Oxygen Requirements of Yeasts

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Type species of 75 yeast genera were examined for their ability to grow anaerobically in complex and mineral media. To define anaerobic conditions, we added a redox indicator, resazurin, to the media to determine low redox potentials. All strains tested were capable of fermenting glucose to ethanol in oxygen-limited shake-flask cultures, even those of species generally regarded as nonfermentative. 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 (μmax, 0.03 and 0.05 h−1, respectively) under anaerobic conditions in mineral medium 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.

Yeasts can be divided into three groups with respect to their fermentative abilities, namely, obligately fermentative, facultatively fermentative, and nonfermentative yeasts. Approximately 40% of the yeast species are listed as nonfermentative (4). 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 nonfermentative yeast species, however, have been shown to produce ethanol to some extent under the conditions of the Durham tube test, and their fermentative capacities often are greatly enhanced under more appropriate conditions (27).

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 (23, 31; M. T. J. Custers, Ph.D. thesis, Delft University of Technology, Delft, The Netherlands, 1940), the Kluysker effect (16, 24), and the Crabtree effect (8, 10, 29) 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 (26). Even when fermentation does occur under anaerobic conditions, as reported for Pachysolen tannophilus, the actual growth of the organism on D-xylose as well as on D-glucose is still dependent on the availability of oxygen (19). The addition of ergosterol and unsaturated fatty acids, which are considered to be essential medium components for anaerobic growth of Saccharomyces cerevisiae (1, 2), does not eliminate this oxygen requirement. Whether yeast species other then S. cerevisiae are able to grow anaerobically is in general unknown or the results are contradictory. For example, anaerobic growth of Candida utilis has been reported by several authors (3, 13), whereas others have stated that C. utilis is not able to grow anaerobically (27).

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 (15).

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

MATERIALS AND METHODS


Media. Complex medium contained, per liter of deminer- alized water, 10 g of yeast extract (Oxoid) and 20 g of glucose. The initial pH of the medium was set at 5. Mineral medium, supplemented with vitamins and trace elements, was prepared as described by Bruinenberg et al. (7), except that the concentration of NaMO₃ · 2H₂O was increased 10-fold. Glucose was added as the sole source of carbon and energy at a concentration of 20 g · liter⁻¹. Ergosterol and Tween 80, dissolved in pure ethanol, were sterilized by heating the solution for 10 min in a nonpressurized auto- clave. These components were added to the medium at concentrations of 6 and 600 mg · liter⁻¹, respectively. Resa- zurin was added to both media at a concentration of 0.002% to indicate low redox potentials (E°F⁻⁻ = -42 mV).

Inocula. Inocula for anaerobic growth tests were prepared by growing the yeasts in 100-mL cotton wool-plugged Erlen- meyer flasks containing 20 ml of 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 because of oxygen limita- tion (28). Therefore, cells from these shake-flask cultures could be considered to be adapted to serve as inocula for anaerobic growth tests.

Anaerobic growth tests. Anaerobic growth tests were conducted in 30-ml serum flasks under static incubation at 25°C. To prevent the entrance of oxygen, we firmly closed the flasks with 4-mm-thick butyl rubber septa. The flasks were almost completely filled with medium and autoclaved at 110°C. During autoclaving, reducing agents in the complex medium converted the redox indicator to colorless dihy- drosororufin. The mineral medium, treated similarly, did not become colorless. It was therefore deoxygenated prior to being autoclaved by including 8 mg of Aspergillus niger glucose oxidase (grade III; Boehringer, Mannheim, Federal Republic of Germany) liter⁻¹; this enzyme preparation con- tains, as an impurity, a catalase activity that is adequate for removal of H₂O₂ formed in the reaction. The redox indicator in the serum flask 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 recolorization within a few hours because of a high rate of oxygen diffusion. After sterilization, the flasks were not opened to prevent the entrance of oxygen. A small amount (5 to 10 µl) of the inoculum was injected into each flask with a 2-ml syringe.

The syringe was left in place during the incubation and thus served as an indicator of CO₂ production. The gas production and turbidity of the inoculated flasks were checked twice daily for 1 month; prolonged incubation after this period did not lead to the onset of anaerobic growth. 

Batch cultivation in fermentors. Comparative studies were performed by use of a laboratory fermentor with a 1-liter working volume and of the type described by Harder et al. (12). The mineral medium described above was used. To prevent foaming, we added 50 µl of silicone antifoaming agent per liter. pH was kept 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. For exclusion of a possible infection, the identity of the organism was checked afterwards by the CBS.

Levels of dissolved-oxygen tension (DOT) were measured with a polarographic oxygen electrode (Ingold type 322 756702/74247) connected to an Ingold type 170 O₂ amplifier. The signal of this amplifier (percent air) was monitored with a Kipp BD 41 datum recorder (Kipp & Zonen, Delft, The Netherlands).

The fermentor was continuously flushed with pure nitrogen gas containing less than 5 ppm of oxygen (Air Products, Waddinxveen, The Netherlands) at a flow rate of 1 liter · min⁻¹. The tubing of the fermentor was made of Norprene (Cole-Parmer Instruments Corp., Chicago, Ill.).

Analytical methods. Ethanol concentrations were determined by gas-liquid chromatography on a Varian type 3400 gas chromatograph (Varian Benelux B. V., Amsterdam, The Netherlands) with a Hayesp Q column (Chrompack, Middelburg, The Netherlands) at a temperature range of 150 to 225°C, increasing by 15°C · min⁻¹. Glucose concentrations were determined by the GOD-PAP method of Boehringer.

Dry weight was determined by filtration of the culture sample with a weighed polysulfone filter (Supor 450, pore size, 0.45 µm; Gelman Sciences Inc., Ann Arbor, Mich.). The filter was washed with demineralized water, dried in an R-7400 magnetron oven (Sharp Inc., Osaka, Japan) for 20 min at medium power, and reweighed.

Carbon analyses were done with a model 915B Tocamam- ter total organic carbon analyzer (Beckman Industrial Corp., La Habra, Calif.). Organic acids were determined by high- pressure liquid chromatography with an HPX-87H column (300 by 7.8 mm; Bio-Rad, Richmond, Calif.) at room tempera- ture. The column was eluted with 0.01 N H₂SO₄ at a flow rate of 0.6 ml · min⁻¹. The detector was a Waters 441 UV meter (used at 210 nm) coupled to a Waters 741 datum module (Waters, Milford, Mass.).

Electron microscopy. Twenty-two milliliters of cell culture was prefixed with 3 ml of 25% (vol/vol) glutaraldehyde for 10 to 30 min at room temperature. After centrifugation, the cells were fixed again with 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 1 h, washed three times with the same buffer, and postfixed with 2% (wt/vol) aqueous KMnO₄ for 2 to 24 h. After fixation, the cells were stained with 1.5% (wt/vol) aqueous uranyl acetate during dehydration at the 50% (vol/vol) ethanol step and embedded in Spurr resin. Ultrathin sections, poststained 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). Silicone antifoaming agent was obtained from BDH (Poole, England).
FIG. 1. Distribution of ethanol production in shake-flask cultures of fermentative (■) and nonfermentative (□) strains. The concentrations of ethanol in the cultures were determined at the end of the exponential growth phase. ■, Number of strains growing anaerobically in serum flask tests.

RESULTS

Ethanol production in oxygen-limited shake-flask cultures. When grown in shake-flask cultures, all strains produced ethanol, even those presently considered nonfermentative on the basis of taxonomic tests (4). However, in such strains, ethanol production was generally rather low (0 to 20 mM; Fig. 1). In some cases (for example, F. neoformans), the amount of ethanol in shake-flask cultures was as high as with typical facultatively fermentative yeasts such as E. nana and D. hansenii.

Screening for anaerobic growth. When transferred to anaerobic conditions in serum flasks, only a few species exhibited clear anaerobic growth, with concomitant ethanol production, both in complex medium (yeast extract containing) and in mineral medium (supplemented with Tween 80 and ergosterol). These were A. telluris, C. tropicalis, C. utilis, C. matriensis, 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, and W. saturnus. In many cases, long lag times (5 to 10 days) elapsed before growth started, despite the fact that the yeasts had been pregrown under oxygen-limited conditions. The number of generations in the flasks with positive growth was 8 to 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 fermentors. 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 dissolved-oxygen concentration in microbial cultures has been described by Wimpenny (32) and Wimpenny and Necklen (33). The redox potential is taken as an appropriate parameter for the definition of anaerobic circumstances, especially for the cultivation of strictly anaerobic bacteria (14). 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 DOT values down to 0.001% air saturation. We found that one must be very careful in the conversion of redox potentials into oxygen levels. For example, it was not possible to obtain a redox potential low enough to decolorize the resorufin simply by purging the medium with nitrogen gas, although a DOT of 0.005% air saturation was reached. On the other hand, in a culture of C. utilis such a redox potential was obtained at a DOT of 0.01 to 0.02% 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, the redox potential as well only indicate the actual activity of the oxygen in the medium. They do not provide information about the actual oxygen flux. Evidence in this respect is our
observation that resorufin was colorless even in a cotton-stoppered shake-flask culture of *C. utilis*, conditions still regularly considered aerobic (17). The decolorization was due to the rapid oxygen consumption of *C. utilis* and therefore did not indicate strictly anaerobic growth. To define anaerobic conditions, we therefore found it necessary to determine the actual flux of oxygen into the system. To investigate the magnitude of the oxygen influx, we flushed the fermentor with pure nitrogen until a DOT of 0.005% air saturation was reached. Subsequently, the outlet gasflow was blocked and an extra pressure of 0.1 atm (ca. 10 kPa) was built up by use of a 1-m-high water column. The diffusion of oxygen into the fermentor is shown in Fig. 2. It could be calculated from this figure that, even when all tubing was closed as closely to the fermentor as possible, the diffusion of oxygen was still approximately $2 \times 10^{-3}$ μmol·h$^{-1}$. We confirmed that gas leakage did not occur. These data clearly show that maintaining strictly anaerobic conditions is not possible in this way because of the diffusion of oxygen.

To keep the concentration of oxygen at a very low level, we found it necessary to flush the fermentor 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 continuously decreased during the next 30 h. The actual DOT finally reached depended on the flow rate of the nitrogen gas (Table 1). Whenever the airflow was reestablished, the DOT returned to the initial value of 100.0 ± 0.1% air saturation, indicating the low drift of the electrode.

**Anaerobic growth in fermentors.** To obtain some quantitative information on growth rates, we attempted to grow various yeasts anaerobically in fermentors. Prior to inoculation, the fermentors were flushed vigorously with nitrogen gas until the DOT did not decrease anymore. This procedure usually took 30 to 35 h. After inoculation, the nitrogen flow was kept at 1 liter·min$^{-1}$ to prevent the 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* was the only species capable of rapid anaerobic growth, with a $\mu_{\text{max}}$ of 0.4 h$^{-1}$ (Table 2). The other species tested (*C. utilis*, *T. delbrueckii*, and *C. tropicalis*) grew poorly under these conditions ($\mu_{\text{max}} < 0.05$ h$^{-1}$). In all cases, according to the indicator, the redox potential in the culture decreased as soon as growth started. It could be calculated that the total amount of oxygen that had entered the culture vessel during the growth period was $<10$ μmol·h$^{-1}$.

**Ultrastructure of anaerobically grown cells.** The ultrastructure of the four yeasts shown in Fig. 3 was investigated with regard to the presence of mitochondria (Fig. 4). Special attention was paid to the staining procedures, because inadequate staining often precludes the visualization of mitochondrial structures (9). Indeed, fixation with potassium permanganate alone did not reveal mitochondrial structures in *S. cerevisiae* (results not shown), whereas these structures were clearly visible when glutaraldehyde-potassium permanganate fixation was used (Fig. 4a). The other species also contained mitochondrial structures, although the fine

![Graph](image_url)

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

**TABLE 1.** Influence of the nitrogen flow rate on the DOT in a 2-liter fermentor

<table>
<thead>
<tr>
<th>N$_{2}$ flow rate (liters·min$^{-1}$)</th>
<th>DOT (% air saturation)</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>0.075</td>
</tr>
<tr>
<td>1.0</td>
<td>0.023</td>
</tr>
<tr>
<td>1.5</td>
<td>0.020</td>
</tr>
<tr>
<td>2.0</td>
<td>0.015</td>
</tr>
<tr>
<td>3.0</td>
<td>0.012</td>
</tr>
<tr>
<td>5.0</td>
<td>0.010</td>
</tr>
<tr>
<td>10.0</td>
<td>0.007</td>
</tr>
<tr>
<td>20.0</td>
<td>0.005</td>
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</tbody>
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FIG. 3. Growth curves (---) and D.O.T. (...) of anaerobically grown yeasts in controlled batch fermentations. (A) S. cerevisiae; (B) C. utilis; (C) T. delbrueckii.

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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.

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

<table>
<thead>
<tr>
<th>Species</th>
<th>$\mu_{\text{max}}$ (h$^{-1}$)</th>
<th>Yield (g/g of glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>0.40</td>
<td>0.10</td>
</tr>
<tr>
<td>C. utilis</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>T. delbrueckii</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

structure was definitely less developed than 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 this yeast were relatively large membranous structures, either in the form of a laminar membrane system branching out at the ends or in the form of a ring structure of concentric membranes. The membranous structures seemed to be interconnected in the cells (Fig. 4f). Single membranes were also found (Fig. 4d), and these were also connected to the larger
structures. The membranous structures were not found in cells grown aerobically with glucose (results not shown).

DISCUSSION

For the screening of the property of anaerobic growth of yeasts, it was necessary to select representative strains. We decided to choose type species of genera rather than randomly selected strains.

Although all strains tested were able to produce some ethanol, only 18 grew anaerobically to some extent in mineral medium supplemented with ergosterol and Tween 80 or in complex medium. All of these belonged to the group of facultatively fermentative yeasts as listed by Barnett et al. (4).

It is important to note that a good fermentative capacity is a prerequisite for anaerobic growth, since none of the so-called nonfermentative yeasts grew under strictly anaerobic conditions. However, a good fermentative capacity alone is not sufficient to fulfill all the requirements of anaerobically grown cells, since many rapidly fermenting species lacked the ability to grow anaerobically. The latter property is relatively rare among yeasts. Even when growth occurred, it was rather slow. S. cerevisiae, however, seemed to be 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 (6). It seems unlikely, however, that the absence of anaerobic growth on glucose is generally due to redox problems. In the case of P. tannophilus, it has been shown that hydrogen acceptors such as diacetyl or acetoin cannot replace oxygen (19). P. tannophilus is unable to grow unless oxygen is available. Although yeasts such as C. utilis can slowly grow anaerobically (Fig. 3), the rates of growth and alcoholic fermentation are greatly enhanced in oxygen-limited shake-flask cultures. Under these conditions, growth and alcoholic fermentation are as rapid as with S. cerevisiae (28). 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 was stated by several authors that S. cerevisiae showed a complete absence of mitochondria when grown under anaerobic conditions (18, 30). This observation, however, is now known to be the result of inadequate electron microscopy techniques (9, 22). Therefore, the theory of de novo synthesis of mitochondria in cells grown anaerobically and subsequently transferred to high levels of oxygen (18, 30) had to be rejected. However, the mitochondria in anaerobically grown cells do not have the same ultrastructure as do those in aerobically grown cells; however, upon aeration they start to become fully organized (21). 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 (18). These authors suggested that such structures should be regarded as precursors of mitochondria. This idea seems unlikely, however, in view of the fact that these structures were not observed in the other yeasts (Fig. 4).

Whether promitochondria fulfill a physiological function remains to be elucidated. It is known that some of the assimilatory processes required for cell synthesis take place within the mitochondria (20); hence, the transport of certain intermediates over the mitochondrial membrane remains a necessity under anaerobic conditions. These transport processes must be energized in the absence of electron transfer. The results of Šubik et al. (25) and Gbelská et al. (11) strongly suggest that under anaerobic conditions, transport processes and other energy-requiring reactions in mitochondria are energized by the import of cytoplasmic ATP via the 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 mitochondrial inner mem-
brane. This inhibition could not be relieved by the addition of a variety of growth factors.

So far, it is unclear why in a variety of yeasts the role of mitochondria in anaerobic reactions is apparently more important than it is 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 here can be considered a useful system for a qualitative estimation of the anaerobic behavior of yeasts. However, for quantitative aspects, fermentor 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. We therefore cannot exclude the possibility that in addition to *S. cerevisiae*, other yeasts may possess the capacity for fast anaerobic growth. However, on the basis of the limited amount of data presented here it seems likely that this property is not widespread among yeasts.

**ACKNOWLEDGMENTS**

We are indebted to the Centraalbureau 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 Foundation for Biological Research, which is subsidized by The Netherlands Organization for Scientific Research.

**LITERATURE CITED**