genera have been examined for their ability to grow anaerobically in complex and mineral media. To define anaerobic conditions a redox indicator, resazurin, was added to the media to signify low redox potentials. All strains tested were capable of fermenting glucose to ethanol in oxygen-limited shake-flask cultures, even those species generally regarded as "non-fermentative". However, only 23 % of the yeast species tested grew under anaerobic conditions. A comparative study with a number of selected strains revealed that *Saccharomyces cerevisiae* stands out as a yeast capable of rapid growth at low redox potentials. Other yeasts, such as *Torulaspora delbrueckii* and *Candida tropicalis*, grew poorly (μ_{max} 0.03 and 0.05 h^{-1}, respectively) under anaerobic conditions in mineral media supplemented with Tween 80 and ergosterol. The latter organisms grew rapidly under oxygen limitation and then displayed a high rate of alcoholic fermentation. It can be concluded that these yeasts have hitherto unidentified oxygen requirements for growth.

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Introduction

Yeasts can be divided into three groups with respect to their fermentative abilities, namely: obligately fermentative, facultatively fermentative, and non-fermentative yeasts. Approximately 40 % of the yeast species is listed as non-fermentative (Barnett *et al*, 1983). The listing is based upon a standard test with Durham tubes, in which visible gas production is used as the criterion for alcoholic fermentation. Nearly all non-fermentative yeasts, however, have been shown to produce ethanol to some extent under the conditions in the Durham-tube test, and their fermentative capacities often are greatly enhanced under more appropriate conditions (Van Dijken and Scheffers, 1986).

One of the most important parameters with respect to the occurrence of alcoholic fermentation in yeasts is the concentration of oxygen in the culture medium. For example, the Pasteur effect, the Custers effect (Scheffers, 1966; Wijsman *et al*, 1984; Custers, 1940), the Kluyver effect (Kluyver and Custers, 1940; Sims and Barnett, 1978) and the Crabtree effect (Crabtree, 1929; Fiechter *et al*, 1981; Van Urk *et al*, 1988) are all closely related to the availability of oxygen.

Fermentation, in principle, can provide enough energy for growth. However, the ability to grow anaerobically depends not only on the fermentative capacity. Many species require a limited amount of oxygen to ferment glucose, for example *Hansenula nonfermentans* (Van Dijken and Scheffers, 1984). Even when fermentation does occur under anaerobic conditions, as reported for *Pachysolen tannophilus*, actual growth of the organism on D-xylose as well as on D-glucose is still dependent on the availability of oxygen (Neirinck *et al*, 1984). The addition of ergosterol and unsaturated fatty acids, which are considered to be essential medium components for anaerobic growth of *Saccharomyces cerevisiae* (Andreasen and Stier, 1953; Andreasen and Stier, 1954), does not eliminate this oxygen requirement.

Whether or not yeast species other then *S. cerevisiae* are able to grow anaerobically is in general unknown, or the results are contradictory. For example, for *Candida utilis* anaerobic growth has been reported by several authors (Babij *et al*, 1969; Haukeli and Lie, 1971), whereas others have stated that *C. utilis* is not able to grow anaerobically (Van Dijken and Scheffers, 1986).

A major problem in comparing the results of different studies with respect to anaerobic growth of yeasts is the definition of anaerobiosis. Also, the preparation of the inoculum is important: when aerobically grown cells are used as an inoculum for anaerobic cultures, rapid growth may occur for a number of generations even in the absence of ergosterol and unsaturated fatty acids (Kirsop, 1982).

The purpose of the present study was to examine the capacity of various yeasts for anaerobic growth under standardized conditions. In order to obtain a broad spectrum of the yeasts, type species of the genera, as listed by the CBS List of Cultures (Centraal Bureau voor Schimmelcultures, Delft, the Netherlands) were studied.

Materials and methods

Organisms.
Type strains of the yeast genera were obtained from the Centraal Bureau voor Schimmelcultures (CBS) at Delft and are cited as listed in the CBS List of Cultures 31st edition 1987 or 32nd edition 1990 (in press):
Ambrosiozyma monospora CBS 2554, *Apiotrichum porosum* CBS 2040, *Arthroascus javanensis* CBS 2555, *Arxiozyma telluris* CBS 2685, *Botryoascus synnaedendrus* CBS 6161, *Brettanomyces bruxellensis* CBS 72, *Bullera alba* CBS 501, *Candida tropicalis* CBS 94 , *Candida utilis* CBS 621 (not a type species), *Citeromyces matritensis* CBS 2764, *Clavispora lusitaniae* CBS 6936, *Cryptococcus albidus* CBS 142, *Debaryomyces hansenii* CBS 767, *Debaryozyma yamadae* CBS 7035, *Dekkera bruxellensis* CBS 74, *Eeniella nana* CBS 1945, *Endomycopsella vini* CBS 4110, *Fellomyces polyborus* CBS 6072, *Fibulobasidium inconspicuum* CBS 8237, *Filobasidiella neoformans* CBS 132, *Filobasidium floriforme* CBS 6241, *Guilliermondella selenospora* CBS 2562, *Hanseniaspora valbyensis* CBS 479, *Hasegawaea japonica* CBS 354, *Holleya sinecauda* CBS 8199, *Holtermannia corniformis* CBS 6979, *Hormoascus platypodis* CBS 4111, *Hyphopichia burtonii* CBS 2352, *Issatchenkia orientalis* CBS 5147, *Kloeckera apiculata* CBS 287 (not a type species), *Kluyveromyces polysporus* CBS 2163, *Leucosporidium scottii* CBS 5930, *Lipomyces starkeyi* CBS 1807, *Lodderomyces elongisporus* CBS 2605, *Malassezia furfur* CBS 1878, *Mastigomyces philippovii* CBS 7047, *Metschnikowia bicuspidata* CBS 5575, *Mrakia frigida* CBS 5270, *Myxozyma melibiosi* CBS 2102, *Nadsonia fulvescens* CBS 2596, *Nematospora coryli* CBS 2608, *Octosporomyces octosporus* CBS 371, *Oosporidium margaritiferum* CBS 2531, *Pachysolen tannophilus* CBS 4044, *Pachytichospora transvaalensis* CBS 2186, *Phaffia rhodozyma* CBS 5905, *Pichia membranaefaciens* CBS 107, *Rhodosporidium toruloides* CBS 14, *Rhodotorula glutinis* CBS 20, *Saccharomyces cerevisiae* CBS 1171, *Saccharomycodes ludwigii* CBS 821, *Saccharomycopsis capsularis* CBS 2519, *Sarcinosporon inkin* CBS 5585, *Schizoblastosporion starkeyi-henricii* CBS 2159, *Schizosaccharomyces pombe* CBS 356, *Schwanniomyces occidentalis* CBS 819, *Sirobasidium magnum* CBS 6803, *Sporidiobolus johnsonii* CBS 5470, *Sporobolomyces roseus* CBS 486, *Sporopachydermia lactativora* CBS 6989, *Stephanoascus ciferrii* CBS 6699, *Sterigmatomyces halophilus* CBS 4609, *Sterigmatosporidium polymorphum* CBS 8088, *Sympodiomyces parvus* CBS 6147, *Torulaspora delbrueckii* CBS 1146, *Trichosporon beigelii* CBS 2466, *Trigonopsis variabilis* CBS 1040, *Waltomyces lipofer* CBS 944, *Wickerhamia fluorescens* CBS 4565, *Wickerhamiella domercqiae* CBS 4351, *Williopsis saturnus* CBS 5761, *Yarrowia lipolytica* 599, *Zygoascus hellenicus* CBS 5839 (mating type α), *Zygoascus hellenicus* CBS 6736 (mating type a), *Zygosaccharomyces rouxii* CBS 732, *Zygozyma oligophaga* CBS 7107.

Media.
Complex medium contained per liter demineralized water: Yeast extract (Oxoid) 10 g, glucose 20 g. The initial pH of the medium was set to 5.
The mineral medium, supplemented with vitamins and trace elements, was prepared according to Bruinenberg (Bruinenberg *et al*, 1983[b]), except that the concentration of $NaMoO_4 \cdot 2H_2O$ was increased tenfold. Glucose was added as sole source of carbon and energy, in a concentration of 20 $g \cdot l^{-1}$. Ergosterol and Tween 80, dissolved in pure ethanol, were sterilized by heating the solution during 10 min in a non-pressurized autoclave. These components were added to the medium to a concentration of 6 $mg \cdot l^{-1}$ and 660 $mg \cdot l^{-1}$, respectively. Resazurin was added to both media to a concentration of 0.002 %, to indicate low redox potentials (E_o'= -42 mV).

Inocula.
Inocula for anaerobic growth tests were prepared by growing the yeasts in 100 ml cottonwool-plugged Erlenmeyer flasks containing 20 ml medium. Cultures were incubated on a rotary shaker at 25° C and 50 rpm. Under these conditions, alcoholic fermentation can be triggered in facultatively fermentative yeasts, due to oxygen limitation (Van Dijken *et al*, 1986). Therefore, cells from these shake-flask cultures can be considered to be adapted to serve as an inoculum for anaerobic growth tests.

Anaerobic growth test.
The anaerobic tests were conducted in 30 ml serum flasks under static incubation at 25 °C. In order to prevent the entrance of oxygen the flasks were firmly closed with 4 mm thick butyl rubber septa. The flasks were almost completely filled with the medium and autoclaved at 110 °C. During autoclaving reducing agents in the complex medium converted the redox indicator to colorless dihydroresorufin. The mineral medium treated similarly did not become colorless. It was therefore deoxygenated prior to autoclaving by including 8 $mg \cdot l^{-1}$ *Aspergillus niger* glucose oxidase/catalase (grade III, Boehringer, Mannheim, FRG). The redox indicator in the serum flasks septa, treated this way, remained colorless for at least 4 months, whereas the use of ordinary rubber septa (red rubber, BGA class 1 FDA) caused re-colorization within a few hours, due to a high rate of oxygen diffusion. After sterilization the flasks were not opened to prevent re-entrance of oxygen. A small amount (approx 5 µl) of the inoculum was then injected into the flasks with a 2 ml syringe. The syringe was left in place during the incubation and thus served as an indicator of CO_2 production. Gas production and turbidity of the inoculated flasks were checked twice a day for 1 month; prolonged incubation after this period did not lead to the onset of anaerobic growth.

Batch cultivation in fermenters.
Comparative studies were performed in a laboratory fermenter with a 1 liter working volume, of the type described by Harder (Harder *et al*, 1974). The mineral medium as described above was used. To prevent foaming, 50 µl antifoam per liter were added. pH Was controlled at 5.0 by automatic titration with sterile 1 M KOH, the temperature was kept at 30 °C and the cultures were stirred at 450 rpm. To exclude a possible infection, the organism was checked afterwards by the identification service of the Centraal Bureau voor Schimmelcultures.

Levels of dissolved-oxygen tension (DOT) were measured with a polarographic oxygen electrode, type Ingold 322 756702/74247, connected to an Ingold O_2 amplifier type 170 (% air). The signal of this amplifier was monitored via a Kipp BD 41 data recorder (Kipp & Zonen, Delft, The Netherlands).
The fermenter was continuously flushed with pure nitrogen gas, containing less than 5 ppm oxygen (obtained from Air Products, Waddinxveen, The Netherlands), at a flow rate of 1 $l \cdot min^{-1}$. The tubing of the fermenter was made of Norprene (Cole-Parmer Instruments Corp., Chicago, USA).

Analytical methods.
Ethanol concentrations were determined by gas liquid chromatography on a Varian 3400 type gas chromatograph (Varian Benelux B.V., Amsterdam, the Netherlands), using a Hayesep. Q column (Chrompack, Middelburg, The Netherlands) in a temperature range of 150-225 °C, increasing 15 $°C \cdot min^{-1}$).
Glucose concentrations were determined by the GOD-PAP method of Boehringer (Mannheim, FRG).
Dry weight was determined by filtration of the culture sample over a weighted polysulfon filter (pore size 0.45 μm, Gelman Sciences Inc., Michigan, USA). The filter was washed with demineralized water and dried in an R-7400 magnetron oven (Sharp Inc., Osaka, Japan) for 20 min at medium power, and re-weighted.
Carbon analyses were done with a Model 915B Tocamaster Total Organic Carbon Analyzer (Beckman Industrial Corp., La Habra, CA, USA).
Organic acids were determined by HPLC on a HPX-87H column (300 x 7.8 mm, Bio-Rad, Richmond, CA, USA) at room temperature. The column was eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 $ml \cdot min^{-1}$. The detector was a Waters 441 UV-meter at 210 nm, which was coupled to a Waters 741 data module (Waters, Milford, MA, USA).

Electron microscopy.
Twenty-two ml of cell culture was pre-fixed with 3 ml 25% (v/v) glutaraldehyde for 10-30 min at room temperature. After centrifugation the cells were fixed again with 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0, for 1 h, washed 3 times with the same buffer, and post-fixed with 2% (w/v) aqueous $KMnO_4$ for 2-24 h. After fixation the cells were stained with 1.5 % (w/v) aqueous uranyl acetate during dehydratation at the 50% (v/v) ethanol step and finally embedded in Spurr resin. Ultrathin sections, post-stained with Reynolds lead citrate for 10 s, were examined in a Philips EM 201 electron microscope at 60 kV.

Chemicals.
Resazurin was obtained from Janssen Chimica, Beerse, Belgium.
Tween 80 was obtained from E. Merck Nederland B.V. (Amsterdam, the Netherlands).
Antifoam was obtained from BDH (Poole, England).

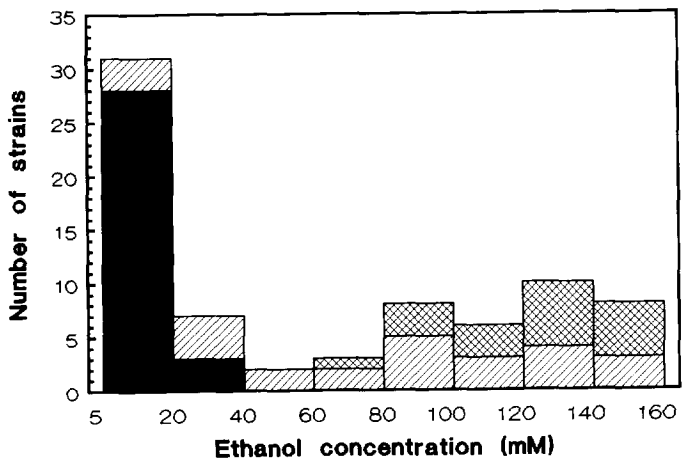

Fig. 1. Distribution of ethanol production in shake-flask cultures over fermentative (▨ and ▩) and non-fermentative (■) strains. The concentration of ethanol in the cultures was determined at the end of the exponential growth phase. ▩*, Number of the strains growing anaerobically in the serum flask tests.*

Results

Ethanol production in oxygen-limited shake-flask cultures.
When grown in shake-flask culture all strains produced ethanol, even those presently considered as non-fermentative on the basis of taxonomic tests (Barnett *et al*, 1983). However, in such strains ethanol production was generally rather low (0-20 Mm, Fig. 1). In some cases (for example *Filobasidiella neoformans*) the amount of ethanol in shake-flask cultures was as high as with typical facultatively fermentative yeasts such as *Eeniella nana* and *Debaryomyces hansenii*.

Screening for anaerobic growth.
When transferred to anaerobic conditions in serum flasks, only few species exhibited clear anaerobic growth with concomitant ethanol production, both in complex (yeast extract-containing) and in mineral medium (supplemented with Tween 80 and ergosterol). These were *A. telluris, C. tropicalis, C. utilis, C. matritensis, C. lusitaniae, E. nana, H. valbyensis, I. orientalis, K. apiculata, K. polysporus, N. coryli, P. transvaalensis, S. cerevisiae, S. ludwigii, S. pombe, T. delbrueckii, W. fluorescens, W. saturnus*. In many cases long lag times (5-10 d) elapsed before growth started, despite the fact that the yeasts had been pre-grown under oxygen-limited conditions. The number of generations in the flasks with positive growth was 8-10, according to dry-weight measurements. In this screening it was not possible to determine growth rates, but *S. cerevisiae* finished growth in 24 h, leading

to an estimated specific growth rate of approximately 0.3 h^{-1}. *S. cerevisiae* therefore stood out as a strong fermenter that readily adapts to anaerobic growth conditions.

Maintenance of anaerobic conditions in small fermenters.
The maintenance of anaerobic conditions in microbial cultures is usually accomplished via the addition of reducing agents such as sulfide or sulfite. For growing yeasts such compounds cannot be included in the media, since they strongly inhibit growth. As a consequence, anaerobic conditions in yeast cultures are often qualified as "flushed with nitrogen", and it is implicitly assumed that the actual concentration of oxygen under those circumstances is zero (e.g. 5).

Generally the problems in establishing anaerobic yeast cultures are (i) how to define and measure anaerobic conditions? and (ii) how to achieve and maintain anaerobic conditions? The first problem can be solved either by measuring the oxygen concentration directly with an oxygen probe or by measuring the redox potential of the medium. The last measurement is indirect, since the redox potential is only partially dependent on the oxygen concentration. The relationship between the redox potential and the concentration of dissolved-oxygen in microbial cultures has been described by Wimpenny and co-workers (Wimpenny, 1969; Wimpenny and Necklen, 1971). The redox potential is taken as an appropriate parameter for the definition of anaerobic circumstances, especially for the cultivation of strictly anaerobic bacteria (Hungate, 1969). It can be measured *in situ* by an appropriate probe or can be indicated by a redox dye, e.g. resazurin.

Direct measurement of low concentrations of oxygen has become possible with the development of sensitive and stable polarographic oxygen sensors, which are able to detect dissolved-oxygen tension (DOT) values down to 0.001% of air saturation. It turned out that one has to be very careful in the conversion of redox potentials into oxygen levels. For example, it was not possible to obtain redox potentials low enough to decolorize the resorufin simply by purging the medium with nitrogen gas, although a DOT of 0.005% was reached. On the other hand, in a culture of *C. utilis* such a redox potential was obtained at DOT values of 0.01-0.02 % of air saturation, indicating that the redox potential and DOT show different relationships when the conditions are changed. Furthermore, the oxygen probe, and to some extent also the redox potential, only indicate the actual activity of oxygen in the medium. They do not provide information about the actual oxygen flux. Illustrating in this respect is our observation that resorufin was colorless even in a cotton-stoppered shake-flask culture of *C. utilis*, presenting culture conditions still regularly considered as aerobic (Ligthelm *et al*, 1988). The decolorization is due to the rapid oxygen consumption of the yeast and hence is not indicating strictly anaerobic growth. In order to define anaerobic conditions it is therefore necessary to determine the actual flux of oxygen into the system.
To investigate the magnitude of the oxygen influx, the fermenter was flushed with pure nitrogen until a value was reached of 0.005% air saturation. Subsequently the outlet gasflow was blocked and an extra pressure of 0.1 atm was built up by using a water column of 1 m height. The diffusion of oxygen into the fermenter is demonstrated in Fig. 2.

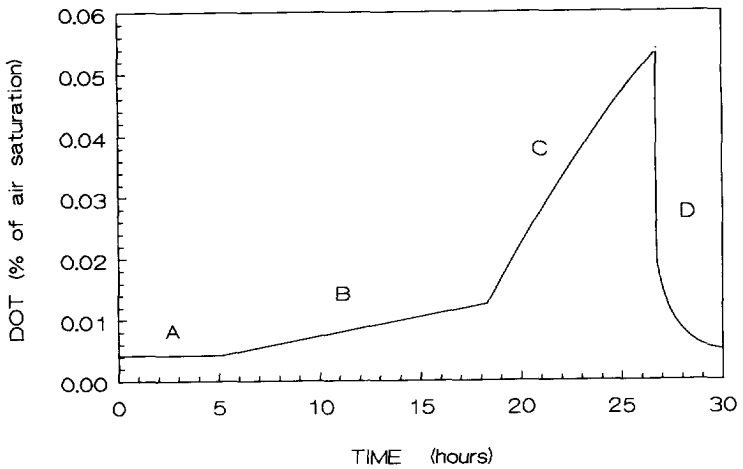

Fig. 2. Oxygen diffusion into a 2 liter laboratory fermenter indicated by recorder tracings of the oxygen probe. The measurements were determined in a 1.5-liter volume of mineral medium. (A) Equilibrium situation whith flushing with nitrogen (1 liter·min^{-1}). (B) Fermentor pressurized to 1.1 atm (ca. 111 kPa) and all tubing closed. (C) Fermenter kept under nitrogen atmosphere by use of a 1-m-high water column. (D) Flushing with nitrogen resumed.

It can be calculated from this figure that, even when all tubing is closed as closely to the fermenter as possible, the diffusion of oxygen is still approximately $2 \cdot 10^{-3}$ µmol·h^{-1}. It was checked that gas leakage did not occur. These data clearly show that maintaining strictly anaerobic conditions is not possible in this way because of diffusion of oxygen.

To keep the concentration of oxygen at a very low level it is necessary to flush the

Table 1. Influence of the nitrogen flow rate on the dissolved-oxygen tension in a 2 l fermenter.

Flow rate N_2 (l·min^{-1})	DOT (% air saturation)
0.5	0.075
1.0	0.023
1.5	0.020
2.0	0.015
3.0	0.012
5.0	0.010
10.0	0.007
20.0	0.005

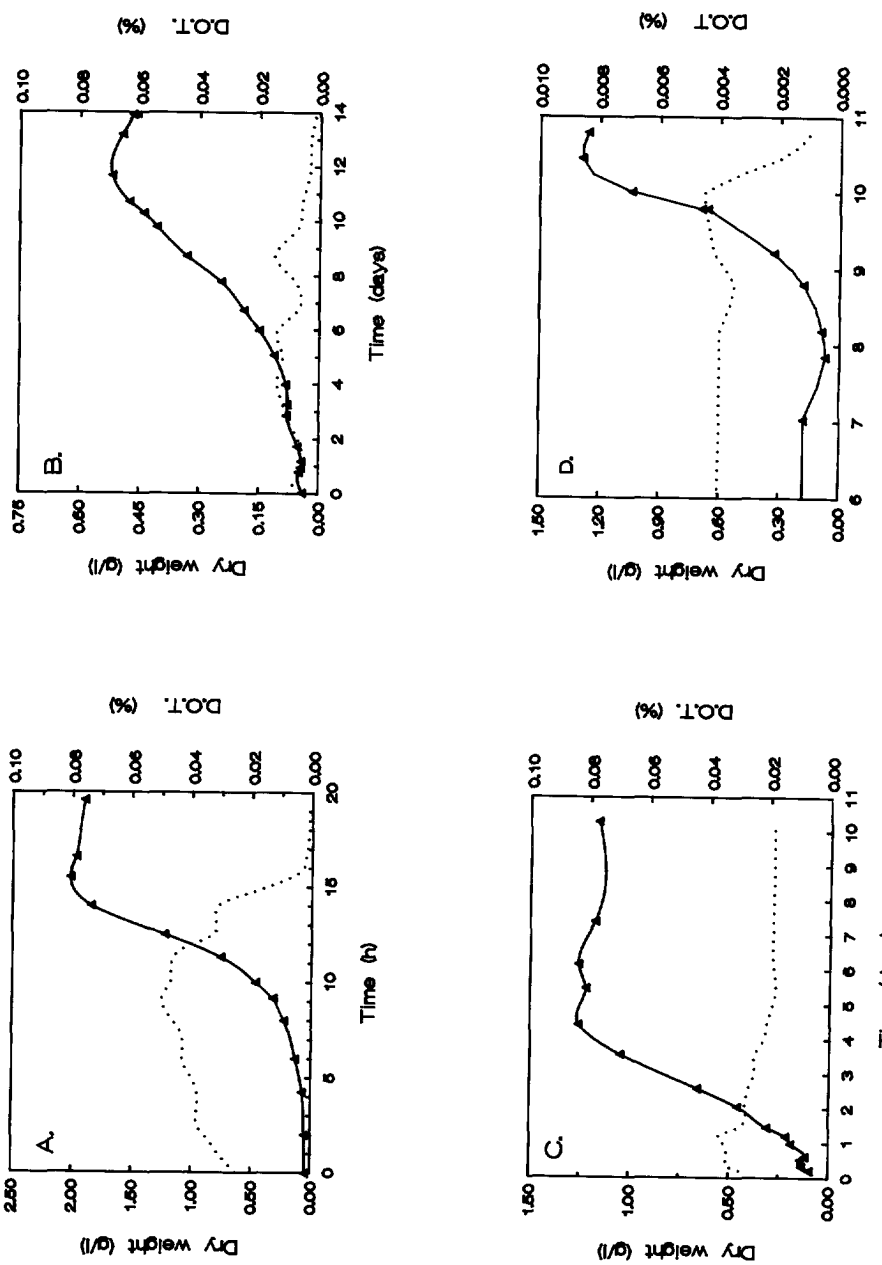

Fig. 3. Growth curves (▲) and DOTs (.....) of anaerobically grown yeasts in controlled batch fermentors. (A) S. cerevisiae. (B) C. utilis. (C) T. delbrueckii. (D) C. tropicalis.

Table 2. Maximal growth rates and cell yields in anaerobic batch cultures

Species	μ_{max} (h^{-1})	Yield (g/g of glucose)
S. cerevisiae	0.40	0.10
C. utilis	0.01	0.03
T. delbrueckii	0.03	0.06
C. tropicalis	0.05	0.07

fermenter continuously with nitrogen. We examined the effectiveness of such a procedure by monitoring the DOT as a function of time and flow rate. Although in a very short time most of the oxygen was removed (95% within 3 min), the concentration of oxygen nevertheless was continuously decreasing further during the next 30 h. The actual value of the DOT finally reached depended on the flow rate of the nitrogen gas (Table 1). Whenever during this experiment the air flow was re-established, the DOT value returned to the initial 100.0 ± 0.1 %, indicating the low drift of the electrode.

Anaerobic growth in fermenters.

In order to obtain some quantitative information on growth rates it was attempted to grow various yeasts anaerobically in fermenters. Prior to inoculation the fermenters were flushed vigorously with nitrogen gas until the DOT was not decreasing anymore. Usually this procedure took 30-35 h. After inoculation the nitrogen flow was kept at 1 $l \cdot min^{-1}$ to prevent diffusion of oxygen into the medium.

The growth curves of the organisms tested are shown in Fig. 3. As in the serum flask tests, *S. cerevisiae* turned out to be the only species capable of rapid anaerobic growth, with a μ_{max} of 0.4 h^{-1} (Table 2). The other yeasts tested (*C. utilis*, *T. delbrueckii* and *C. tropicalis*) grew poorly under these conditions (μ_{max} less than 0.05 h^{-1}). In all cases, according to the indicator, the redox potential in the culture decreased as soon as growth started. It can be calculated that the total amount of oxygen that had entered the culture vessel during the growth period was less than 10 $\mu mol \cdot h^{-1}$.

Ultrastructure of anaerobically grown cells.

The ultrastructure of the four yeasts was investigated with regard to the presence of mitochondria (Fig. 4). Special attention was given to the staining procedures, because inadequate staining often precludes the visualization of mitochondrial structures (Damsky *et al*, 1969). Indeed, fixation with potassium permanganate alone did not reveal mitochondrial structures in *S. cerevisiae* (results not shown), whereas these structures were clearly visible using glutaraldehyde/potassium-permanganate fixation (Fig. 4A). The other strains also showed mitochondrial structures, although the fine structure was definitely less developed compared to that in aerobically grown cells (results not shown), in which the cristae were clearly visible. Most remarkable was the difference between the cells of *C. utilis* and the cells of the other species. Coexisting with the mitochondrial structures in

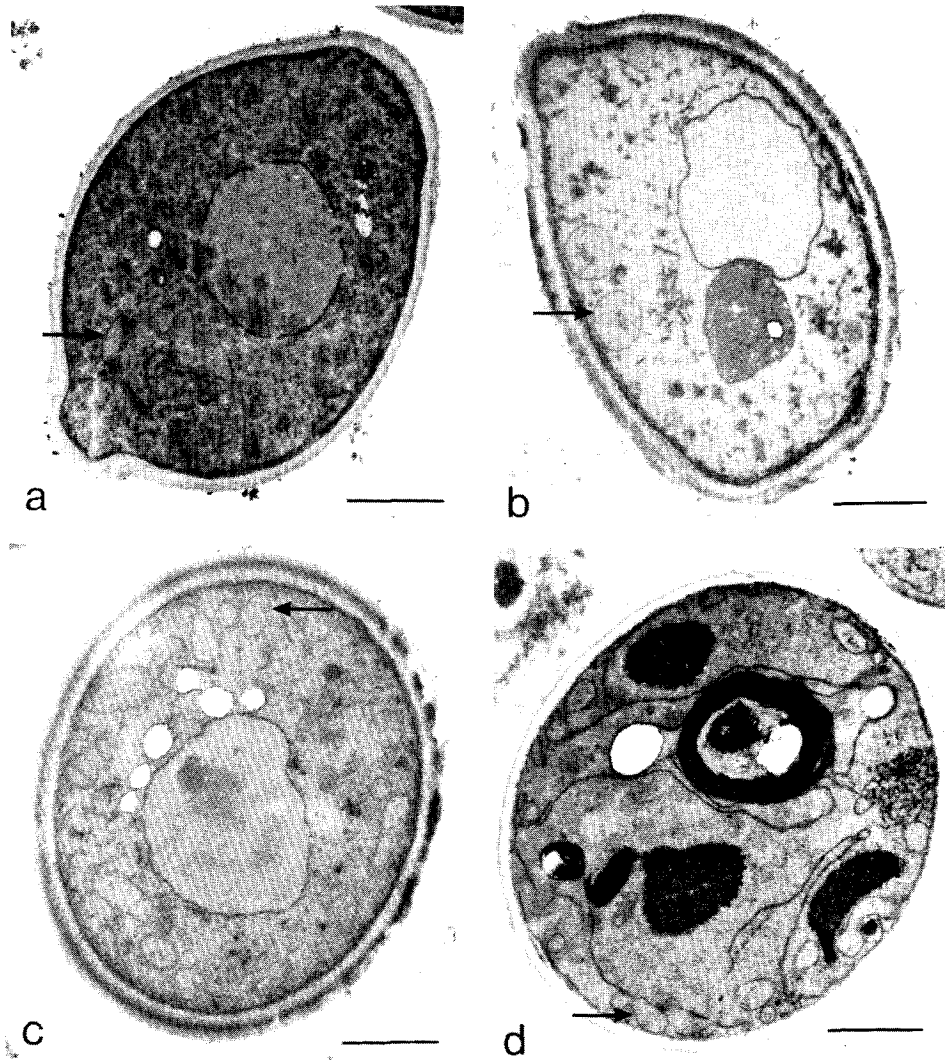

Fig. 4. Electron micrographs of anaerobically grown yeasts (bars, 1 μm). (a) S. cerevisiae; (b) T. delbrueckii; (C) C. tropicalis; (d, e and f) C. utilis. Note the presence of promitochondria (small arrows). The large arrow (panel f) indicates a connection of the ring structure to other membrane structures in the cell.

this yeast were relatively large membranous structures, either in the form of a laminar membrane system branching out at the ends, or a ring structure of concentric membranes. The membranous structures seemed to be interconnected in the cell (Fig. 4F). Single membranes were also found (Fig. 4D), and these also were connected to the larger structures. These membranous structures were not found in aerobically glucose-grown cells (results not shown).

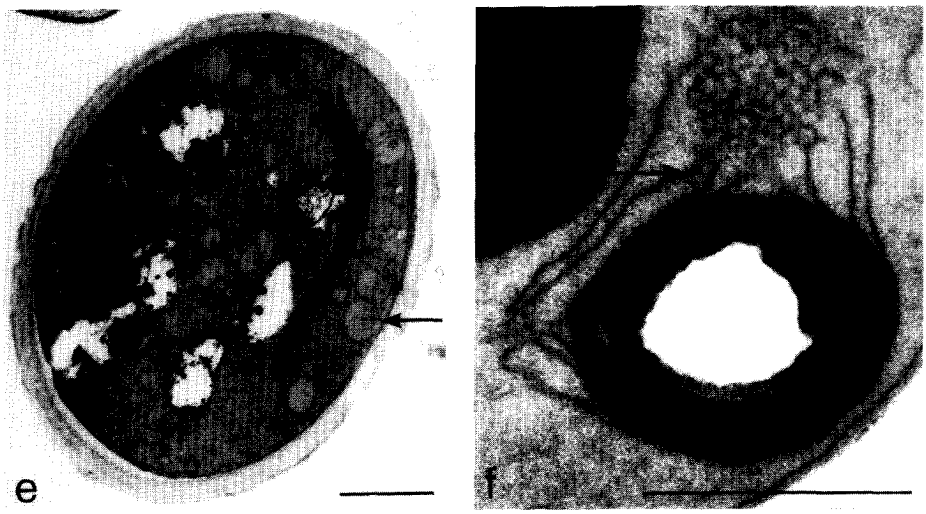

Fig 4--Continued

Discussion

For the screening of the property of anaerobic growth of yeasts it was required to make a representative selection of strains. It was decided to choose type species of genera rather than an at random selection of strains.

Although all strains tested are able to produce some ethanol, only 18 grew "anaerobically" to some extent in mineral media supplemented with ergosterol and Tween 80, or in complex media. All of these belong to the group of the facultatively fermentative yeasts as listed by Barnett (1983).

It is important to note that a good fermentative capacity is a prerequisite for anaerobic growth, since none of the "non-fermentative" yeasts grew under strictly anaerobic conditions. However, a good fermentative capacity alone is not sufficient to fulfill all the requirements of the anaerobically grown cells, since many rapidly fermenting species lack the ability for anaerobic growth. The latter property is relatively rare among yeasts. Even when growth occurred it was rather slow. *S. cerevisiae*, however, seems a positive exception in this respect (Fig. 3).

The inability of many facultatively fermentative yeasts to grow anaerobically may be caused by a variety of factors. For example, anaerobic alcoholic fermentation of xylose may be prevented by a disturbed redox balance (Bruinenberg *et al*, 1983[a]). It seems unlikely, however, that the absence of anaerobic growth on glucose is generally due to redox problems. In the case of *Pachysolen tannophilus* it has been shown that hydrogen acceptors such as diacetyl or acetoin cannot replace oxygen (Neirinck *et al*, 1984). *P. tannophilus* is unable to grow unless oxygen is available. Although yeasts such as *C. utilis* can slowly grow anaerobically (Fig. 3), the rate of growth and alcoholic fermentation is greatly enhanced in oxygen-limited shake-flask cultures. Under these conditions growth and alcoholic fermentation is as rapid as with *S. cerevisiae* (Van Dijken *et al*, 1986).

Hence it seems likely that in many facultatively fermentative yeasts an unimpaired mitochondrial function is required for growth.

The existence of mitochondria in anaerobically-grown cells has been disputed in the literature for a long time. It has been stated by several authors that *S. cerevisiae* showed a complete absence of mitochondria when grown under anaerobic conditions (Linnane *et al*, 1962; Wallace and Linnane, 1964). This observation, however, is now known to be due to inadequate electron microscopy techniques (Damsky *et al*, 1969; Plattner and Schatz, 1971). Therefore the theory of *de novo* synthesis of mitochondria in cells grown anaerobically and subsequently transferred to high levels of oxygen (Linnane *et al*, 1962; Wallace and Linnane, 1964) had to be rejected. However, the mitochondria in anaerobically grown cells do not have the same ultrastructure as those in aerobically grown cells, but upon aeration start to develop into fully organized mitochondria (Plattner *et al*, 1971). Indeed, in the four yeasts studied here, promitochondria could be detected (Fig. 4). Remarkable are the membranous structures in the yeast *C. utilis* as already reported by Linnane and co-workers (Linnane *et al*, 1962). These authors suggested that such structures should be regarded as precursors of mitochondria. This seems unlikely, in view of the fact that these structures were not observed in the other yeasts (Fig. 4).

Whether or not promitochondria fulfill a physiological function remains to be elucidated. It is known that part of the assimilatory processes required for cell synthesis are located within the mitochondria, (Perlman and Mahler, 1970) and hence transport of certain intermediates over the mitochondrial membrane remains a necessity under anaerobic conditions. This raises the problem of energizing these transport processes in the absence of electron transfer. The results of Šubík, Gbelská and co-workers (Šubík *et al*, 1972; Gbelská *et al*, 1983) strongly suggest that under anaerobic conditions transport processes and other energy-requiring reactions in mitochondria are energized by the import of cytoplasmic ATP via reversal of adenosine nucleotide translocation. Anaerobic growth of *S. cerevisiae* was shown to be arrested in the presence of bongkrekic acid, a specific inhibitor of the ATP/ADP translocator of the inner mitochondrial membrane. This inhibition could not be relieved by addition of a variety of growth factors.

So far, it is unclear why in a variety of yeasts the role of mitochondria in anabolic reactions is apparently more important than in *S. cerevisiae*. Our results demonstrate that in studies on alcoholic fermentation by yeasts great care should be taken with respect to culture conditions. The serum flask test used herein can be considered as a useful system for a qualitative estimation of anaerobic behavior of yeasts. However, for quantitative aspects fermenter cultures are required, even though the entrance of oxygen cannot be totally prevented (Fig. 2). Nevertheless, it is clear that yeasts like *C. utilis*, *C. tropicalis* and *T. delbrueckii* do not grow as well as *S. cerevisiae* when only traces of oxygen are available. The biochemical basis for this difference between *S. cerevisiae* and the other yeasts remains to be elucidated.

In our study only type species of genera were tested. It can therefore not be excluded that in addition to *S. cerevisiae* other yeasts may possess the capacity for fast anaerobic growth. However, from the limited amount of data presented herein it seems likely that this property is not widespread amongst yeasts.

Acknowledgments

We are indebted to the Centraal Bureau voor Schimmelcultures for providing us with the strains tested and for carrying out identification tests. We thank Maudy Smith for stimulating discussions and Marc Rijneveen for performing part of the experimental work. The investigations were supported by the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

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CHAPTER 3

Involvement of mitochondria in the assimilatory metabolism of anaerobic *Saccharomyces cerevisiae* cultures

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Abstract

The possible physiological role of mitochondria in anaerobically grown *Saccharomyces cerevisiae* was investigated via enzyme localization and inhibitor studies. Almost all of the activity of citrate synthase (EC 4.1.3.7) was recovered in the mitochondrial fraction after differential centrifugation of spheroplast lysates. The enzyme exhibited a high degree of latency which was demonstrated by sonication of the mitochondrial fractions.

Since citrate synthase is an important enzyme in anabolic reactions, a consequence of this localization is the requirement for transport of metabolites across the mitochondrial membranes. Such transport is likely to require energy which, as a result of anaerobiosis, cannot be supplied by respiration. It was therefore investigated whether ATP translocation into the mitochondria by an ADP/ATP translocase might be involved in anaerobic mitochondrial energy metabolism. It was shown that addition of the ADP/ATP translocase inhibitor bongkrekic acid to anaerobic cultures indeed inhibited growth, although only partially. It is concluded that mitochondria of *S. cerevisiae* fulfil a vital role in anaerobic sugar metabolism.

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Introduction

The existence of mitochondria in anaerobically grown yeast cells has been disputed for a long period in the literature. Early reports (Wallace and Linnane, 1964; Linnane, 1965; Chapman and Bartley, 1968) claim that such cells are complete devoid of mitochondria and that adaptation to aerobic conditions involves "*de novo*" synthesis of these organelles. These reports were based on electron microscopic studies. Later it was shown that inadequate staining or fractionation procedures had led to these conclusions (Damsky *et al.*, 1969; Cartledge *et al.*, 1972, Cartledge and Lloyd, 1972; 1973, Jenkins *et al.*, 1984). Since the membranous structures found under anaerobic conditions were quite different from the well-known aerobic mitochondria, the term "promitochondria" was proposed to emphasise the relationships to the aerobic organelles, as well as the differences between these organelles and fully functional mitochondria.

In all reports on the disappearance of mitochondria under anaerobic conditions, a discussion of the physiological consequences was restricted to the respiratory system., i.e. cytochromes and oxidases and the F1-ATPase (Groot *et al.*, 1971). However, it is well-established that most enzymes of the citric acid cycle as well as some enzymes for sterol biosynthesis (Shimizu *et al.*, 1973) and amino acid synthesis (Ryan and Kohlhaw, 1974; Jauniaux *et al.*, 1978) are localised inside the mitochondria and hence disappearance of the organelle could be lethal during growth under anaerobic conditions. Evidence for the indispensability of mitochondria is the fact that yeast proteins mediating protein import into mitochondria are essential for cell viability (Jensen and Yaffe, 1988; Baker and Schatz, 1991; Stuart *et al.*, 1994).

Studies on the localization of mitochondrial enzymes under anaerobic conditions are complicated due to the very fragile structure of these mitochondria (Criddle and Schatz, 1969; Damsky *et al.*, 1969). Hence, in this study, great attention has been given to the isolation of intact mitochondria. Citrate synthase activity was taken as a marker enzyme in view of its key role in the TCA-cycle and since earlier reports showed that its activity under anaerobic conditions was not found in a particulate fraction (Wales *et al.*, 1980). Under anaerobic conditions, all ATP is produced in the cytoplasm during glycolysis. If promitochondria also fulfil a role in biosynthesis of cell material during anaerobic growth, energy must be supplied to the organelle both for driving transport processes across its membranes and for the energy-demanding reactions within (Groot *et al.*, 1971). Import of ATP into mitochondria may occur via an ADP/ATP translocator in exchange for ADP. The possible physiological function of yeast mitochondria under strictly anaerobic conditions was therefore also studied by testing the sensitivity of anaerobic cells to bongkrekic acid, a well-known inhibitor of the ADP/ATP translocator.

Methods

Organisms.

Saccharomyces cerevisiae CBS 8066 was obtained from the Centraalbureau voor Schimmelcultures (Delft, the Netherlands) and maintained on malt agar slopes.

Media.

The mineral medium, supplemented with vitamins and trace elements, was prepared according to Verduyn *et al.* (1992). Glucose was added as sole source of carbon and

energy, to a concentration of 30 $g \cdot l^{-1}$ unless stated otherwise. Ergosterol and Tween 80, supplements required for anaerobic growth of *S. cerevisiae* (Andreasen and Stier, 1953, 1954) were dissolved in pure ethanol and sterilized by heating the solution for 10 min at 100 °C. These components were added to the medium to a concentration of 6 $mg \cdot l^{-1}$ and 660 $mg \cdot l^{-1}$, respectively. To prevent foaming, 50 µl silicone antifoam per liter was added to the reservoir medium.

Growth conditions.

The yeast was grown in continuous cultures, using a 2-liter laboratory fermenter of the type described by Harder *et al.* (1974), with a 1 liter working volume. The pH was controlled at 5.0 by automatic titration of 1 M KOH. The stirrer speed was maintained at 1000 rpm and the cells were grown at 30 °C at a dilution rate of 0.1 h^{-1} unless stated otherwise.

To maintain anaerobic conditions, the fermenter was flushed with 1 $l.min^{-1}$ pure nitrogen gas, containing less than 5 ppm oxygen (obtained from Air Products, Waddinxveen, The Netherlands). To minimize diffusion of oxygen Norprene tubing (Cole-Parmer Instruments Corp., Chicago, USA) was used. Since dissolved oxygen in the growth medium will significantly contribute to the overall influx of traces of oxygen, the medium was stripped of oxygen using a second fermenter in which it was vigorously flushed with the nitrogen. From this fermenter the medium was pumped directly into the fermentation vessel. The outlet gas passed a cooled condenser (4 °C) and was subsequently led through a glycerol trap to prevent back diffusion of oxygen into the fermenter.

The dissolved-oxygen tension was measured continuously with an autoclavable polarographic (Clark type) oxygen electrode, type Ingold 322 756702/74247, connected to an Ingold O_2 amplifier type 170. As a result of the precautions mentioned above the actual dissolved oxygen tension in the anaerobic cultures was below 0.005 % air saturation.

For comparative purposes, cells were also grown aerobically in chemostat cultures. All parameters were kept the same, only the nitrogen gas was replaced with air and the glucose concentration in the reservoir was reduced to 5 $g \cdot l^{-1}$.

Analytical methods.

Dry weight was determined by filtration of culture samples over weighed polysulfone filters (pore size 0.45 µm, Gelman Sciences Inc., Michigan, USA). The filter was washed with demineralized water and dried in a microwave (Sharp Inc., Osaka, Japan) for 20 min at medium power, and re-weighed.

Cultures were routinely analyzed for metabolites. Organic acids were determined by HPLC on a HPX-87H column (300 × 7.8 mm, Bio-Rad, Richmond, CA, USA) at 30°C. The column was eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 $ml \cdot min^{-1}$. The detector was a Waters 441 UV-meter at 210 nm, which was coupled to a Waters 741 data module (Waters, Milford, MA, USA). Glycerol and ethanol were also measured using this column by the use of a refraction index detector which was coupled in line with the UV detector.

Isolation of mitochondria from anaerobic cells.

For the isolation of mitochondria the procedure of Bruinenberg *et al.* (1985) was adopted. To prevent adaptation to aerobic conditions all buffers and reagents were made anaerobic by flushing with argon and all incubations were carried out under an atmosphere of this gas. When the entrance of oxygen could not be avoided the samples were kept at 0 °C to prevent adaptation to aerobic conditions. Per gram cells 1000 U Zymolyase were used for spheroplasting. During incubation small samples were taken and diluted 200-fold in water. The osmotic shock caused lysis of the spheroplasts, which was determined by measuring the optical density of the solution at 660 nm. Incubation was stopped when the optical density was ten percent of the starting value. A crude mitochondrial fraction was obtained from the spheroplast lysates via differential centrifugation as described by Bruinenberg *et al.* (1985). The mitochondrial fractions were stabilized by the addition of 1 $mg \cdot ml^{-1}$ bovine serum albumin (BSA). The fractions P1 and P2 were obtained after centrifugation in a SS34 rotor of a Sorvall RC5B at 10,000 rpm at 10 min and 20,000 rpm at 20 min respectively.

When appropriate the fractions were disrupted by ultrasonic treatment at 4 °C by the use of a MSE sonicator. Full power treatment (30 s, unless indicated otherwise, at 150 W) was alternated with equal periods of cooling in ice-water.

Respiration measurements.

Respiration measurements on cells and mitochondria were performed with a polarographic oxygen electrode (Clark type) in a stirred vessel at 30 °C (Biological Oxygen Monitor, Yellow Springs, USA). Cells were diluted in mineral medium without glucose. After determination of the endogenous respiration glucose was added to a final concentration of 20 mM. The oxygen uptake rate in the presence of glucose was taken as maximum respiratory capacity of the cells. Activity is expressed as $\mu mol\ O_2 \cdot min^{-1} \cdot g$ cell $protein^{-1}$ and are based on a protein content of whole cells of 47% (Verduyn *et al.*, 1990).

Oxygen consumption of mitochondria was measured in 25 mM potassium-phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$, 0.65 M sorbitol and 0.17 mM ADP using 0.25 mM NADH as substrate. In order to verify whether the oxygen consumption by whole cells and isolated mitochondria was mediated by cytochrome oxidase, activity was tested by adding cyanide to the solution to a final concentration of 1 mM.

Enzyme assays.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed according to Bruinenberg *et al.* (1983). The activity of NADH dehydrogenase (EC 1.6.99.3) was determined as described by Bruinenberg *et al.* (1985). Citrate synthase was assayed in a 100 Mm Tris/HCl buffer (pH 8.0) containing 0.10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.05 mM acetyl CoA. After addition of the sample the reaction was started with oxaloacetate (final concentration 0.25 mM). The appearance of coenzyme A was followed spectrophotometrically at 412 nm (ϵ = 13.6 $mM^{-1} \cdot cm^{-1}$). Protein determinations in mitochondrial fractions were disturbed by the addition of BSA and therefore not performed.

Chemicals.
Tween 80 was obtained from E. Merck Nederland B.V. (Amsterdam, the Netherlands). Zymolyase (100,000T) was purchased from the Kirin brewery, Japan. Antifoam was obtained from BDH (Poole, England).
Bongkrekic acid was a gift of Prof. J.A. Duine from our department.

Results

Isolation of mitochondria.
S. cerevisiae cells were grown under strictly anaerobic conditions and glucose limitation at a dilution rate of 0.1 h^{-1}. The procedure for the isolation of promitochondria was based on the method of Bruinenberg *et al.* (1985) and van Urk *et al.* (1989), developed for the isolation of mitochondria from aerobically-grown cells of *Candida utilis* and *S. cerevisiae* respectively. The essentials of these procedures are enzymatic degradation of the cell wall with Zymolyase in hypertonic medium, followed by dialysis of the spheroplast suspension to lower its osmotic value gently. Spheroplasts are then mechanically disrupted by a few strokes in a Potter-Elvejhem homogenizer. Mitochondrial fractions are subsequently isolated by differential centrifugation. These fractions were named P1, P2 and S according to earlier experiments (Wales *et al.*, 1980; Bruinenberg *et al.*, 1985; van Urk *et al.*, 1989). The P1 fraction contains mostly mitochondria, the P2 fraction contains the remaining membranes and the S fraction is the final supernatant or soluble fraction.

In the method of van Urk *et al.* (1989) pretreatment of the aerobically-grown cells with dithiothreitol (DTT) and EDTA was necessary to obtain spheroplasts at a satisfactory rate. DTT, however, interfered with our measurements of citrate synthase activity due to remaining residues after washing. These traces of DTT reacted with the DTNB used in the citrate synthase assay. Further checking showed that pretreatment of cells with DTT, EDTA or the combination of the two did not enhance the rate of spheroplasting of anaerobically-grown *S. cerevisiae* with Zymolyase and hence this pretreatment was omitted from the procedure.

When promitochondria of anaerobically-grown cells are to be studied, special care has to be taken to prevent adaptation of the cells to aerobic surroundings during the isolation procedures, which were therefore carried out under an atmosphere of argon. Purging argon or nitrogen through the liquid was avoided since high shear forces resulted in premature lysis of the spheroplasts (results not shown).

When anaerobiosis could not be maintained (e.g. during centrifugation steps), cells were cooled down to 0 °C under argon gas prior to further handling.

Since it has been shown that premature lysis of spheroplasts will lead to damaged mitochondria (Bruinenberg, 1985) we optimized the concentration of the osmotic stabilizer sorbitol. Measurements of the activity of (cytoplasmic) glucose-6-phosphate dehydrogenase released in the supernatant of the incubation mixture showed that 2 M sorbitol was optimal with respect to spheroplast stability and the time required to reduce the sorbitol concentration to 0.65 M by dialysis. The optimum concentration of Zymolyase was found to be 2000 U in 20 ml cell suspension (100-120 mg cells/ml). Electron microscopic examination of the fractions obtained after differential centrifugation revealed that the P1-fraction mainly consisted of relatively intact

mitochondria. Micrographs of the P2-fraction showed only irregular membranous structures and some vesicles (Fig. 1).

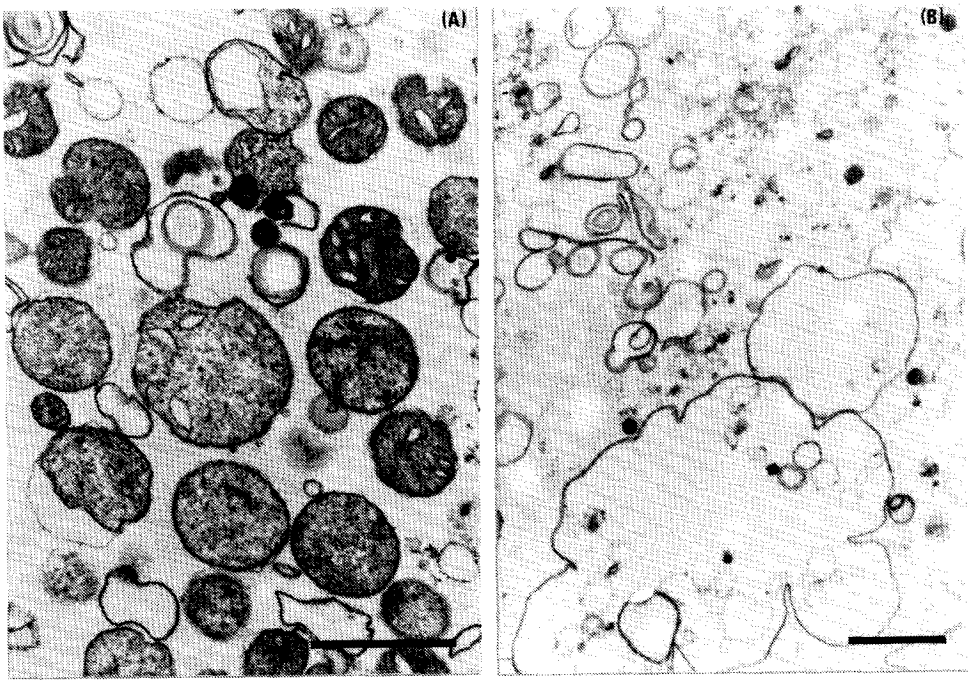

Fig. 1. Electron micrographs of subcellular fractions from anaerobically-grown, glucose-limited Saccharomyces cerevisiae cells (bars, 1 μm). Cells were grown at a dilution rate of 0.10 h^{-1}. (a) P1-fraction, mainly containing promitochondria. (b). P2-fraction, mainly containing membranes and vesicles.

Table 1. Maximum respiratory capacity of anaerobically and aerobically grown cells of *S. cerevisiae*, and of mitochondrial preparations of these cultures, measured in the presence and absence of 1 mM KCN.

Growth conditions	QO_2^{max} [μmol $O_2 \cdot min^{-1} \cdot (g\ protein)^{-1}$]			
	Cells		Mitochondria*	
	-CN	+CN	-CN	+CN
Aerobic	150	0	360 ¶	NT
Anaerobic	10	5	ND	ND

ND, Not detectable; NT, not tested. * In mitochondrial fractions respiration was measured with NADH instead of glucose as a substrate, in the presence of ADP. ¶ Value obtained from van Urk *et al.*, (1989).

Respiration of mitochondria of anaerobically-grown cells.

The possible, undesirable, aerobic adaptation during handling of anaerobically-grown cells would be reflected in the ability of the cells to consume oxygen. In order to validate the isolation procedure with respect to this adaptation, cells were incubated under conditions equivalent to those in the isolation procedure, i.e. under argon atmosphere or kept at 0 °C, and were subsequently checked for the maximal respiratory capacity. Zymolyase and sorbitol were not added in these control experiments. Cells incubated this way for at least four hours did not acquire a higher respiratory activity. This remained constant at a low value of 10 $\mu mol \cdot min^{-1} \cdot g$ cell $protein^{-1}$. Approximately 50 % of this activity was insensitive towards cyanide, whereas the respiration of aerobically grown cells was blocked completely by this inhibitor (Table 1). No respiration of NADH was found with the mitochondria of anaerobically-grown cells, indicating that no adaptation of cells to aerobic conditions had occurred during the isolation procedure.

Table 2. Subcellular localization of citrate synthase and glucose-6-phosphate dehydrogenase in anaerobically-grown *Saccharomyces cerevisiae.*

Distributions are expressed as percentages of recovered activity. This recovery was based upon the total activity in the cell free extract (fraction T), being 3 and 20 $\mu moles \cdot min^{-1}$ for citrate synthase and glucose-6-phosphate dehydrogenase, respectively. Data ± SD are based on four independent isolations of mitochondria. P1: mitochondrial pellet fraction; P2: rest of particulate material.

Fraction	Citrate synthase	Glucose-6-P-dehydrogenase
P1	79.0 ± 3.3	0.8 ± 0.6
P2	10.3 ± 4.1	1.0 ± 1.0
Total particulate	89.2 ± 7.3	1.7 ± 1.2
Soluble	10.8 ± 7.3	98.3 ± 1.2
Recovery	87.3 ± 15.6	88.4 ± 8.4

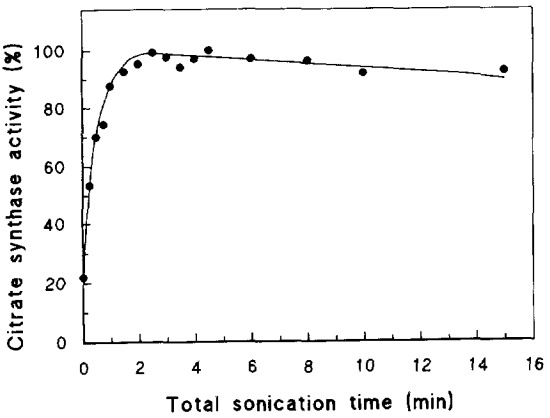

Fig. 2. The release of citrate synthase activity following ultrasonic treatment of the mitochondria isolated from anaerobically-grown Saccharomyces cerevisiae cells. Results are presented as percentage of the maximal activity after sonication.

Subcellular localization of citrate synthase.

Glucose-6-phosphate dehydrogenase is a well-known cytosolic marker enzyme. As can be concluded from the distribution of the activity of this enzyme over the fractions examined (Table 2), the mitochondrial fractions isolated were hardly contaminated with cytosolic enzymes.

Citrate synthase in the mitochondrial fraction P1 exhibited latency. Only after sonication was the maximum activity reached. This is demonstrated in Fig. 2, in which the activity of the enzyme in the P1-fraction is depicted as a function of the sonication time. The sonication procedure hardly damaged the enzyme, since prolonged exposure time did not significantly reduce the activity. The citrate synthase activity in the P1-fraction increased approximately five-fold after the sonication procedure, whereas the small amount of enzyme in the P2-fraction did not exhibit significant latency (less than 10%).

Effect of bongkrekic acid on anaerobic cultures.

Bongkrekic acid is a well-known specific inhibitor of the mitochondrial ADP/ATP-translocase of mammalian cells (beef heart and rat liver), both in intact mitochondria (Erdelt *et al.*, 1972) and in a reconstituted system (Krämer and Klingenberg, 1979). The same was demonstrated for the yeast mitochondrial translocase by following transport activity in proteoliposomes containing reconstituted, purified translocase of aerobically grown *S. cerevisiae* (haploid strain D 273-10B α) cells with ^{14}C- labeled ATP (Knirsch *et al.*, 1989).

In order to investigate the *in vivo* role of mitochondrial translocase during anaerobic growth, inhibition of growth was followed by recording the washout kinetics of steady state cultures after the addition of bongkrekic acid. This translocase inhibitor was added at zero time to both the medium reservoir and the culture vessel to a concentration of 5 μM. In this way the concentration of bongkrekic acid remained constant during the washout experiment. From the washout profile after the addition of the inhibitor it can

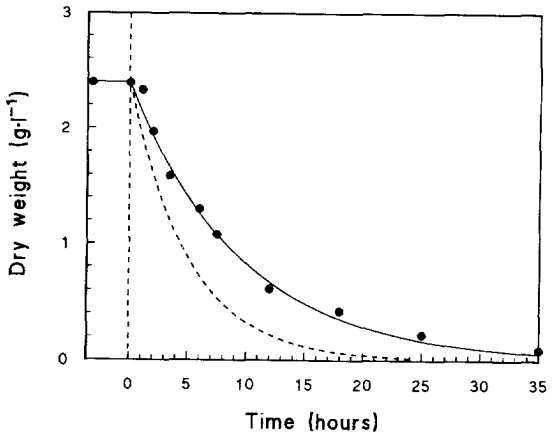

Fig. 3 Washout curve of an anaerobic continuous culture of Saccharomyces cerevisiae (D= 0.2 h^{-1}, S_R= 27.5 $g \cdot l^{-1}$ glucose) after addition of bongkrekic acid (5 μM), as a specific inhibitor of the ADP/ATP-carrier of the mitochondrial membrane, at T = 0 h. (●, Dry weight ($g \cdot l^{-1}$)). Dashed curve refers to washout kinetics when μ = 0. The solid drawn line through the data points is the result of a curve fit procedure assuming that the organism is washed out according to $x = x_0 \cdot e^{(\mu - D)t}$ (x = biomass, μ = specific growth rate, D = dilution rate). The data show that anaerobic growth in the presence of bongkrekic acid still proceeds with μ=0.1 h^{-1} for at least five generations.

be calculated that the growth rate decreased by 50% from 0.2 to 0.1 h^{-1} (Fig. 3). Increasing the concentration of the bongkrekic acid from 5 to 50 μM did not enhance the inhibitory effect (data not shown).

It has been reported (Sûbik, 1972) that addition of bongkrekic acid to growing cells of *S. cerevisiae* under anaerobic conditions induced the formation of respiratory deficient mutants. Whether a similar phenomenon would occur in our continuous-culture experiment was checked by plating the samples from the washout culture on appropriate agar media, but no petite colonies were found.

Discussion

Anabolic functions of mitochondria during anaerobiosis.

Citrate synthase is a key-enzyme of the TCA-cycle (Lowenstein, 1967; Walsh and Koshland, 1985) catalyzing the condensation of oxaloacetate and acetyl-CoA to produce citrate. The TCA cycle is important under aerobic conditions, as it generates reduced coenzymes to supply the electron transport chain with substrates. The second role of the cycle is to provide the cell with anabolic precursors, such as 2-oxoglutarate, the precursor for the glutamate family of amino acids (Fig. 4). Thus, although under anaerobic conditions the first role is of no significance, the latter anabolic function is still of vital importance.

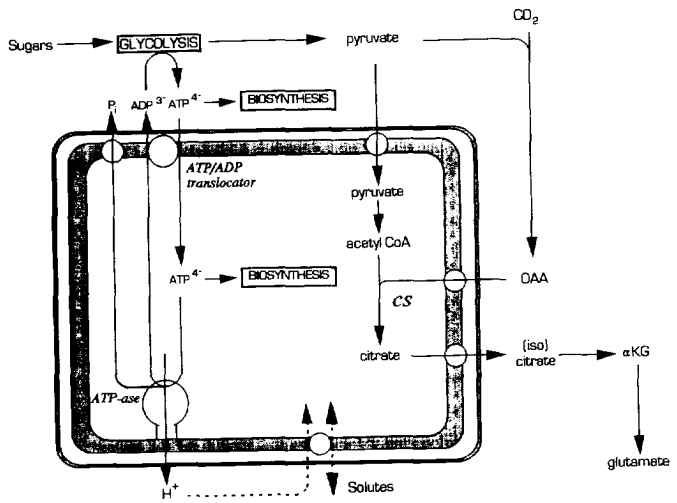

Fig. 4. Vectorial processes in mitochondria during anaerobic growth, showing the coupling of the intra- and extramitochondrial nucleotide pools by the ADP/ATP-translocase in the inner mitochondrial membrane. Note that ATP-ase, when functional in anaerobic mitochondria, would build up a proton motive force by consumption of ATP, instead of dissipating the proton motive force for synthesis of ATP as occurring in aerobic cells. The proton symport (antiport) symbolizes energy-dependent transport processes across the mitochondrial membrane, for example of the metabolites shown, pyruvate, oxaloacetate (OAA), (iso)-citrate, etc. CS: citrate synthase.

The localization of several TCA-cycle enzymes under anaerobiosis has been studied for *Saccharomyces carlbergensis* by Wales *et al.* (1980). They found that citrate synthase was almost entirely recovered in the soluble fraction, whereas with aerobically-grown cells only 6 % of this activity was found in the supernatant. The low recovery of activity in the mitochondrial fraction of anaerobically-grown cells was explained by leakage of enzyme from the more fragile (Criddle and Schatz, 1969; Damsky, 1969) promitochondria. Therefore, in this study a very gentle procedure for the isolation of the mitochondria was used.

The results, shown in Table 2 and Fig. 2, clearly show that with this procedure most of the enzyme is particulate, also under anaerobic conditions. Isoenzymes, functioning in different locations in the cell, always complicate localization studies. When, for example, yeast cells are grown on ethanol or acetate, the glyoxylate cycle is active. It has been shown that the enzymes of this route are localized in the peroxisomes. Under these conditions two different genes coding for citrate synthase are active, *CIT1* coding for the mitochondrial enzyme and *CIT2* for the peroxisomal protein (Lewin *et al.*, 1990). In cells grown on glucose under anaerobic conditions, however, peroxisome synthesis is repressed (Veenhuis and Harder, 1987) and peroxisomal enzymes are not synthesized (Rogers and Stewart, 1973).

Therefore, it seems unlikely that the particulate localization of citrate synthase is partially or totally due to contamination of the mitochondrial fraction with peroxisomes.

Furthermore, since it is well-known that peroxisomes are very fragile, the observed latency of citrate synthase (Fig. 2) is also not in accordance with a peroxisomal localization of the enzyme.

ATP requirements for anabolic functions of mitochondria.

The majority of the mitochondrial enzymes are coded on the nuclear DNA and citrate synthase is no exception in this respect (Schatz and Mason, 1974). Import of these enzymes or precursor proteins requires not only a membrane potential but also hydrolysis of matrix ATP (Eilers *et al.*, 1987; Pfanner *et al.*, 1987; Stuart *et al.*, 1994). The subsequent refolding of imported proteins by mitochondrial chaperones (e.g. hsp60) requires ATP within the mitochondrial matrix (Baker and Schatz, 1991; Stuart *et al.*, 1994). Anabolic reactions also will depend on the supply of energy, whether this is direct as in phosphorylation reactions, or indirect as active transport processes.

Whereas under aerobic conditions ATP is produced within the mitochondria by respiration, under anaerobic conditions the energy requirement for anabolic processes necessarily implies extramitochondrial sources of energy, e.g. import of ATP into the mitochondrion.

The only possible route for mitochondrial uptake of ATP is mediated by the ADP/ATP translocator (see Fig. 4). This carrier is the most abundant protein in the mitochondrial membrane (Klingenberg, 1985) and under respiratory conditions it will export ATP and import ATP at high rates. Under these conditions, the ATP/ADP ratio in the cytosol is considerably higher than in the mitochondrial matrix. Transport therefore occurs against the chemical gradient of the substrates ATP and ADP. The driving force for this process is the membrane potential due to the fact that the exchange of ATP^{4-} against ADP^{3-} includes charge movement across the membrane. Thus, the direction of the fluxes and the relative contribution of the transport modes carrying ADP and ATP, respectively, depend on the energy state of the membrane (Krämer and Klingenberg, 1980).

Since heterologous exchange (ATP^{-4}, ADP^{-3}) is electrogenic, the mitochondrial import of ATP in anaerobically grown cells creates a membrane potential in the physiological direction, i.e. positive outside (see Fig. 4). On the other hand, once in the matrix, ATP may be hydrolyzed by the ATP-ase, leading to extrusion of protons thereby generating an electrochemical proton gradient. It has to be taken into account, however, that this would also decrease further uptake of ATP by the electrogenic heterologous exchange mode of the ADP/ATP translocase to some extent.

A highly specific and effective inhibitor of the ADP/ATP-translocator is bongkrekic acid. It has been shown that aerobic growth of respiration-deficient mutants of *S. cerevisiae* could be arrested by addition of this drug, indicating the vital importance of mitochondrial import of ATP for anabolic purposes (Šubík *et al.*, 1972 and Gbelská *et al.*, 1983).

Since anaerobic conditions of wild type *S. cerevisiae* will lead to a similar mitochondrial energy demand as in the experiments of Šubík *et al.* (1972) and Gbelská *et al.* (1983), it was expected that bongkrekic acid would be as effective in blocking growth under these conditions. Our results show that anaerobic growth was indeed inhibited, but the effect was not complete. Although our results seem contradictory to published studies on the effect of bongkrekic acid on the ADP/ATP-translocase in respiratory deficient mutants (Šubík *et al.*, 1972 and Gbelská *et al.*, 1983), it should be born in mind that so far no

studies have been performed on the effect of this inhibitor on cells that were grown under strict anaerobic conditions. In this respect it is relevant that different genes (*AAC1*, *AAC2* and *AAC3*) code for ADP/ATP-translocase in *S. cerevisiae*. *AAC1* and *AAC2* are expressed under aerobic conditions whereas *AAC3* is specifically expressed under anaerobic conditions (Gawaz *et al.*, 1990; Kolarov *et al.*, 1990). We therefore hypothesize that this third gene product is only partly inhibited by the drug and thus supports anaerobic growth in its presence.

In conclusion, the present study has provided experimental support for the contention that the mitochondria play a vital role in the anabolic metabolism of anaerobically growing cells. The term "non-functional mitochondria", often used in the literature in the sense of 'non-respiring mitochondria', should therefore cease to be used.

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CHAPTER 4

Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*

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Abstract

Effects of growth conditions on mitochondrial morphology were studied in living *Saccharomyces cerevisiae* cells by vital staining with the fluorescent dye dimethylaminostyryl-methylpyridinium iodine (DASPMI), fluorescence microscopy, and confocal-scanning laser microscopy. Cells from respiratory, ethanol-grown batch cultures contained a large number of small mitochondria. Conversely, cells from glucose-grown batch cultures, in which metabolism was respiro-fermentative, contained small numbers of large, branched mitochondria. These changes did not significantly affect the fraction of the cellular volume occupied by the mitochondria. Similar differences in mitochondrial morphology were observed in glucose-limited chemostat cultures. In aerobic chemostat cultures, glucose metabolism was strictly respiratory and cells contained a large number of small mitochondria. Anaerobic, fermentative chemostat cultivation resulted in the large, branched mitochondrial structures also seen in glucose-grown batch cultures. Upon aeration of a previously anaerobic chemostat culture, the maximum respiratory capacity increased from 10 to 70 μmole·min^{-1}·g dry weight^{-1} within 10 h. This transition resulted in drastic changes of mitochondrial number, morphology and, consequently, mitochondrial surface area. These changes continued for several hours after the respiratory capacity had reached its maximum. Cyanide-insensitive oxygen consumption contributed ca. 50 % of the total respiratory capacity in anaerobic cultures, but was virtually absent in aerobic cultures. The response of aerobic cultures to oxygen deprivation was qualitatively the reverse of the response of anaerobic cultures to aeration. The results indicate that mitochondrial morphology in *S. cerevisiae* is closely linked to the metabolic activity of this yeast: conditions that result in repression of respiratory enzymes generally lead to the mitochondrial morphology observed in anaerobically grown, fermenting cells.

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Introduction

Yeasts are easy to grow under a variety of culture conditions and are therefore well suited for studies into the relation between structure and function of eukaryotic cells and organelles. The morphology of yeast mitochondria, the key organelles in respiratory metabolism, has been extensively studied over the past three decades (see e.g. Agar and Douglas, 1957).

When *Saccharomyces cerevisiae*, a facultatively fermentative yeast, is grown under anaerobic conditions, its sugar metabolism is strictly fermentative. However, alcoholic fermentation also occurs under aerobic conditions when cells are exposed to excess sugar (Fiechter *et al.*, 1981; Van Urk *et al.*, 1988). Only when the supply of the sugar is growth limiting and the sugar concentration in the culture is thus poised at a low value, fully respiratory growth can be observed. Because of this dependence of respiratory metabolism on environmental conditions, a large number of studies have been performed on the effect of culture conditions on yeast mitochondrial morphology.

Electron microscopy is an excellent tool for studying subcellular organization. Yeast cells grown on non-fermentable carbon sources clearly show mitochondrial structures (Marquardt, 1962, 1963; Yotsuyanagi, 1962). Under anaerobic growth conditions however, detection of mitochondria is more difficult, which has led to a dispute in the early literature as to whether or not anaerobically grown yeast cells contain mitochondria. This controversy has been resolved by the development of adequate staining and fixation techniques. It is now generally accepted that anaerobically grown yeast cells do contain mitochondria, sometimes called promitochondria (Schatz, 1965; Damsky *et al.*, 1969; Plattner and Schatz, 1969; Plattner *et al.*, 1971).

In the literature, apparent controversies exist with respect to the number of mitochondria per yeast cell and their morphology. Descriptions range from numerous ovoid-shaped mitochondria to a single branched mitochondrion and suggest that mitochondrial structure may be species dependent (Kawakami, 1961; Prusso and Wells, 1967; Hoffman and Avers, 1973; Keddie and Barajas, 1969). Hoffman and Avers (1973) showed that the many small mitochondria seen in thin-section electromicrographs may represent cross-sections of a single, branched organelle. Stevens (1977, 1981) reported that the mitochondrial number and morphology depended on the growth conditions.

Most experiments on mitochondrial morphology have been performed with batch cultures grown on glucose as a carbon and energy source. In such cultures, it is not possible to discriminate between effects of growth rate and glucose repression (van Dijken and Scheffers, 1986; Alexander and Jeffries, 1990). Furthermore, during cultivation in standard shake-flask cultures, oxygen limitation almost invariably occurs due to poor oxygen-transfer characteristics. The use of chemostat cultures makes it possible to control growth rate and dissolved-oxygen concentration and enables growth at low, derepressing glucose concentrations. The aim of the present study was to investigate the effect of environmental conditions on mitochondrial morphology in living *S. cerevisiae* cells grown in batch and chemostat cultures.

Although electron microscopy appears an obvious choice to study these morphological changes, it has a number of disadvantages, including the necessity of sample dehydration and the dependency of yeast-cell staining characteristics on growth conditions (Damsky *et al.*, 1969). To circumvent these problems, we used the vital stain dimethyl-aminostyryl methylpyridinium iodine (DASPMI), a fluorescent, non-toxic stain for mitochondria (Bereiter-Hahn, 1976; Bereiter-Hahn *et al.*, 1983), combined with confocal scanning laser

microscopy (CSLM) (Brakenhoff *et al.*, 1979, 1985, 1989). CSLM allows the generation of high resolution, three-dimensional images of living cells, without the disadvantage of conventional fluorescence microscopy; i.e., the strong out-of-focus fluorescence light which reduces the contrast of images made at high magnifications.

Material and Methods

Microorganism and growth conditions

Diploid *Saccharomyces cerevisiae* was obtained by crossing the strains X2180-1A and X2180-1B (Yeast Genetic Stock Centre, Berkeley, California) and was maintained on malt-agar slopes.

Shake-flask cultivation

Repeated batch cultures were grown overnight at 28 °C on an orbital shaker (200 rpm) in 250 ml erlenmeyer flasks containing 50 ml of a synthetic medium (Wickerham, 1946) with either 4 $g \cdot l^{-1}$ ethanol or 4 $g \cdot l^{-1}$ glucose as a carbon and energy source. Cultures were grown for 8-10 generations until the OD_{450} read 0.2, at which time the exponentially growing cells were transferred to a new culture. Samples for microscopy were taken during exponential growth at an OD_{450} of approximately 0.2.

Chemostat cultivation

Chemostat cultivation was performed in 2-liter laboratory fermenters (Applikon Dependable Instruments b.v., Schiedam, the Netherlands) with a 1-liter working volume, at a dilution rate of 0.10 h^{-1}. The mineral medium, supplemented with vitamins, trace elements and the anaerobic growth factors ergosterol (5,7,22-ergostatrien-3β-ol; Sigma E-6510) and Tween-80 (polyoxyethylene-sorbitanmonooleate; Merck 822187) (see Andreasen and Stier, 1953, 1954) was prepared as described by Weusthuis *et al.* (1993). Glucose was added to the sterile mineral medium after separate sterilization at 110 °C. The pH was maintained at 5.0 by automatic addition of 2M KOH.

The stirrer speed was maintained at 1000 rpm and the growth temperature was 30 °C. The dissolved-oxygen concentration was measured with an Ingold autoclavable oxygen electrode. For anaerobic cultivation, the fermenter was flushed with 1 $l \cdot min^{-1}$ pure nitrogen gas, containing less than 5 ppm oxygen (Air Products, Waddinxveen, The Netherlands). The medium was made anaerobic before entering the culture by passing it through a second, sterile fermenter which was vigorously flushed with nitrogen. To minimize diffusion of oxygen, Norprene tubing (Cole-Parmer Instruments Corp., Chicago, USA) was used. For aerobic cultivation, the fermenter was sparged with air (1 $l \cdot min^{-1}$).

Transient-state experiments

Transient-state experiments were performed by switching the gas supply of steady-state chemostat cultures from nitrogen to air or *vice versa*. This resulted in a rapid change in the dissolved-oxygen concentration: within 5 min, the dissolved-oxygen concentration had increased to above 30 % air saturation or to below 0.1 % air saturation, respectively. After the switch to aerobic conditions, the dissolved-oxygen concentration remained above 30 % of air saturation throughout the experiment. Samples were collected from the effluent at appropriate time intervals and analysed for biomass dry weight, metabolite

concentrations and mitochondrial morphology. Each transient-state experiment was performed in duplicate and showed good reproducibility. In this paper, data from two experiments are presented.

Analytical procedures

Culture dry weights were determined using a microwave oven and 0.45 μm membrane filters as described by Postma *et al.* (1989). Parallel samples varied by less than 1%. Concentrations of ethanol, glycerol and acetate were determined by HPLC (Weusthuis *et al.*, 1993).

Oxygen-consumption measurements

The maximum respiratory capacity of culture samples was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA) at 30 °C. Cyanide-insensitive respiration was assayed in the presence of 1 mM KCN. Higher cyanide concentrations did not enhance the observed inhibitory effect.

Staining techniques for fluorescence microscopy

Cells were harvested by centrifugation (10 min at 4,000 × *g*) at room temperature, washed and resuspended in 0.1 M Tris-HCl buffer (pH 8.0). After 30 min incubation at room temperature with 10^{-6} M DASPMI, excess stain was removed by washing the cells with buffer.

Cell-volume measurements

Cell volumes (if not measured by CSLM; see below) were measured in an Olympus photomicroscope with a 100/1.3 lens. Images were collected with a video camera and transferred to a Macintosh IIci computer (Apple Computer Inc., Cupertino, California, USA). Measurements were made with a modified version of the program *Image* 1.35 (supplied by the Natl. Inst. of Health, Maryland) as described by Huls *et al.* (1992).

Conventional fluorescence microscopy

Conventional fluorescence microscopy was used when observing a large number of cells in a short period of time was more important than making three-dimensional reconstructions of a small number of cells, for instance during transient-state experiments and for determining the percentage of dividing cells. DASPMI-stained cells were placed between coverslips and observed in an Olympus photomicroscope, equipped for epifluorescence with a 100-W high-pressure mercury arch lamp and a 100/1.3 lens. Filters for fluorescein-isothiocyanate (FITC) excitation were used. Photographs were made on Kodak TMAX-400 film and developed in Kodak D-76, thus pushing the sensitivity of the film to 1600 ASA.

Confocal-scanning laser microscopy (CSLM) techniques

For making optical sections, samples were photographed with a CSLM (prototype; University of Amsterdam), with its krypton ion laser (Spectra-Physics model 2020) tuned to 483 nm and a 510 nm blocking filter. In order to avoid rapid photobleaching, the output of the laser beam was kept constant at 10 mW and the illumination pinhole in the lightpath of the confocal microscope was kept as small as possible. To increase the signal-to-noise ratio, the detector pinhole of the microscope was slightly opened, thereby trading

a small amount of confocality against a reduction of noise in the image. For the same reason 4 images per section were integrated, reducing this ratio further by a factor of 2. Yeast cells that were visualized in the CSLM were sectioned optically, usually in 16 layers, and stored as digital data on magnetic disk. After complete scanning of one section, the scanning stage containing the specimen was automatically raised by means of computer-controlled piezo-electric elements to prepare for the next section.

Image processing

In order to further decrease the amount of noise in the image, a 3-dimensional equivalent of a median filter was used on the images. The 3-D data were visualized by reprojection, a method simulating the optical characteristics (absorption, transmission, fluorescence) of these points, which are actually little volumes.
The success of this method critically depends on the signal-to-noise ratio of the image and the possibility of finding a threshold which separates objects from their surroundings. The suggestion of depth was enhanced by calculating the shadow which the object casts on an imaginary wall behind it. For a technical treatment of these aspects the reader is referred to Wu and Hesselink (1988) and Van der Voort *et al.* (1989).

Coulter counter

For electronic particle-counting, cells were lightly sonicated (1 s at 75 W output with a Branson sonifier) to avoid clumps. A 70-μm orifice tube (Coulter Electronics Ltd., Luton, England) was used at a current of 0.55 mA and with a solution of 0.9% (w/v) NaCl and 0.24 % (v/v) formaldehyde as diluent.

Results

Mitochondrial morphology in repeated batch cultures growing on glucose or ethanol

For an initial comparison of mitochondrial structure in respiring and fermenting *S. cerevisiae*, confocal-scanning laser microscopy (CSLM) was performed on DASPMI-stained cells grown in aerobic repeated batch cultures on ethanol or glucose, respectively. This method ensures that the cells are continuously growing exponentially. The mitochondrial morphology in these cultures was very different: short, round or oval mitochondria were observed in ethanol-grown cells (Fig. 1A), whereas large, branched mitochondrial structures were observed in glucose-grown cells (Fig. 1B).

Light microscopy can be used for reliable measurement of the volume of large numbers of yeast cells (Huls *et al.*, 1992). The cellular volume and the percentage of dividing cells in the ethanol- and glucose-grown cultures were determined by measuring about 250 cells. In a smaller number of cells (ca. 20), the number of mitochondria per cell and the mitochondrial volume were measured by CSLM optical sectioning. The most striking observation concerned the number of mitochondria, which was approximately ten-fold higher in ethanol-grown cells than in cells grown on glucose (Table 1). Although the number of mitochondria was different, the mitochondrial volume in ethanol- and glucose-grown cells was approximately the same (Table 1). This implies that mitochondria in ethanol-grown cultures have a much larger surface area than those in glucose-grown cells.

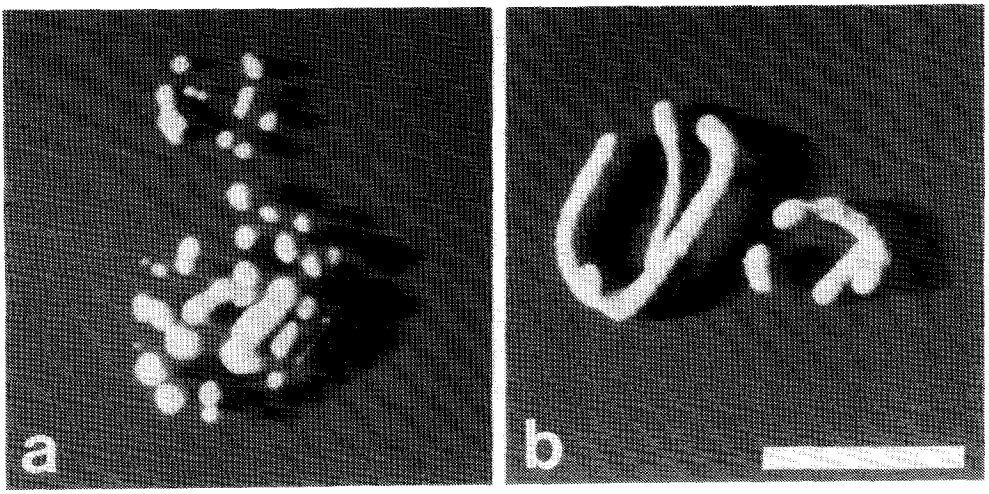

Fig. 1 Reprojected (Wu and Hesselink, 1988, Van der Voort et al., 1988) CSLM images of the mitochondria of budding S. cerevisiae cells grown in repeated batch cultures with ethanol as a non-fermentable carbon source (A) and with a relatively high concentration of glucose (B). Bar = 5 μm.

Table 1. Effect of growth substrate on morphology of *S. cerevisiae* cells and mitochondria. Exponentially growing cells were sampled from repeated batch cultures grown on the fermentable substrate glucose or the non-fermentable substrate ethanol.

Growth substrate	Growth phase	Cells			Mitochondria	
		%[1]	volume[2]	N[3]	Number per cell[4]	Relative volume[5]
Glucose	non-budding	48	55	9	2.3	7.4
Glucose	budding	52	79	13	3.2	7.4
Ethanol	non-budding	53	43	11	20-30	6.3
Ethanol	budding	47	63	10	20-30	6.2

1. The percentage of (non)-budding cells was calculated from ca. 250 measurements with an ordinary light microscope.
2. The average volume (μm^3) was calculated from ca. 250 measurements with an ordinary light microscope.
3. Number of cells analyzed by CSLM
4. Average number of mitochondria per cell as measured by CSLM optical sectioning.
5. Relative volume of mitochondria as percentage of cell volume.

Table 2. Changes in cell size, biomass concentration and cell mass during a switch from anaerobic to aerobic conditions in a glucose-limited chemostat culture. Cell-size measurements were performed with a Coulter counter (see Materials and Methods).

Time (h)	Average size (% of initial value)	Dry weight ($g \cdot l^{-1}$)	Cell number (ml^{-1})	Cellular mass (mg dry weight·$cell^{-1}$)
0	100	1.27	56.10^6	$2.3 \cdot 10^{-11}$
2	112	1.40	78.10^6	$1.8 \cdot 10^{-11}$
25	140	6.16	293.10^6	$2.1 \cdot 10^{-11}$

Glucose-limited chemostat cultures.

Theoretically, the large mitochondrial surface area observed in ethanol-grown cells may be related to the high respiration rates in such cultures as compared to the glucose-grown batch cultures, in which respiratory enzymes are largely repressed. To eliminate influences of growth rate and glucose catabolite repression, the relation between respiratory capacity and mitochondrial morphology was further investigated in both aerobic and anaerobic glucose-limited chemostat cultures. The dilution rate was fixed at 0.10 h^{-1} to avoid the mixed respiro-fermentative metabolism that is exhibited by aerobic, sugar-limited chemostat cultures of *S. cerevisiae* at high dilution rates (von Meyenburg, 1969; Postma *et al.*, 1989).

In anaerobic, glucose-limited chemostat cultures, glucose metabolism was strictly fermentative. Under these conditions, all cells exhibited a mitochondrial morphology similar to that shown in Fig. 1B. In contrast, cells from aerobic glucose-limited chemostat cultures, in which glucose metabolism was strictly respiratory, contained many small mitochondria, similar to the morphology found with cells grown on ethanol in batch cultures (Fig. 1A).

The transition from anaerobic, fermentative growth to aerobic, respiratory growth results in drastic changes in the metabolism of *S. cerevisiae*. To investigate these changes and their impact on mitochondrial morphology, transient-state experiments were performed, in which anaerobic chemostat cultures were switched to aerobic conditions and *vice versa*.

Transition from anaerobic to aerobic conditions

In aerobic and anaerobic steady-state chemostat cultures, the specific growth rates are equal to the dilution rate. However, the biomass yield of *S. cerevisiae* on glucose in aerobic, sugar-limited cultures is approximately five-fold higher than in anaerobic cultures (Verduyn, 1991). This difference, which is due to the contribution of oxidative phosphorylation to energy transduction, implies that the biomass concentration will increase during a switch from anaerobic to aerobic conditions and, consequently, that the growth rate will increase during the transient state. The expected increase in biomass concentration was indeed observed: during the transition from anaerobic to aerobic growth, the growth rate transiently increased to 0.2 - 0.3 h^{-1} (Fig. 2A). Although the dry weight per cell appeared to be fairly constant during the transition, the cell volume increased by ca. 40 %, suggesting that the water content of the cells increased (Table 2).

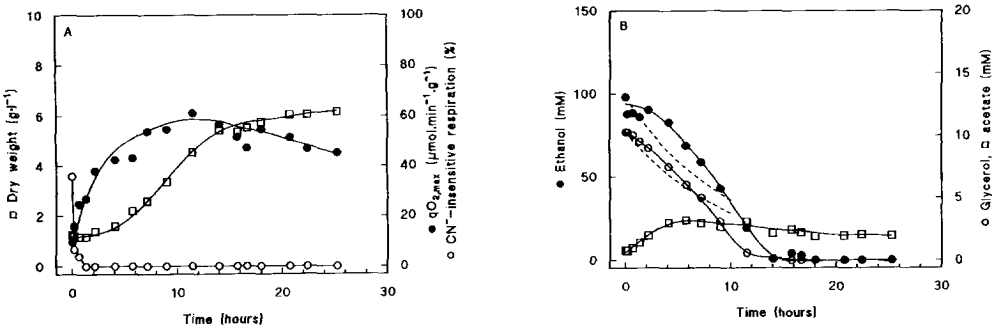

Fig. 2 Transient response of an anaerobic, glucose-limited chemostat culture of S. cerevisiae after a shift to aerobic conditions. A. Biomass concentration, maximum respiratory capacity and contribution of cyanide-insensitive respiration to the maximum respiratory capacity. B: Concentrations of ethanol, glycerol and acetate. The dashed lines show wash-out kinetics (no net consumption or production) for glycerol and ethanol. Growth conditions: D = 0.10 h^{-1}, pH 5.0, T = 30 °C, reservoir concentration of glucose 11.5 $g \cdot l^{-1}$.

After shifting an anaerobic, fermentative culture to aerobic conditions, the culture can still produce ATP from fermentative metabolism, but also from respiration of glucose, ethanol, glycerol and other fermentation products. To follow the change from fermentative to respiratory metabolism, concentrations of relevant metabolites were measured in the fermenter effluent. In chemostat cultures, the concentration of metabolites is the net result of production, consumption and dilution (wash-out). Immediately after addition of oxygen to the culture, the concentrations of ethanol and glycerol in the culture medium decreased (Fig. 2B). However, during the first two hours after the shift, the decrease of the glycerol concentration was slower than calculated on the basis of wash-out kinetics (Fig. 2B). Apparently, glycerol formation continued during this period, albeit at a lower rate than in the anaerobic culture. Similarly, alcoholic fermentation appeared to continue for approximately 4 h after the shift. After this period, utilization of ethanol and glycerol occurred (Fig. 2B).

Maximum respiratory capacities (qO_2^{max}) increased rapidly after switching from anaerobic to aerobic conditions (Fig. 2A), reaching a maximum approximately 8 h after the switch. It is well known that alternative, cyanide-insensitive respiratory systems are often expressed in yeasts when normal respiration is limited (Alexander and Jeffries, 1990). Indeed, cyanide-insensitive oxygen consumption made up approximately half of the total respiratory capacity in anaerobically grown cells (Fig. 2A). Upon the switch from anaerobic to aerobic conditions, cyanide-insensitive respiratory capacity decreased very sharply during the first hour, and was virtually absent in cells from aerobic cultures (less than 1 $\mu mol \cdot min^{-1} \cdot g^{-1}$; Fig. 2A).

During the shift from anaerobic to aerobic conditions, mitochondrial morphology was regularly studied by fluorescence microscopy (Fig. 3). The various stages show that the change in mitochondrial morphology became evident about 8 h after the shift to aerobic

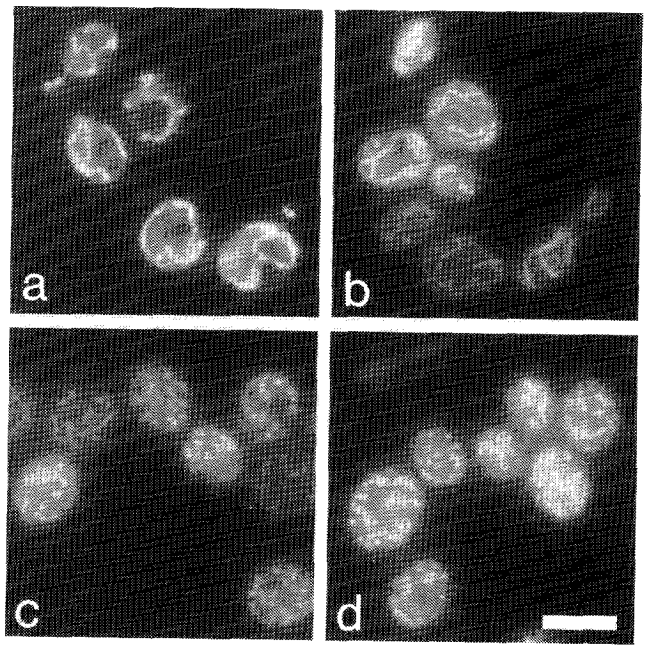

Fig. 3 Fluorescence microscopy of DASPMI-stained mitochondria in S. cerevisiae. Photographs were taken at various time intervals after a shift from anaerobic to aerobic growth conditions in a sugar-limited chemostat culture: A, anaerobic steady-state culture; B, 7 h after the shift; C, 11.5 h after the shift; D, 18 h after the shift: at this stage the morphology was indistinguishable from that of an aerobic steady-state culture. Growth conditions as in Fig. 2.

conditions. Since these transient states were performed in continuous partially new population. Nevertheless, the cultures remained homogeneous with respect to mitochondrial structure throughout the transient-state experiments (data not shown). This indicates mitochondrial morphology was changing within existing cells, rather than by the appearance of daughter cells with a different mitochondrial structure.

A remarkable difference with respect to time scale was observed when the changes in respiratory capacity and in mitochondrial morphology were compared (Fig. 2A and 3). After 7 h the cells still contained large, branched mitochondria, although the respiratory capacity was already near its maximum.

Transition from aerobic to anaerobic conditions.

When an aerobic chemostat culture is shifted to anaerobic conditions, respiration ceases as a result of the absence of oxygen and energy transduction becomes critically dependent on fermentation (i.e. substrate-level phosphorylation). The lower ATP yield of alcoholic fermentation was expected to lead to a temporary reduction of the growth rate. Indeed,

microscopy revealed a decrease in the percentage of budding cells (data not shown). Furthermore, the biomass concentration decreased during the first hours due to wash-out (Fig. 4A). After the shift to anaerobiosis, ethanol appeared virtually immediately (Fig. 4B). Also glycerol formation set in immediately after the shift. Glycerol is produced by *S. cerevisiae* when the surplus NADH that is formed during biomass synthesis cannot be reoxidized by respiration (Weusthuis *et al.*, 1994).

When aerobic cells were deprived of oxygen, a rapid decrease of the oxygen-uptake capacity was observed during the first hours (Fig. 4A). The capacity of cyanide-insensitive oxygen consumption increased from 1 to 7 $\mu mol \cdot min^{-1} \cdot g^{-1}$ and eventually, in the anaerobic steady-state culture, contributed 50 % of the total oxygen consumption.

Fluorescence microscopy of DASPMI-stained samples taken during the switch to anaerobic conditions demonstrated that the adaptation of mitochondrial morphology shown in Fig. 3 was reversible. The many small mitochondria that were observed in aerobic cultures evolved into a few, large and branched structures over a period of approximately 10 h (data not shown).

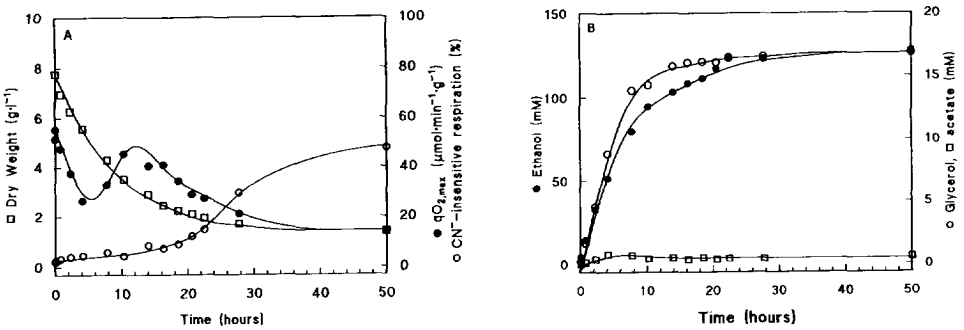

Fig. 4 Transient response of an anaerobic, glucose-limited chemostat culture of S. cerevisiae after a shift to aerobic conditions. A. Biomass concentration, maximum respiratory capacity and contribution of cyanide-insensitive respiration to the maximum respiratory capacity. B: Concentrations of ethanol, glycerol and acetate. Growth conditions: D = 0.10 h^{-1}, pH 5.0, T = 30 °C, reservoir concentration of glucose 14.6 $g \cdot l^{-1}$.

Discussion

Most studies on the effect of environmental conditions on yeast mitochondria have focused on the expression of key respiratory enzyme activities, including TCA-cycle enzymes and components of the respiratory chain. In *S. cerevisiae*, regulation of the expression of many of these enzymes has been studied in detail at the molecular level. It is generally dependent on the presence of oxygen and subject to glucose catabolite repression (for a review see De Winde and Grivell, 1993).

Investigations into the relation between environmental conditions and mitochondrial morphology has yielded conflicting results. This may be due to technical problems related to visualization of (pro)mitochondria and the maintenance of well-defined growth

conditions. Many studies have been performed under poorly defined growth conditions in shake-flask cultures, which implies that conditions that are of key importance in the regulation of mitochondrial activity (in particular glucose-concentration and dissolved-oxygen concentration) cannot be controlled. Perhaps for this reasons, clear correlations between environmental conditions and mitochondrial number, volume and shape were often not observed.

In the present study, low-cell density repeated batch cultivation and chemostat cultivation have been used to avoid some of the problems inherent to the use of standard shake-flask cultures. Vital staining of mitochondria with the fluorescent dye DASPMI, in combination with confocal-scanning laser microscopy (CSLM) obviated the use of the fixation and dehydration steps that are required for electron microscopy.

The extreme situations that were encountered with respect to mitochondrial morphology are illustrated in Fig. 1. The exclusively respiratory metabolism in ethanol-grown cells was accompanied by the presence of many small mitochondria. A similar morphology was observed in aerobic, glucose-limited chemostat cultures. One or a few large, branched mitochondria were observed in cells from exponentially growing, aerobic batch cultures on glucose, in which metabolism is predominantly fermentative due to glucose repression of respiratory enzymes (De Winde and Grivell, 1993; Alexander and Jeffries, 1990). This morphology was also observed in cells from anaerobic, glucose-limited chemostat cultures. These differences in mitochondrial morphology were also readily apparent with conventional fluorescence microscopy, which was therefore used to study mitochondrial morphology during transient-state experiments in chemostat cultures.

Our data indicate that, similar to the regulation of the key enzymes of mitochondrial respiration, the morphology of *S. cerevisiae* mitochondria is influenced by a combination of oxygen availability and glucose concentration (Table 3). Situations that are known to lead to repression of respiratory enzymes (anaerobic conditions and/or presence of excess glucose) were invariably accompanied by the presence of one or a few large, branched mitochondria per cell. In contrast, many small mitochondria were observed in cells grown under derepressing conditions (aerobic growth on ethanol or in aerobic, glucose-limited chemostat cultures). The relation between mitochondrial morphology and growth conditions observed in the present study is generally in compliance with that observed by Stevens (1977, 1981), who studied mitochondrial morphology by electron microscopy and serial sectioning. A strikingly different pattern has been reported by Gélinas and Goulet (1991). Using freeze-etching techniques, these authors found large, branched mitochondria in highly aerated, fed-batch grown cells and small mitochondria in cells lacking oxygen. In our study, the different mitochondrial number and morphology observed in ethanol- and glucose-grown cells did not significantly affect the fraction of the cellular volume occupied by the mitochondria (6-7%, Table 1). In contrast, Stevens (1977) observed that mitochondrial volumes for glucose-grown cells and cells grown on a nonfermentable carbon source were different (3% and 13 %, respectively). The mitochondrial volumes found in the present study were ca. 50 % lower than those observed by Grimes *et al.*, (1974).

Table 3. Mitochondrial morphology under different cultivation conditions as observed with fluorescence microscopy of DASPMI-stained cells. Substrate excess refers to cells grown in batch cultures on 4 $g \cdot l^{-1}$ carbon source. Substrate-limited conditions refer to chemostat cutures ($D = 0.10\ h^{-1}$) fed with a medium in which the carbon source was the growth-limiting factor. The glucose concentration in such cultures was below 100 $mg.l^{-1}$. Absence of oxygen refers to a dissolved oxygen concentration below the detection limit of commercially available oxygen probes. The terms repressed/derepressed refer to well-documented (Gancedo and Serrano, 1989) regulation of various TCA-cycle and respiratory enzymes. Mitochondrial morphology refers to the differences shown in Fig 1.

Growth Substrate	Concentration	Oxygen	Respiratory enzymes	Mitochondrial morphology
glucose	excess	present	repressed	few, large
ethanol	excess	present	derepressed	many, small
glucose	excess	absent	repressed	few, large
glucose	limiting	absent	repressed	few, large
glucose	limiting	present	derepressed	many, small

With a constant mitochondrial volume, a change in morphology from a single, large mitochondrion to a large number of small organelles leads to an increase of the total mitochondrial surface area. This might reflect an increased membrane-area requirement, necessary to accommodate newly synthesized components of the respiratory chain. However, during a shift from anaerobic to aerobic conditions, changes in mitochondrial morphology continued for several hours after the respiratory capacity had reached its maximum (Fig. 2 and 3). This indicates that presence of many small mitochondria is not a prerequisite for the increased respiration rate. In this respect, it is important to bear in mind that the membrane area available for respiratory enzymes is also dependent on the fine-structure of the mitochondrial inner membrane, which starts developing when cells are shifted to aerobic conditions (Damsky *et al.*, 1976).

The present study indicates that chemostat cultivation, in combination with vital staining, is a useful tool for studies into the relation between growth conditions an mitochondrial morphology. This method can be extended to other situations that are particularly interesting with respect to the regulation of respiratory capacity in yeasts. A number of options are available to manipulate respiratory activity in yeasts, including aerobic, glucose-limited growth at high dilution rates, which leads to a mixed respiro-fermentative metabolism (Von Meyenburg, 1969; Postma *et al.*, 1989), growth under a double limitation of glucose and oxygen (Weusthuis *et al.*, 1994) and growth in the presence of uncoupling weak acids, which leads to very high respiration rates and an increase of the mitochondrial volume (Verduyn *et al.*, 1992). In view of the conflicting results with some earlier reports and in order to obtain more detailed information on mitochondrial structure, in particular with respect to inner-membrane surface area (Damsky *et al.*, 1976), it is essential that such work should also include the use of electron microscopic techniques.

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CHAPTER 5

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

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Abstract

Growth and metabolite formation was 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.

Also *C. utilis* could be grown under dual limitation of glucose and oxygen. However, at very low oxygen feed rates (i.e. below 4 $mmol \cdot l^{-1} \cdot h^{-1}$) 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 exceptional: alcoholic fermentation did not occur in such cultures 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* 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* 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.

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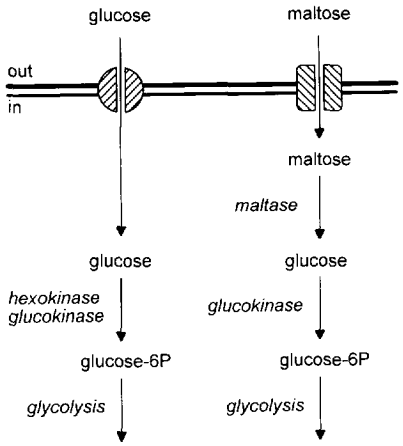

Figure 1. *Differences between glucose and maltose metabolism of yeasts. In this scheme it has been assumed that phosphorylation of glucose units derived from maltose occurs by glucokinase only, while glucose that is taken up from the medium can be phosphorylated by both glucokinase and hexokinase (Clifton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

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 carbon dioxide and water. The majority of the yeast species described sofar are also capable of fermenting sugars to ethanol and carbon dioxide (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).

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 substrate. The Pasteur effect has been defined as an inhibition of fermentative sugar metabolism by oxygen (Lagunas, 1986; Lagunas *et al.*, 1982; 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, 1986; Postma *et al.*, 1988; 1989a, b; Käppeli *et al.*, 1985a, b; Käppeli & Sonnleitner, 1986; van Urk *et al.*, 1988; 1989).

A fourth phenomenon, the Kluyver 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 Kluyver 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 Kluyver effect must be caused by differences between monosaccharide and disaccharide metabolism. As shown in figure 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 Kluyver effect. Many of these directly or indirectly involve oxygen as a key factor. Kluyver and 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 figure 1 may cause the Kluyver 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 Kluyver effect are capable of rapid anaerobic growth (Visser *et al.*, 1990), occurrence of the Kluyver effect must be confined to oxygen-limited growth conditions. Although oxygen has often been implicated as a key factor in its occurrence, the Kluyver 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 Kluyver effect. For this purpose, the effect of the oxygen feed rate on sugar metabolism was studied in sugar-limited chemostat cultures. *C. utilis* CBS 621 was chosen as a model organism that exhibits the Kluyver effect for maltose (Sims & Barnett, 1978), whereas *S. cerevisiae* CBS 8066 is Kluyver-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.

Material and Methods

Organisms and maintenance

S. cerevisiae CBS 8066 and *C. 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, Schiedam, The Netherlands) at a dilution rate of 0.10 h^{-1}, a temperature of 30 °C and a stirrer speed of 750 rpm. 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 differed 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-3ß-ol, Sigma E-6510) and Tween-80 (polyoxyethylene-sorbitanmono-oleate, 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 con-centration 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 $g \cdot l^{-1}$. The purity of the chemostat cultures was routinely checked by phase contrast microscopy at 1000× 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 $ml \cdot min^{-1}$. The temperature of the ingoing air was kept constant at 20 °C. The overall gas flow into the cultures was maintained at 0.5 $l \cdot min^{-1}$ by the supplementary addition of nitrogen gas, using a Brooks 5876 mass flow controller (Brooks, The Netherlands). 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 (Cole Parmer Inc., U.S.A.), and the reservoir vessel was flushed with nitrogen gas. The dissolved-oxygen concentration in the cultures was monitored with a polarographic oxygen electrode (Ingold, Switzerland).

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 into the cultures (N_2 and air) 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 were 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, the carbon dioxide content with a Beckman infrared CO_2 analyser. The exhaust gas entering both analysers was dried with a Perma Pure Dryer (PD-625-12P). Specific rates of carbon dioxide production and oxygen consumption were calculated according to van Urk *et al.* (1988). The amount of CO_2 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 enzymatic 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-μm 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; Laplace *et al.*, 1991; Furukawa *et al.*, 1983; Nishizawa *et al.*, 1980; Cysewski & Wilke, 1976; Moss *et al.*, 1969). 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 (Grosz & Stephanopoulos, 1990; Kuriyama & Kobayashi, 1993; Oura, 1972). The effect of oxygen feed rate on growth and metabolism is strongly influenced by the gas transfer characteristics of the culture, 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 (qO_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 qO_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 qO_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), it was tried 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 is ca. 5-fold higher than that under strictly anaerobic conditions (Ver-duyn *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 amount of sugar in the feed, 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 ethanol, carbon dioxide, 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 metabolised by respiration. This fraction is equal to the ratio of the actual specific oxygen uptake rate (qO_2) and the specific oxygen uptake rate during fully respiratory growth (qO_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.

$$\frac{1}{Y} = \frac{1}{Y_F}\left(1 - \frac{qO_2}{qO_{2,R}}\right) + \frac{1}{Y_R}\frac{qO_2}{qO_{2,R}} \qquad (1)$$

Equation 1 is based on the assumption that the fraction of sugar that is fermented ($1/Y_F$) decreases linearly with increasing qO_2. Correspondingly, with increasing qO_2, the specific ethanol production rate ($q_{ethanol}$) will decrease linearly from the rate that is observed during anaerobic growth linearly from the rate that is observed during anaerobic growth ($q_{ethanol,F}$) to zero, according to equation 2.

$$q_{ethanol} = q_{ethanol,F}\left(1 - \frac{qO_2}{qO_{2,R}}\right) \qquad (2)$$

Dissimilatory production of CO_2 occurs both during respiration and fermentation. During complete respiratory dissimilation of glucose, the rate of carbon dioxide production is equal to the oxygen consumption rate (the respiratory quotient, RQ, is being one). Fermentative CO_2 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 CO_2 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 CO_2 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 $qCO_{2,A}$ of 0.58 $mmol \cdot g^{-1} \cdot h^{-1}$ has been reported by Verduyn *et al.* (1990). When the three sources of carbon dioxide are combined, this results in equation 3:

$$qCO_2 = qCO_{2,A} + qO_2 + q_{ethanol} \quad (3)$$

Substitution of equation 2 in equation 3 gives equation 4, which indicates that also the specific carbon dioxide production rate by the cultures is a linear function of qO_2.

$$qCO_2 = qCO_{2,A} + qO_2 + q_{ethanol,F}\left(1 - \frac{qO_2}{qO_{2,R}}\right) \quad (4)$$

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 $mmol \cdot l^{-1} \cdot h^{-1}$ and sugar concentrations below 5 $g \cdot l^{-1}$, growth of *S. cerevisiae* was not oxygen-limited: a further increase in air supply did not result in higher biomass yields (tables 1 & 2). Growth was fully respiratory, as was evident from the absence of ethanol in the culture supernatants (tables 1 & 2) and an RQ of approximately 1 (figure 3). The qO_2 of these oxygen-sufficient, glucose-limited chemostat cultures was ca. 2.5 $mmol \cdot g^{-1} \cdot h^{-1}$ (figure 2). When qO_2 was reduced by decreasing the oxygen feed rate,

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

oxygen ($mmol \cdot l^{-1} \cdot h^{-1}$)		glucose ($g \cdot l^{-1}$)		dry weight	ethanol
in[a]	out[b]	in[c]	out[d]	($g \cdot l^{-1}$)	(mM)
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

[a] oxygen feed rate; [b] oxygen leaving the culture; [c] reservoir sugar concentration; [d] residual sugar concentration.

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

oxygen ($mmol \cdot l^{-1} \cdot h^{-1}$)		glucose ($g \cdot l^{-1}$)		dry weight	ethanol
in	out	in	out	($g \cdot l^{-1}$)	(mM)
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

alcoholic fermentation set in (tables 1 & 2; figure 3). This coincided with a decrease of the biomass yield (figure 2). Over a range of oxygen consumption rates, respiratory and fermentative glucose metabolism occurred simultaneously. In these cultures, the bio-mass concentration could be increased either by increasing the oxygen feed rate (figure 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 (figure 2). However, the apparent biomass yield of 0.63±0.02 $g \cdot g\ sugar^{-1}$ (figure 2) in the oxygen-sufficient cultures was substantially higher than the 0.51 $g \cdot g^{-1}$ 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 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 (figure 3). Also oleic acid, added as Tween-80, may have a slight carbon-sparing effect and thus contribute to the biomass yield.

Both with glucose and maltose, $q_{ethanol}$ and qCO_2 increased with decreasing qO_2, to reach a maximum in the anaerobic cultures (figure 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 (figure 2) and higher $q_{ethanol}$ (figure 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

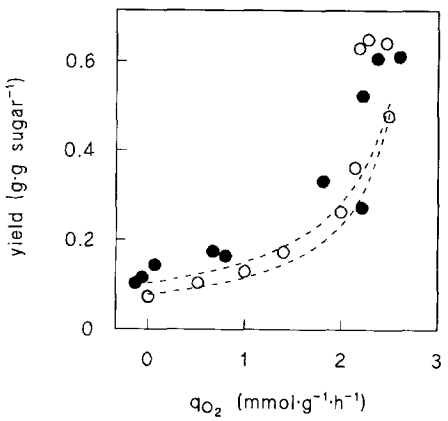

Figure 2. *Relation between the specific oxygen uptake rate (qO_2) and biomass yield of Saccharomyces cerevisiae CBS 8066, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}), with glucose (●) or maltose (○) as a carbon and energy source. The data are fitted with equation 1 (dashed lines). Note that in this and the following figures also some negative qO_2 values are presented. This is due to the inaccuracy of the gas analysis at very low oxygen feed rates (see materials and methods).*

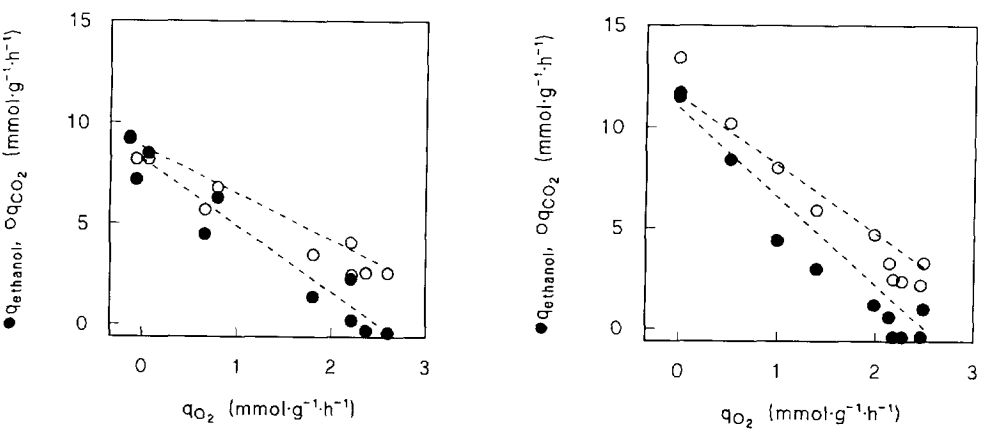

Figure 3. *Relation between the specific oxygen uptake rate (qO_2) and the specific production rates of ethanol ($q_{ethanol}$) and carbon dioxide (qCO_2) of Saccharomyces cerevisiae CBS 8066, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}), with glucose (A) or maltose (B) as a carbon and energy source. The ethanol data are fitted with equation 2, the carbon dioxide data with equation 4 (dashed lines).*
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}.

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

oxygen ($mmol \cdot l^{-1} \cdot h^{-1}$)		glucose ($g \cdot l^{-1}$)		dry weight	eth.	pyr.
in	out	in	out	($g \cdot l^{-1}$)	(mM)	(mM)
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

during anaerobic growth on maltose and glucose (figures 2 & 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 (figure 2).

Effects of oxygen feed on growth of C. utilis on glucose

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

At oxygen feed rates between 4.3 and 30 $mmol \cdot l^{-1} \cdot h^{-1}$, virtually all glucose was consumed (table 3), with respiration and fermentation occurring simultaneously (figure 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, figures 4 & 5). However, when the oxygen feed rate was decreased below 4.3 $mmol \cdot l^{-1} \cdot h^{-1}$, the residual glucose concentration in the cultures increased from below 0.1 $g \cdot l^{-1}$ to 28.3 $g \cdot l^{-1}$, 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 $mmol \cdot g^{-1} \cdot h^{-1}$ 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).

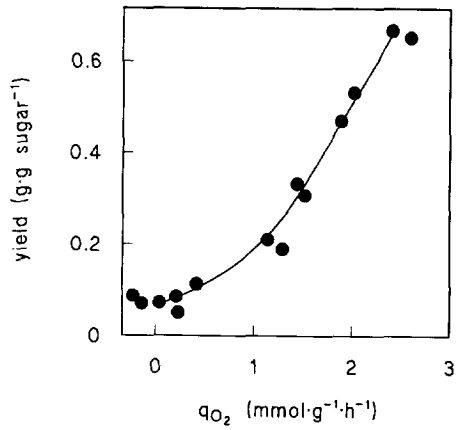

Figure 4. *Relation between the specific oxygen uptake rate (qO_2) and the biomass yield of Candida utilis CBS 621, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}), with glucose as a carbon and energy source.*

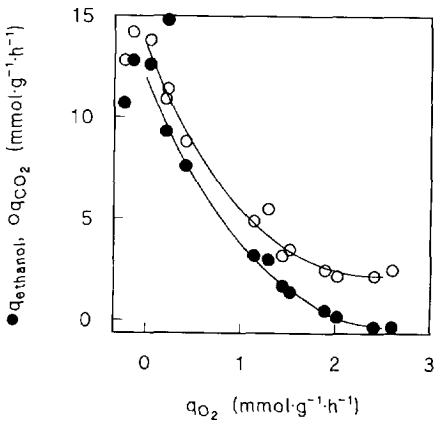

Figure 5. *Relation between the specific oxygen uptake rate (qO_2) and the specific production rates of ethanol ($q_{ethanol}$) and carbon dioxide (qCO_2) of Candida utilis CBS 621, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}), with glucose as a carbon and energy source.*

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 the equations 1-4. However, it is clear from figure 5 that during dual substrate-limited growth,

$q_{ethanol}$ and qCO_2 did not increase linearly with decreasing qO_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 (figures 4 & 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 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).

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 (figure 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 of oxygen feed rates tested, as indicated by the constant qCO_2 and qO_2 (figure 6). Since the qO_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).

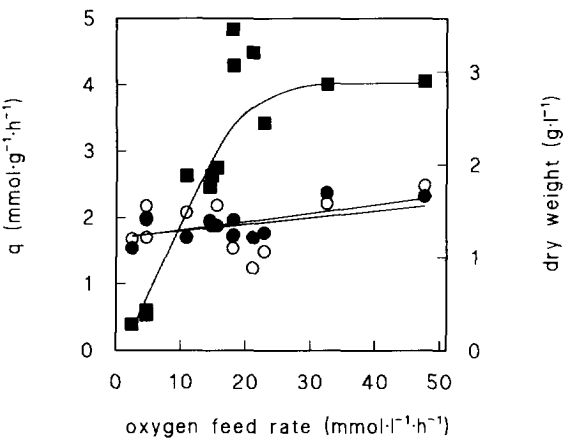

Figure 6. *Relation between oxygen feed rate and the dry weight (■), specific production rate of carbon dioxide (qCO_2, ●) and specific consumption rate of oxygen (qO_2, ○) of Candida utilis CBS 621, grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}), with maltose as a carbon and energy source. Note that on the x-axis the oxygen feed rate is plotted and not, as in the other graphs, qO_2.*

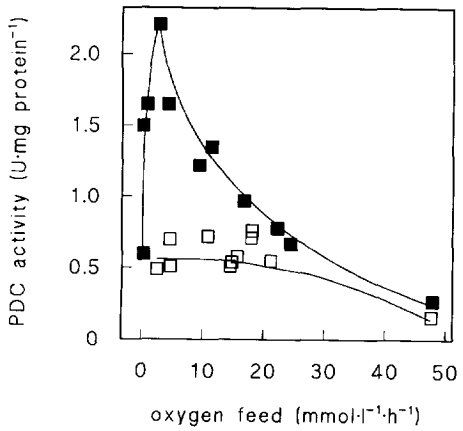

Figure 7. *Relation between oxygen feed rate and pyruvate decarboxylase activity of Candida utilis CBS 612, grown in chemostat cultures (D = 0.10 h^{-1}) with glucose (■) or maltose (□) as a carbon and energy source.*

Table 4. Effect of oxygen feed rate on oxygen and substrate utilization and production of biomass, ethanol and pyruvate by *Candida utilis* CBS 621 grown in chemostat (D = 0.10 h^{-1}) with maltose as a carbon and energy 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. nd = not determined; eth. = ethanol; pyr. = pyruvate.

oxygen ($l \cdot h^{-1}$)	(mmol·l⁻	maltose ($g \cdot l^{-1}$)		dry weight	eth.	pyr.
in	out	in	out	($g \cdot l^{-1}$)	(mM)	(mM)
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

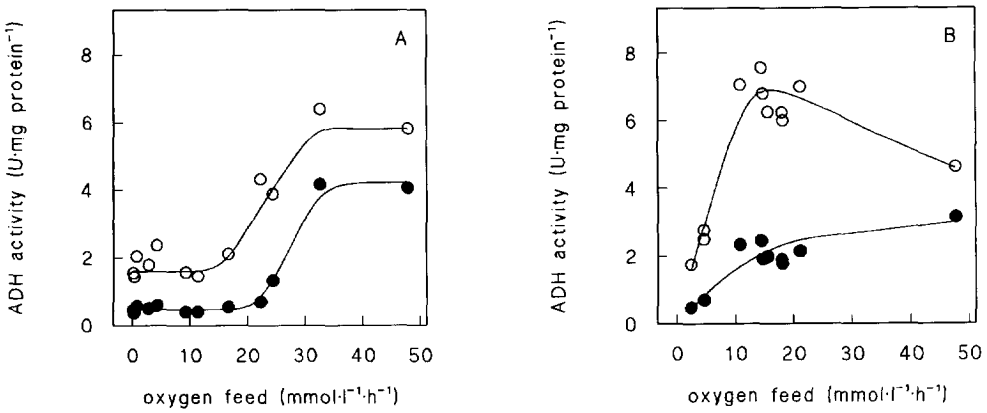

Figure 8. *Relation between oxygen feed rate and ethanol (○)- and pentanol (●)-dependent alcohol dehydrogenase activity of Candida utilis CBS 621, grown in chemostat cultures (D = 0.10 h^{-1}) with glucose (A) or maltose (B) as a carbon and energy source.*

At oxygen feed rates below 30 mmol·l^{-1}·h^{-1}, residual maltose concentrations in the cultures increased and the amount of biomass decreased (table 4, figure 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, 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 mmol·l^{-1}·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 g·l^{-1} biomass. 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} (figure 7). The maximum

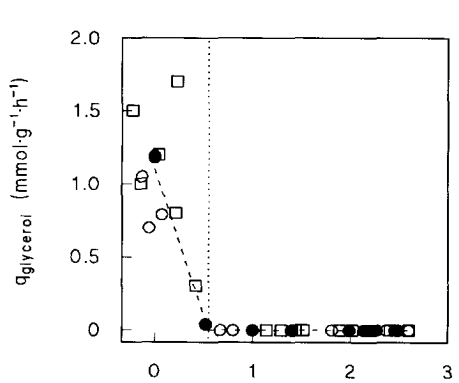

Figure 9. *Relation between specific oxygen uptake rate (qO_2) and specific glycerol production rate (qglycerol) of Saccharomyces cerevisiae CBS 8066 (circles) and Candida utilis CBS 621 (squares), grown at different oxygen feed rates in chemostat cultures (D = 0.10 h^{-1}) with glucose (open symbols) or maltose (closed symbols) as a carbon and energy source. The dashed line indicates the qO_2 value below which glycerol formation must occur to obtain a closed redox balance.*

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 weight^{-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 discerned from the other two ADH activities by its inability to use pentanol as a substrate. 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 co-metabolism. Under oxygen-limited condi-tions, the ADH activities of maltose-grown cells were equal to or higher than those of glucose-grown cells (figure 8).

Production of glycerol

In the 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 additional to ethanol, other metabolites may be excreted by yeasts. Quantitatively, glycerol is one of the major byproducts of alcoholic fermentation under anaerobic conditions (Verduyn *et al.*, 1990).

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 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 one mole of glycerol from glucose requires the net hydrolysis of one mole of ATP. In contrast, respiratory re-oxidation 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 $5.5 \times 0.1 = 0.55$ mmol·g^{-1}·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 (figure 9). Cultures in which qO_2 was decreased below this threshold invariably produced glycerol (figure 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*, can not 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 pyruvate decarboxylase 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 pyruvate decarboxylase 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 (figure 7), suggesting that oxygen may be a key factor in the regulation of pyruvate decarboxylase activity in this yeast. In fact, also in cultures in which growth was only limited by glucose (i.e., at dissolved-oxygen concentrations above ca. 1 % air saturation) the actual dissolved-oxygen concentration has a significant effect on pyruvate decarboxylase 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, 1985). Theoretically, therefore, yeasts can optimize their sugar metabolism under oxygen-limited growth conditions by preferentially using oxygen for the regeneration of 'excess' NADH. The data presented in figure 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 can also fulfil an essential role in assimilatory oxygenation reactions. Cellular constituents, the synthesis of which requires 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 & Stier, 1953; 1954) (table 1, figures 2 & 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, figure 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 are 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 flushed 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 respiratory on maltose, without the occurrence of alcoholic fermentation (figure 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 (figure 5). The fermentation rates in these cultures (figure 5) were equal to or even exceeding those of the Kluyver-negative yeast *S. cerevisiae*, which exhibited a respiro-fermentative metabolism during oxygen-limited growth on both glucose and maltose (figure 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 CO_2 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 CO_2 production measured in the experiments of Sims & Barnett (1978) was due to respiratory rather than fermentative maltose metabolism.

In earlier reports, low pyruvate decarboxylase activities have been reported for Kluyver-positive yeasts grown on disaccharides (Sims & Barnett, 1991; Sims *et al.*, 1991). In the present study, the pyruvate decarboxylase and alcohol dehydrogenase activities in *C. utilis* grown on maltose in oxygen-limited chemostat cultures (figures 7 & 8) were theoretically sufficient to sustain a $q_{ethanol}$ of 11 $mmol \cdot g^{-1} \cdot h^{-1}$. This theoretical flux is even higher than that observed in *S. cerevisiae* grown under the same conditions. Apparently, in *C. utilis* the Kluyver effect for maltose is not caused by an insufficient capacity of pyruvate decarboxylase and alcohol dehydrogenase. 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 Kluyver 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 Kluyver effect (Sims & Barnett, 1978; Schulz & Höfer, 1986). However, low, but significant disaccharide uptake rates have been reported for Kluyver-positive yeasts (Sims & Barnett, 1978; Schulz & Höfer, 1986). Barnett & Sims (1982) therefore concluded that the Kluyver 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 was stoichiometric-ally 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 Kluyver effect (Sims & Barnett, 1978) discriminated between aerobic and anaerobic utilization of sugars. However, *C. utilis* and other yeasts exhibiting the Kluyver effect are not capable of anaerobic growth (Visser *et al.*, 1990). Furthermore, the data presented here indicate that occurrence of the Kluyver 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 Kluyver effect: 'The inability to ferment certain disaccharides to ethanol and carbon dioxide, even though respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexose(s) are possible'.

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Oxygen requirements of fermentative yeasts

Summary

Wiebe Visser

One of the most important parameters influencing the metabolism of yeasts is the availability of oxygen. Oxygen-limited growth conditions trigger alcoholic fermentation in most yeasts, thereby providing the organism with the energy required for growth. However, the ability to grow anaerobically depends not only on the fermentative capacity: most yeast species are able to perform alcoholic fermentation, yet few can grow in the complete absence of oxygen.

In chapter 2, type species of 75 yeast genera have been examined for their ability to grow anaerobically in complex and mineral media. All strains tested were capable of fermenting glucose to ethanol in oxygen-limited shake-flask cultures, even those species generally regarded as "non-fermentative". To define anaerobic conditions a redox indicator, resazurin, was added to the media to signify low redox potentials. However, only 23 % of the yeast species tested grew under anaerobic conditions. A comparative study with a number of selected strains revealed that *Saccharomyces cerevisiae* stands out as a yeast capable of rapid growth at low redox potentials. Other yeasts, such as *Torulaspora delbrueckii* and *Candida tropicalis*, grew poorly (μ_{max} 0.03 and 0.05 h^{-1}, respectively) under anaerobic conditions in mineral media supplemented with Tween 80 and ergosterol. The latter organisms grew rapidly under oxygen limitation and then displayed a high rate of alcoholic fermentation. It can be concluded that these yeasts have hitherto unidentified oxygen requirements for growth.

Since oxygen requirements may relate to the physiological function of mitochondria in anaerobically grown cells, this function was investigated as described in chapter 3, using *S. cerevisiae* as a model organism.

Mitochondria of anaerobically grown *S. cerevisiae* cells are known to be very fragile. Hence, in this study, attention has been given to the isolation of intact mitochondria. Citrate synthase (EC 4.1.3.7) activity was taken as a marker enzyme in view of its key role in the TCA-cycle. Almost all of the activity of citrate synthase was recovered in the mitochondrial fraction after differential centrifugation of spheroplast lysates. The enzyme exhibited a high degree of latency which was demonstrated by sonication of the mitochondrial fractions.

Since citrate synthase is an important enzyme in anabolic reactions, a consequence of

its mitochondrial localization is the requirement for transport of metabolites across the mitochondrial membranes. Under anaerobic conditions, all ATP is produced in the cytoplasm during glycolysis. If promitochondria also fulfil a role in biosynthesis of cell material during anaerobic growth, energy must be supplied to the organelle both for driving transport processes across its membranes and for the energy-demanding reactions within the mitochondria.

Import of ATP into mitochondria may occur via an ADP/ATP translocator in exchange for ADP. The possible physiological function of yeast mitochondria under strictly anaerobic conditions was therefore also studied by testing the sensitivity of anaerobic cells to bongkrekic acid, a well-known inhibitor of the ADP/ATP translocator. It was shown that addition of this inhibitor to anaerobic cultures indeed inhibited growth, although only partially. It is concluded that mitochondria of *S. cerevisiae* fulfil a vital role in anaerobic sugar metabolism.

In chapter 4, the effects of growth conditions on mitochondrial morphology are described for living *S. cerevisiae* cells. Vital staining with the fluorescent dye dimethylaminostyryl-methylpyridinium iodine (DASPMI), fluorescence microscopy, and confocal-scanning laser microscopy were used for this *in vivo* study. Cells from respiratory, ethanol-grown batch cultures contained a large number of small mitochondria. Conversely, cells from glucose-grown batch cultures, in which metabolism was respiro-fermentative, contained small numbers of large, branched mitochondria. These changes did not significantly affect the fraction of the cellular volume occupied by the mitochondria. Similar differences in mitochondrial morphology were observed in glucose-limited chemostat cultures. In aerobic chemostat cultures, glucose metabolism was strictly respiratory and cells contained a large number of small mitochondria. Anaerobic, fermentative chemostat cultivation resulted in the large, branched mitochondrial structures also seen in glucose-grown batch cultures.

Upon aeration of a previously anaerobic chemostat culture, the maximum respiratory capacity increased from 10 to 70 μmole·min^{-1}·g dry weight^{-1} within 10 h. This transition resulted in drastic changes of mitochondrial number, morphology and, consequently, mitochondrial surface area. These changes continued for several hours after the respiratory capacity had reached its maximum. Cyanide-insensitive oxygen consumption contributed ca. 50 % of the total respiratory capacity in anaerobic cultures, but was virtually absent in aerobic cultures. The response of aerobic cultures to oxygen deprivation was qualitatively the reverse of the response of anaerobic cultures to aeration. The results indicate that mitochondrial morphology in *S. cerevisiae* is closely linked to the metabolic activity of this yeast: conditions that result in repression of respiratory enzymes generally lead to the mitochondrial morphology observed in anaerobically grown, fermenting cells.

The fermentation experiments described in the preceding chapters were all carried out using glucose as a carbon and energy source, since all fermenting yeasts will use this sugar. However, the main sugar constituent of media used in industrial fermentations is usually not glucose but disaccharides and oligosaccharides like maltose, lactose, maltotriose etc.. Many yeasts are not able to ferment these sugars, despite the fact that, under aerobic conditions, they grow rapidly using these substrates. This phenomenon is known as the Kluyver effect. For example, *Candida utilis* is able to grow on maltose under aerobic conditions, but cannot ferment this sugar under oxygen-limited growth conditions. The industrial impact of this Kluyver effect prompted us to study the

phenomenon in more detail, which is described in chapter 5.

Growth and metabolite formation were studied in oxygen-limited chemostat cultures of *S. cerevisiae* CBS 8066 and *C. 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.

The formation of yeast biomass leads to an excess of NADH, which will be re-oxidized by oxygen under respiratory conditions. Under anaerobic conditions, this surplus of reduction equivalents can be oxidized by the reduction of dihydroxyacetone phosphate to glycerol. It was shown by growing *S. cerevisiae* and *C. utilis* under dual limitation of glucose and oxygen, that both species produce glycerol when the q_{O2} is decreased below the same threshold value of 0.55 $mmol \cdot g^{-1} \cdot l^{-1}$. Since this value equals the net production rate of NADH, it could be concluded that under oxygen limiting growth conditions the redox metabolism in these yeasts is regulated to minimize ATP expenditure for glycerol formation.

In contrast to *S. cerevisiae* with *C. utilis*, growth at low oxygen feed rates (i.e. below 4 $mmol \cdot l^{-1} \cdot h^{-1}$) was limited by oxygen only as indicated by the high residual glucose concentration in the cultures. Furthermore, *C. utilis* could not be grown anaerobically at a dilution rate of 0.1 h^{-1}. Consequently, 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 exceptional: alcoholic fermentation did not occur in such cultures 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. Apparently, maltose is a non-fermentable sugar for *C. utilis* 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, since 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 in *C. utilis* the Kluyver effect for 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.

Zuurstofbehoefte van fermentatieve gisten

Samenvatting

Wiebe Visser

Een van de meest belangrijke parameters die het metabolisme van gisten beïnvloedt is de beschikbaarheid van zuurstof in het kweekmedium. Kweekcondities waarbij zuurstof limitatie optreedt leiden bij de meeste gisten tot een alcoholische fermentatie die de voor de groei benodigde energie levert. Echter, het vermogen om onder anaërobe omstandigheden te groeien hangt niet alleen af van de eigenschap om te kunnen fermenteren. Zo zijn de meeste gistsoorten in staat om suikers alcoholisch te vergisten, terwijl er maar enkele in staat zijn om te groeien in de volledige afwezigheid van zuurstof.

Hoofdstuk 2 betreft het onderzoek naar de vermogens tot anaërobe groei, zowel in complexe als minerale media, van de type soorten van 75 gistgeslachten. Een redox-indicator, resazurine, werd aan de media toegevoegd om de anaërobe condities te controleren. Het bleek dat alle geteste stammen in staat waren om glucose te vergisten tot ethanol als ze onder zuurstof-limiterende condities in schudflessen werden gekweekt, zelfs al die stammen die algemeen als "niet-gistend" bekend staan. Daarentegen vertoonde slechts 23% van de geteste stammen enige groei onder anaërobe omstandigheden. Bij een vergelijkende studie naar een aantal geselecteerde stammen bleek dat *Saccharomyces cerevisiae* de enige was die bij lage redox potentiaal nog snel kon groeien. Andere gisten, zoals *Torulaspora delbrueckii* en *Candida utilis* groeiden onder anaërobe omstandigheden slecht in minerale media waaraan Tween 80 en ergosterol was toegevoegd (μ_{max} respectievelijk 0,03 en 0,05 uur^{-1}). Dit ondanks de snelle groei van deze gisten onder zuurstof limitatie en hun hoge snelheid van alcoholische gisting onder deze omstandigheden. Er kan worden geconcludeerd dat deze gisten om te groeien een nog niet opgehelderde specifieke behoefte aan zuurstof hebben.

Aangezien zo'n behoefte gerelateerd kan zijn aan de fysiologische functie van mitochondriën in anaëroob gekweekte cellen werd deze functie onderzocht zoals beschreven in hoofdstuk 3. Het is bekend dat de mitochondriën van anaëroob gekweekte cellen erg fragiel zijn.Daarom werd in dit onderzoek veel aandacht gegeven aan de isolatie van intacte mitochondria. De activiteit van het enzym citrate syntase (EC 4.1.3.7) werd voor dit doel als testparameter gebruikt, mede gezien de rol van dit enzym in de citroenzuurcyclus. Na differentiële centrifugering van sferoplast-lysaten werd bijna alle

activiteit van citraat synthase teruggevonden in de mitochondriële fracties. Het enzym bleek latent in de fracties aanwezig, hetgeen aangetoond werd door de mitochondriële fracties ultrasoon te trillen.

Aangezien citrate syntase ook een belangrijk enzym is in anabole reacties heeft deze lokalisatie als consequentie dat de benodigde metabolieten over de mitochondriële membranen moeten worden getransporteerd. Onder anaërobe omstandigheden wordt alle ATP geproduceerd in het cytoplasma tijdens de glycolyse. Indien pro-mitochondria (mitochondria van anaëroob gekweekte cellen) ook een rol hebben in de biosynthese van celmateriaal gedurende anaërobe groei, dan moet aan het organel ook energie worden geleverd, zowel voor het transportprocessen over de membraan alsook voor de energie-vragende reacties binnen de mitochondria. Het importeren van ATP vanuit het cytoplasma kan geschieden via een ADP/ATP translocator in ruil voor ADP. De mogelijke fysiologische rol van gist mitochondria onder strikt anaërobe omstandigheden is daarom ook onderzocht door gevoeligheid van de cellen voor bongkrekzuur te testen, een algemeen bekende remmer van de ADP/ATP translocator.

Het bleek dat de toevoeging van dit bongkrekzuur aan anaërobe cultures inderdaad een remmend effect had op de groei. Uit het onderzoek kon worden geconcludeerd dat mitochondriën van *Saccharomyces cerevisiae* een vitale rol vervullen in het anaërobe suiker metabolisme.

In hoofdstuk 4 zijn, voor levende cellen van *S. cerevisiae*, de effecten van de kweek omstandigheden op de mitochondriële morfologie beschreven. Voor deze *in vivo* studie werd vitale kleuring met de fluorescente stof dimethyl-aminostyryl-methylpyridinium iodine (DASPMI), fluorescentie microscopie en confocale-scanning laser microscopie gebruikt. Cellen van op ethanol gekweekte en dus ademende batch cultures bleken een groot aantal kleine mitochondria te bevatten. Cellen van op glucose gekweekte batch-cultures, waarin zowel ademhaling als gisting optreedt, bevatten daarentegen een klein aantal, grote, vertakte mitochondria. Deze verschillen hadden geen invloed op de fractie van het cel volume dat door de mitochondriën werd ingenomen. Soortgelijke verschillen in mitochondriële morfologie werden waargenomen in glucose-gelimiteerde continu cultures. In aërobe chemostaat cultures was het glucose metabolisme strikt respiratoir en de cellen bevatten een groot aantal klein mitochondria. Anaërobe, fermentatieve chemostaat kweken leverde de grote, vertakte mitochondriële structuren op die ook in de batch cultures op glucose werden waargenomen.

Na het aëreren van een daarvoor anaërobe chemostaat culture neemt de maximale ademhalingssnelheid van de cellen toe van 10 tot 70 $\mu mol \cdot min^{-1} \cdot g$ drooggewicht binnen 10 uur. Deze overgang leidde tot drastische veranderingen in het aantal mitochondriën, hun morfologie en, dientengevolge, de grootte van het mitochondriële oppervlak. Deze veranderingen gingen nog verscheidene uren door nadat de ademhalingscapaciteit zijn maximum had bereikt. Cyanide-ongevoelige zuurstofconsumptie maakte ca. 50 % uit van de totale ademhalingscapaciteit van anaërobe cultures, maar was nagenoeg afwezig in aërobe cultures. De reactie van aërobe cultures op het onthouden van zuurstof was in kwalitatieve zin het tegenovergestelde van de reactie van anaërobe cultures op aëratie. De resultaten geven aan dat mitochondriële morfologie in *S. cerevisiae* nauw samenhangt met de metabole activiteit van deze gist: omstandigheden die de repressie van de ademhalingsenzymen tengevolge hebben leidden in het algemeen tot de mitochondriële morfologie zoals die is waargenomen in anaëroob gekweekte, gistende cellen.

Omdat alle fermenterende gisten de suiker glucose kunnen gebruiken als koolstof- en

energiebron, is deze suiker in de voorafgaande hoofdstukken als zodanig gebruikt. De belangrijkste component in de media die in de industriële fermentaties worden gebruikt is in het algemeen echter niet glucose maar disachariden en oligosachariden zoals maltose, lactose, maltotriose, etc.. Veel gisten zijn niet in staat om deze suikers te vergisten, ondanks het feit dat ze onder aërobe condities snel groeien op deze substraten. Dit fenomeen staat bekend als het Kluyver effect. Zo kan *C. utilis* aëroob groeien op maltose, maar kan deze suiker niet vergisten onder zuurstof limiterende kweekomstandigheden. Het industriële belang van dit Kluyver effect gaf aanleiding om dit fenomeen nader te bestuderen. Dit is beschreven in hoofdstuk 5.

In zuurstof-gelimiteerde chemostaat cultures van *S. cerevisiae* CBS 8066 en *C. utilis* CBS 621 op glucose en maltose als substraat werd zowel de groei als de vorming van metabolieten bestudeerd bij een verdunningssnelheid van 0,1 uur^{-1}. *S. cerevisiae* kon zowel op glucose als op maltose worden gekweekt onder een dubbele limitatie van zuurstof en suiker. Ademhaling en alcoholische gisting vonden tegelijkertijd plaats en de fluxen van de metabolieten van deze processen waren afhankelijk van de grootte van de zuurstoftoevoer.

De vorming van de biomassa van gist leidt tot een overschot aan NADH, dat, onder omstandigheden waarbij de gist ademhaalt, weer wordt geoxideerd door zuurstof. Onder anaërobe omstandigheden kan dit overschot aan reductie-equivalenten worden geoxideerd door de reductie van dihydroxyaceton fosfaat tot glycerol. Door de gisten *S. cerevisiae* en *C. utilis* onder dubbele limitatie van glucose en zuurstof te kweken kon worden aangetoond dat beide gisten glycerol produceren zodra de q_{O2} wordt verlaagd tot onder de zelfde drempelwaarde van 0,55 mmol·g^{-1}·l^{-1}. Aangezien deze waarde gelijk is aan de netto produktiesnelheid van NADH, kon worden geconcludeerd dat het redox metabolisme van deze gisten onder zuurstof limitaties zodanig is gereguleerd, dat voor de vorming van glycerol een minimale hoeveelheid ATP wordt gebruikt. Desondanks, als zuurstof met lage snelheid werd toegevoegd (d.w.z. minder dan 4 mmol·l^{-1}·h^{-1}), werd de groei van *C. utilis* alleen door zuurstof gelimiteerd, hetgeen bleek uit de hoge residuele concentraties glucose in de cultures. In tegenstelling tot *S. cerevisiae*, kon *C. utilis* boven dien niet anaëroob worden gekweekt bij een verdunningssnelheid van 0.1 h^{-1}. De afwezigheid van zuurstof had dan ook tot resultaat dat de culture uitspoelde, ondanks de aanwezigheid van ergosterol en Tween-80 in het kweekmedium.

In zuurstofgelimiteerde cultures was het gedrag van *C. utilis* met betrekking tot het gebruik van maltose opmerkelijk: in zulke cultures vond geen alcoholische fermentatie plaats en de hoeveelheid gemetaboliseerde maltose was afhankelijk van de zuurstof toevoer. Op maltose groeiende, zuurstofgelimiteerde cultures van *C. utilis* bevatten altijd hoge concentraties residuele suikers. Klaarblijkelijk is maltose voor *C. utilis* een niet-fermenteerbare suiker, ondanks het feit dat het kan dienen als groeisubstraat voor deze facultatief-fermentatieve gist. Dit is niet het gevolg van de afwezigheid van de sleutelenzymen voor alcoholische fermentatie, aangezien zowel pyruvaatdecarboxylase als alcoholdehydrogenase in hoge activiteiten aanwezig waren in cellen van *C. utilis* die onder zuurstoflimitatie maltose gebruikten.

In dit hoofdstuk wordt geconcludeerd dat het Kluyver effect voor maltose in *C. utilis* het gevolg is van een regulatoir mechanisme dat voorkomt dat de suiker wordt gefermenteerd. Zuurstof is in dit verschijnsel geen bepalende factor, aangezien de alcoholische gisting niet onder zuurstoflimitatie wordt geïnduceerd.

Nawoord

Dit boekje dient, in het kader van de promotie, als "zodanig bewijs van bekwaamheid tot het zelfstandig beoefenen van de wetenschap" dat ik tot de verdediging van het proefschrift wordt toegelaten.
Zelfstandig is echter niet hetzelfde als alleen: velen er hebben de afgelopen jaren aan bijgedragen. Ik wil hierbij dan ook iedereen daarvoor bedanken.

Hans, bedankt voor je tomeloze inzet en enthousiasme voor de begeleiding van het onderzoek. Daarnaast bleek ook nog ruimte voor het samen doen van andere, ook leuke proefjes (o.a. met lichtgevende bacteriën) en voor de ontwikkelingen op het gebied van fermentoren en labruimtes. Overal stond je voor open, hetgeen ik als heel stimulerend heb ervaren. Verder gaven de reisjes naar de UK niet alleen op wetenschappelijk, maar ook op andere gebieden veel stof tot voor herinnering.

Gijs, in het begin waren onze (formele) contacten wat minder frequent (enkele werkbesprekingen per jaar). Desondanks wist je altijd snel tot de kern door te dringen en met kritische opmerkingen mede richting te geven aan het onderzoek. Het is verbazend hoeveel proefjes jij in 1 uur kunt bedenken (ongeveer voor 1 manjaar?). Grote waardering heb ik voor je begeleiding in de slotfase: de vele uurtjes 's avonds thuis met, afhankelijk van het tijdstip koffie, bier of wijn, waren fantastisch.

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Het Kluyverlab laboratorium zal vermoedelijk na korte tijd krakend tot stilstand komen zonder de niet aflatende ondersteuning door het vaste personeel. Max, Nico, Pascal, Astrid, bedankt voor het steriliseren van de fermentoren en de media en alle verdere ondersteuning. Jos Lispet, je goede humeur, moppen, zang (Anne...) en regelzucht waren onmisbaar!
Ook de werkplaats mag in dit rijtje zeker niet ontbreken: nooit klopte ik tevergeefs aan voor ontwikkeling of reparatie, hetzij mechanisch, hetzij electr(on)isch, en altijd werd

meegedacht over het uiteindelijke doel.

Bij het eigenlijke werk hebben ook Marc Rijneveen, Adriaan van der Baan, Kjeld Bangma en Yvonne van Klei hun steentjes bijgedragen, waarvoor dank!

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Astrid, afgezien van al het bovenstaande maakte jij alle inspanningen al meer dan waard. Dat ik jou heb leren kennen op het Kluyverlab is wellicht mijn mooiste resultaat.

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