Physiological effects of nitrogen starvation in an anaerobic batch culture of *Saccharomyces cerevisiae*

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The effects of nitrogen starvation on the anaerobic physiology of *Saccharomyces cerevisiae* were studied in cells cultivated in a bioreactor. The composition of the mineral medium was designed such that the nitrogen source became depleted while there was still ample glucose left in the medium. The culture was characterized by acoustic gas analysis, flow injection analysis and HPLC analysis of extracellular substrates and metabolites. During the cultivation, the macromolecular composition of the cells was analysed with respect to the cellular content of RNA, protein, trehalose and glycogen. During exponential growth under anaerobic conditions, the maximum specific growth rate ($\mu_{\text{max}}$) of *S. cerevisiae* CBS 8066 (0.46 h$^{-1}$) was identical to the $\mu_{\text{max}}$ determined under aerobic conditions. Depletion of ammonium in the medium led to an abrupt decrease in the flux through glycolysis. Subsequently, a continuous decrease in the carbon dioxide evolution rate, caused by catabolite inactivation of the hexose-transport system, was observed. The apparent half-life of the transport system under nitrogen starvation was 13 h. During the exponential growth phase, the cellular content of RNA and protein was 15% (w/w) and 60% (w/w), respectively. At the end of the cultivation where the cells had been starved of nitrogen for 18 h, the cellular content of RNA and protein had decreased to 4% (w/w) and 22% (w/w), respectively. The intracellular carbohydrate content increased dramatically as trehalose and glycogen accumulated to final concentrations of 7% (w/w) and 25% (w/w), respectively. Glycerol formation during nitrogen starvation was higher than that accounted for by the formation of organic acids, suggesting a protein turnover of approximately 6% h$^{-1}$. The growth energetics of *S. cerevisiae* CBS 8066 also changed as a result of nitrogen starvation, and $\gamma_{\text{ATP}}$ was observed to increase from 80 mmol g$^{-1}$ during the exponential growth phase to more than 130 mmol g$^{-1}$ towards the end of the cultivation. The presented results illustrate the effect of nitrogen starvation on glycerol formation, protein turnover, catabolite inactivation of the sugar-transport system, the cellular composition, the cell cycle and growth energetics.

**Keywords:** *Saccharomyces cerevisiae*, anaerobic batch culture, nitrogen starvation, intracellular metabolites, growth energetics

INTRODUCTION

Although *Saccharomyces cerevisiae* is one of the few yeasts capable of good anaerobic growth (Visser *et al.*, 1990), the anaerobic physiology of this micro-organism has not gained as much attention as the aerobic, even though it is of great industrial importance, e.g. in the production of ethanol and beverages like beer and wine. The focus of previous studies of the anaerobic physiology of *S. cerevisiae* has been on the basic physiology (Schatzmann, 1975; Verduyn *et al.*, 1990a; Visser *et al.*, 1994, 1995) and...
growth energetics (Verduyn et al., 1990b) under conditions of glucose limitation. Limitation by other nutrients, e.g. nitrogen, has not been studied, despite the fact that these kinds of limitations or starvations occur in industrial processes and are known to induce profound changes in the metabolism of cells. Nitrogen limitation leads to changes in the grown energetics of both bacteria (Benthin et al., 1994) and yeast (Larsson et al., 1993). The macromolecular composition of cells will change as a result of nitrogen starvation (Johnston et al., 1977b; Lilie & Pringle, 1980; Lagunas & Ruiz, 1988) and under such conditions aerobically cultivated S. cerevisiae accumulates intracellular carbohydrates in the form of glycogen and trehalose (Lilie & Pringle, 1980; Quain et al., 1981). Nitrogen starvation also leads to catabolite inactivation of the glucose-transport system in S. cerevisiae (Lagunas et al., 1982; Busturia & Lagunas, 1986), an aspect of the anaerobic yeast physiology which can be a great nuisance to oenology (Salmon, 1989). Nitrogen starvation will eventually cause yeast cells to arrest in the G1 portion of the cell cycle (Johnston et al., 1977a; Johnston, 1977), but those cells which have just initiated the DNA division cycle when nitrogen starvation sets in will continue and complete their division cycle with little dependence on continued net cell growth (Johnston et al., 1977b).

The anaerobic conversion of glucose into ethanol by S. cerevisiae is redox neutral, i.e. the NADH consumed initially in the Embden–Meyerhof–Parnas (EMP) pathway is regenerated when ethanol is produced (see Fig. 1). However, when intermediates in the EMP pathway are withdrawn as precursors for synthesis of cellular material, this balance is disturbed since the surplus of NADH produced initially in the EMP pathway is not converted back into NAD+. Thus other processes must exist for the regeneration of NADH. During anaerobic growth, NADH cannot be oxidized by oxygen but must be disposed of by formation of reduced byproducts such as glycerol (Holzer et al., 1963; Oura, 1977). Anabolism is responsible for the major part of the glycerol production (Lagunas & Gancedo, 1973; van Dijken & Scheffers, 1986; Schulze, 1995), but secretion of organic acids like acetic acid, pyruvic acid and especially succinic acid will also require a compensatory formation of glycerol (Genoviois, 1950, 1961; Oura, 1977). Since nitrogen limitation or starvation affects anabolism, it will also influence the formation of glycerol. Turnover of the cellular protein complement has been observed under conditions of nitrogen starvation (López & Gancedo, 1979) and this too can be expected to affect glycerol formation.

The objective of the present work was to study the effects of nitrogen starvation on the anaerobic physiology of S. cerevisiae. This has been achieved by monitoring the growth and product formation under well-defined conditions in a batch cultivation. A detailed picture has emerged of the cellular composition and the product and byproduct formation. The results illustrate the effects of nitrogen starvation on glycerol formation, protein turnover, catabolite inactivation of the sugar-transport system, the cellular composition, the cell cycle and growth energetics.

**METHODS**

Melting-organism and its maintenance. _Saccharomyces cerevisiae_ CBS 8066 was obtained from the Centraal Bureau voor Schimmelcultures (Delft, the Netherlands). The strain was maintained at 4°C on yeast extract/peptone/glucose agar plates, monthly prepared from a lyophilized stock kept at
—80 °C. The bioreactor was inoculated to an initial biomass concentration of 1 mg l⁻¹ with a preculture grown in un baffled shake flasks at 30°C and 100 r.p.m. for 24 h.

**Media.** The yeast was cultivated in a mineral medium prepared according to Verdun et al. (1990a) except for the following changes: the concentration of (NH₄)₂SO₄ was 0.64 g l⁻¹, the concentrations of all vitamins were doubled and glucose was added as sole carbon source to a concentration of 50 g l⁻¹. Growth of S. cerevisiae under anaerobic conditions requires the supplementary addition to the medium of ergosterol and unsaturated fatty acids, typically in the form of Tween 80 (Andreasen & Stier, 1953, 1954). Ergosterol and Tween 80 were dissolved in 96% (v/v) ethanol and the solution was autoclaved at 121°C for 5 min. The final concentrations of ergosterol and Tween 80 in the medium were 15 mg l⁻¹ and 660 mg l⁻¹, respectively. To prevent foaming, 75 μl antifoam l⁻¹ (Sigma; A-5551) was added to the medium.

**Cultivation conditions.** The anaerobic batch fermentations were performed at 30°C and at a stirring speed of 500 r.p.m. in a bioreactor manufactured in-house with a working volume of 5 l. pH was kept constant at 5.00 by addition of 2 M KOH. The anaerobic batch fermentations and gave results identical to those presented in this paper considering the RSD of the individual analyses (see above).

**RESULTS**

**Establishment and maintenance of anaerobic conditions**

When studying the anaerobic physiology of *S. cerevisiae*, it is crucial that oxygen is efficiently excluded from the growth environment. In the presence of oxygen, *S. cerevisiae* can generate much more ATP by respiring glucose to carbon dioxide than that generated by substrate-level phosphorylation. In addition, since oxygen can be used to reoxidize NAD⁺, the presence of oxygen will also reduce glycerol formation. The establishment of strictly anaerobic conditions will require the addition to the medium of oxygen-trapping agents like sulphite, glucose oxidase/catalase or cysteine. Unfortunately, these agents will adversely affect the physiology of *S. cerevisiae* or hamper a quantitative treatment of the data. Hence, anaerobic conditions in bioreactor systems are normally established by sparging the bioreactor with pure nitrogen, typically in the form of commercially available nitrogen gas containing less than 5 p.p.m. oxygen (Schatzmann, 1975; Verdun et al., 1990a; Visser et al., 1990, 1994). Gas of similar quality can also be produced at the site from standard nitrogen, typically containing less than 100 p.p.m. oxygen, by passing the nitrogen through a reactor filled with small copper turnings heated to 350 °C. Oxygen will be trapped in the reactor as CuO and the reactor can readily be regenerated by flushing with hydrogen. We compared the physiological response of *S. cerevisiae* grown in a bioreactor sparged with commercially available nitrogen containing less than 5 p.p.m. oxygen to the response obtained when using the nitrogen produced in our laboratory. We were unable to detect any differences with respect to product concentrations (data not shown).

Samples were immediately frozen in liquid nitrogen and subsequently stored at −40 °C. The protein content of whole cells was determined by a modified Biuret method according to Verdun et al. (1990a) (RSD = 2.5%, n = 3). RNA measurements were determined as described by Benthin et al. (1991b) (RSD = 20%, n = 3). Trehalose and glycerol were determined enzymically as previously described (Schulze et al., 1995) (RSD = 2.5%, n = 3).

**Enzyme assay.** *In vitro* enzyme activities were determined in freshly prepared cell-free extracts (Postma et al., 1989) at 30°C, and it was checked that the reaction rate was proportional to the amount of cell-free extract added. Catalase was assayed as described by Verdun et al. (1984). One unit is defined as the amount of enzyme catalysing the conversion of 1 μmol substrate min⁻¹. The specific activity is expressed as units per mg protein in the cell-free extract as determined by the Lowry method.

**Reproducibility.** All experiments were repeated at least once and gave results identical to those presented in this paper considering the RSD of the individual analyses (see above).

**Analysis of metabolites.** Culture liquid was withdrawn from the bioreactor into an ice-cooled beaker. For each analysis, an adequate amount of sample was centrifuged, washed twice with 0.9% (w/w) NaCl and resuspended in buffer.
to more than 1.5 g l⁻¹ in the presence of these supplements. This further indicated that the conditions were truly anaerobic. Finally, the cells were analysed for the presence of catalase activity, an enzyme known to be active even at low oxygen concentrations (Lee, 1986). No activity (< 1 mU mg⁻¹) of this enzyme was detected under the described conditions. Thus, it was concluded that the described approach was successful for establishing and maintaining anaerobic conditions.

Formation of biomass and extracellular metabolites

In previously published studies, the physiological effects of nitrogen starvation were studied by transferring cells from a nitrogen-containing to a nitrogen-free medium. This was done by collecting exponentially growing cells from a shake-flask culture on a filter, followed by washing and resuspension of the cells in a nitrogen-free medium (Johnston et al., 1977b; Lagunas & Ruiz, 1988). Such a procedure will not be suitable for studying the effects of nitrogen starvation under anaerobic conditions since the cells inevitably will be exposed to air. However, by designing the medium such that the nitrogen source becomes depleted while there is still ample glucose left in the medium, the experiment can be performed in a bioreactor without transfer of cells. Additionally, these conditions will better mimic those which can be encountered in industrial processes and the large volume of the bioreactor will allow for an intensive monitoring of both intra- and extracellular metabolites. The results of such a batch cultivation are shown in Fig. 2. Fig. 2(a) shows the concentration profile of biomass and ammonia and the CER during the cultivation, whereas the concentration profiles of the other extracellular metabolites are shown in Fig. 2(b, c). Based on these data, the cultivation could be divided into four phases.

Phase I (0–5 h) – lag phase. No detectable changes could be determined in any of the measured metabolites. The bioreactor was inoculated with S. cerevisiae CBS 8066 to an initial biomass concentration of 1 mg l⁻¹ at t = 0 h. By extrapolating the exponential growth in phase II back to the initial biomass concentration, the length of the lag phase can be estimated at 5 h. This relatively long lag phase is most likely due to the preculture, which was grown in an un baffled shake flask which corresponds to aerobic growth, although severely oxygen limited. It is well known that the transfer from aerobic to anaerobic conditions will require adaptation of the cell machinery (Luzikov et al., 1970).

Phase II (5–20 h) – exponential growth phase. From Fig. 2(a) it is seen that the culture grew exponentially until t = 20 h. The maximum specific growth rate (μmax) determined by on-line flow injection analysis of biomass (Benthin et al., 1991a) was found to be 0.46 h⁻¹ (RSD = 1.5%, n = 5), which was identical to the value also found for aerobic conditions (RSD = 1.5%, n = 4). Thus, the presence or absence of oxygen does not affect the μmax of S. cerevisiae CBS 8066. At t = 20 h, ammonia became depleted and the CER dropped sharply by more than 30%. The effect of the total consumption of the extra-

![Fig. 2](https://via.placeholder.com/150)

Fig. 2. (a) CER (solid line) and the concentrations of biomass (■) and ammonia (□) in an anaerobic batch culture. The medium initially contained 165 mg ammonia l⁻¹ and 50 g glucose l⁻¹. No detectable changes could be determined in any of the measured metabolites. Nitrogen starvation sets in at 20 h. (b) Concentrations of glucose (○), ethanol (●) and glycerol (▲); (c) concentrations of acetic acid (△), succinic acid (▲) and pyruvic acid (▼).

...cellular ammonia was also reflected in the other concentration profiles; the exponential growth of the biomass ceased and the rates of ethanol and glycerol production were reduced as judged from Fig. 2(b). From analysis of non-nitrogen-limited cultures, it is found that both succinic acid and acetic acid are produced during the exponential growth phase, but in this experiment the concentrations were below the detection limit of the analysis.

Phase III (20–25 h) – transition phase. A highly transient course of the CER and the acetate concentration were observed. Initially the CER decreased, but at approximately 22 h an increase in CER was observed. The acetate concentration decreased by more than 30% during the first 3 h of this phase, followed by an incipient increase. The local minimum of the acetate concentration did not seem to coincide with the local minimum in the CER. The biomass concentration and the concentration of the other
products increased but at reduced rates compared to phase II. The glucose consumption rate was also reduced. Microscopy of the cells at the end of this phase showed that more than 80% existed as single cells.

**Phase IV (25-38 h) – nitrogen-starvation phase.** The linear increase in CER observed in the last part of phase III turned to a continuous decrease at the beginning of this phase. At approximately 38 h, the CER decreased sharply to zero as a consequence of glucose depletion. The rate of glucose consumption seemed to decrease continuously during this phase and this was also reflected in a decreasing production rate of extracellular metabolites and biomass. At the end of the cultivation, virtually all (>98%) cells existed as single cells as judged by microscopy.

Table 1 summarizes the overall yields from phase I plus II and phase III plus IV. It is seen that the biomass and the measured metabolites can account for approximately 98% of the consumed glucose. Thus, it can be concluded that no major product(s) are formed which are not accounted for by the measurements. It is seen that approximately equal amounts of glucose are used for production of ethanol (\(y_{Sc} \approx 0.39 \, \text{g g}^{-1}\)) and carbon dioxide (\(y_{Sc} \approx 0.40 \, \text{g g}^{-1}\)). This is reasonable, since production of ethanol from pyruvate involves a decarboxylation (Fig. 1).

It is observed that the experimentally determined \(y_{Sc}\) is approximately 20% lower than the maximum theoretical yield, \(y_{Sc} = 0.51 \, \text{g g}^{-1}\), which of course is due to the formation of biomass and other products. Both yields increase slightly in phase III plus IV. In phase I plus II, the yields of glycerol (\(y_{gly}\)) and biomass (\(y_{bx}\)) are almost identical but both \(y_{bx} \, y_{gly}\) decrease in the phase with nitrogen starvation. In phase III plus IV, the nitrogen source is depleted and no more net synthesis of nitrogen-containing compounds can take place. \(y_{gly}\) is therefore expected to be lower than during exponential growth, since glycerol is formed to compensate for the NADH formed in biosynthesis. However, \(y_{gly}\) is not reduced to the same extent as \(y_{bx}\).

**Relationship between biomass and glycerol formation**

By plotting the amount of glycerol which is due to secretion of organic acids versus the total glycerol concentration in the medium (Fig. 3), it is possible to obtain a more detailed picture of the relationship between glycerol formation and biomass synthesis. In the calculations, it is assumed that secretion of pyruvic acid, acetic acid and succinic acid leads to production of 1, 2 and 5 mol NADH per mol acid secreted, respectively (these figures are based on the assumption that both isocitrate dehydrogenase and acetaldehyde dehydrogenase use NADH as cofactor). Each mol NADH formed must be disposed of by formation of 1 mol glycerol. Fig. 3 illustrates that in phase II, 18% of the formed glycerol is due to secretion of organic acids, whereas secretion of organic acids accounts for 63% in phase IV. Phase III is an intermediate phase in the change from biomass synthesis to acid secretion as the major source of glycerol formation. In other words, the presence of an extracellular nitrogen source, biomass formation accounts for more than 80% of the formed glycerol, but under nitrogen starvation less than one-third of the formed glycerol can be ascribed to processes other than acid secretion. Since the total cellular protein and RNA content remained constant during the nitrogen-starvation phase (see section on cellular composition), net synthesis of these compounds cannot explain the 37% of the glycerol formation which is not due to acid secretion.

The overall redox balance closed in each phase of the batch cultivation (Schulze, 1995), and this indicates that there is a transfer of electrons from NADH to NADPH.
Since there is no transhydrogenase in S. cerevisiae (Lagunas & Gancedo, 1973; van Dijken & Scheffers, 1986), this electron transfer must be a result of protein turnover, i.e. the degradation and resynthesis of protein. However, this would require that different cofactors (NADH/NADPH) are used in degradation and resynthesis, since a net formation of NADH must take place. From the biochemistry, it is known that de novo synthesis of 1 g protein leads to the formation of 21.9 mmol NADH and the consumption of 15.2 mmol NADPH (Bruinenberg et al., 1983; Verduyn et al., 1990a; Schulze, 1995). If NADPH is used in the degradation pathways where NADH was formed in the synthesis, protein turnover will result in a net synthesis of NADH. Unfortunately, the cofactor specificity of the reactions involved in the degradation of protein is rather poorly described in the literature (Jones & Fink, 1982; Cooper, 1982). However, in mammalian cells, degradation of alanine to pyruvate gives rise to formation of NADH, whereas the synthesis of alanine uses NADPH as electron donor (Stryer, 1988). During phase IV, glycerol formation of 0.804 mmol l^{-1} h^{-1} is observed, which can account for de novo synthesis of 36.9 mg protein l^{-1} h^{-1}, corresponding to a 6.6 h^{-1} turnover of the protein in the cells. This calculation is based on the assumption that no NADH is used in the degradation of protein. The amino acid composition of the protein did not change significantly during the period of starvation as compared to the exponential growth phase (data not shown).

**Carbon dioxide evolution and the uptake of glucose**

The abrupt decrease in the CER observed simultaneously with depletion of ammonia in the medium may indicate that ammonia exerts some type of regulation on the flux through glycolysis. This abrupt decrease in the CER was also observed by Lagunas & Ruiz (1988) in a similar experiment and was ascribed to the function of ammonia as an allosteric activator of both phosphofructokinase 1 and 2 (Ramaiah, 1974) and pyruvate kinase (Rhodes et al., 1986). However, the sudden decrease in CER may also reflect the fact that the cellular requirement for ATP of non-growing cells is lower than for growing cells.

The steady decrease in CER observed during phase IV seems to be correlated with a decrease in the glucose-uptake rate, as judged from Fig. 2(b). By plotting the rate of glucose uptake, based on the total cellular content of nitrogen, as a function of time, it is seen that the rate of glucose uptake decreases exponentially (Fig. 4). The rate constant for the decay in the uptake of glucose is 0.05 h^{-1}, corresponding to a half-life of 13 h or 7 h if the glucose-uptake rate is expressed with respect to the biomass concentration (data not shown). A half-life of 10 h has previously been reported by Cardoso & Leão (1992), but lower half-lives of between 4 and 7 h, based on protein, have also been reported (Lagunas et al., 1982; Riballo & Lagunas, 1994). The differences between reported values may be partly attributed to strain dependence, but may also be caused by the different methods used for measuring the glucose-uptake rate. The short half-lives were obtained from uptake-rate measurements with xylose, in which case it could not be excluded that mainly the inactivation of the high-affinity transport system was determined (Busturia & Lagunas, 1986).

**Cellular composition and intracellular metabolites**

The cellular composition was analysed with respect to the cellular content of trehalose, glycogen, RNA and protein. The cellular content of trehalose and glycogen is shown in Fig. 5(a) and the cellular content of protein and RNA is shown in Fig. 5(b). It was not possible to follow the cellular composition during phase I due to the low biomass concentration.
Phase II (5–20 h). The culture grew exponentially at the highest possible growth rate, \( \mu_{\text{max}} \). The cells had a cellular protein content of 57 % (w/w) (Fig. 5b), which remained constant during the exponential phase, and the cellular content of RNA also stayed constant at 15 % (w/w) during this phase (Fig. 5b). This illustrates that the amount of RNA and protein increased exponentially at a rate equal to \( \mu_{\text{max}} \). This phenomenon is often referred to as balanced growth (Ingraham et al., 1983). If the amount of nitrogen taken up from the medium is plotted against the biomass formed, one obtains a straight line with a slope of 0.12 g g\(^{-1}\) (graph not shown). This shows that, at this very high growth rate, have a cellular nitrogen content of 12 % (w/w). The elemental composition of protein is \( \text{CH}_{1.28}\text{O}_{0.93}\text{N}_{0.02}\text{S}_{0.04} \) and that of RNA is \( \text{CH}_{1.28}\text{O}_{0.70}\text{N}_{0.03}\text{P}_{0.11} \) (Nielsen & Villadsen, 1994), and by using the measured cellular content of RNA and protein, the cellular nitrogen content is calculated to be 11.9 % (w/w). Hence the major part of the cellular nitrogen is bound in these two macromolecular pools. The remaining nitrogen is found in DNA, the pool of free amino acids and other nitrogen-containing compounds. The cellular content of the two carbohydrates trehalose and glycogen is virtually zero in this phase (Fig. 5a).

Phase III (20–25 h). As soon as the extracellular nitrogen source was depleted, the intracellular concentrations of glycogen and trehalose started to increase simultaneously, but with a higher rate for glycogen (Fig. 5a). The RNA content, on the other hand, started to decrease right after depletion of ammonia in the medium (Fig. 5b). However, the total amount of RNA remained constant, implying that the RNA content of the cells decreased as a consequence of an increase in the cell weight due to accumulation of glycogen and trehalose. The same applies for the protein content of the cells (Fig. 5b).

Phase IV (25–38 h). Trehalose and glycogen continued to accumulate during this phase, albeit at a decreasing rate towards the end of the cultivation. Accumulation ceased when the extracellular glucose became depleted. Throughout this phase, the cellular RNA and protein content decreased continuously as a consequence of the accumulation of intracellular carbohydrates. The total amounts of these macromolecular pools remained constant in this phase, except for the last 5 h, when an approximate 10% decrease was observed in the cellular content of both. The amino acid composition of the cellular protein in the exponential growth phase was not significantly different from that at the end of the cultivation. No amino acid concentration changed by more than 20% (data not shown).

Growth energetics

Following the onset of nitrogen starvation, the glucose consumption continued, but the biomass concentration increased at a much slower rate (Fig. 2b, c). Thus, the cost of biomass formation in terms of ATP, \( Y_{\text{ATP}} \), increased as a consequence of nitrogen starvation. When calculating \( Y_{\text{ATP}} \), it is important to correct for the amount of ATP consumed in the formation of glyceraldehyde (Verduyn et al., 1990a). Typically, the energy consumption in terms of ATP is split in two terms, growth associated and non-growth associated (Benthin et al., 1994). However, in the present case, \( Y_{\text{ATP}} \) can be calculated without this distinction and as mean values for the various growth phases. During the exponential growth phase, the mean value of \( Y_{\text{ATP}} \) was 80 mmol g\(^{-1}\), which is considerably higher than the theoretical value of \( Y_{\text{ATP}} \) which can be calculated from the biochemistry and cellular composition to be approximately 36 mmol g\(^{-1}\) (Verduyn et al., 1991; Schulze, 1995). Nitrogen starvation led to a 70% increase in the cost of biomass formation, resulting in a \( Y_{\text{ATP}} \) of 137 mmol g\(^{-1}\) in phase IV. The discrepancy between the theoretically calculated and experimentally determined values of \( Y_{\text{ATP}} \) becomes even larger when considering that much of the biomass increase during nitrogen starvation is due to accumulation of reserve carbohydrates. These can be synthesized at a cost of 123 mmol g\(^{-1}\).

DISCUSSION

Anaerobicity in bioreactors

The supplementary addition of unsaturated fatty acids and sterols to the medium is necessary for optimal growth of \( S. cerevisiae \) (Andreasen & Stier, 1953, 1954; Verduyn et al., 1990a), since oxygen is required in the biosynthesis of these compounds (Hunter & Rose, 1971). However, growth in the absence of these supplements has been reported in both batch (Macy & Miller, 1983) and continuous (Verduyn et al., 1990a) cultures. Macy & Miller (1983) reported that \( S. cerevisiae \) could grow under strictly anaerobic conditions even in the absence of these compounds, although growth was slow and associated with long lag phases and low biomass yields. The medium contained the reducing agent cysteine (0.03%, w/w) and the colour redox indicator resazurin, which is colourless for redox potentials less than −42 mV. The redox indicator remained colourless during the growth experiment, which was interpreted by the authors as a proof of strict anaerobic conditions at all times. However, a redox indicator measures the oxygen concentration in the solution and, as pointed out by Visser et al. (1990), it is the oxygen flux that is important for maintaining anaerobic conditions. Visser et al. (1990) showed that it is possible to have a decolourized resazurin solution and still not have anaerobic conditions. The affinity for oxygen may be very high and the oxygen-uptake rate of the micro-organisms can therefore be sufficient to maintain a very low dissolved oxygen concentration, despite a continuous oxygen transfer to the solution. That growth can be observed in the absence of sterols and unsaturated fatty acids does not indicate that oxygen is dispensable in the synthesis of these compounds but merely demonstrates how difficult it is to prevent even minute amounts of oxygen from entering bioreactor systems. The absence of catalase activity was taken as an indication that anaerobic conditions prevailed in our bioreactor. In addition, this indicated that the nitrogen gas produced at site is comparable to commercial nitrogen gas containing less than 5 p.p.m. oxygen.
Exponential growth under anaerobic conditions

In the present work, it was found that the $\mu_{\text{max}}$ (0.46 h$^{-1}$) of S. cerevisiae was identical for aerobic and anaerobic growth. In a previous study, the anaerobic $\mu_{\text{max}}$ of S. cerevisiae CBS 8066 was estimated to be 0.31 h$^{-1}$ from a Hanes plot of the residual glucose concentration in a glucose-limited continuous culture (Verduyn et al., 1990a) and thus was not directly measured. It was also reported that no steady state could be obtained for dilution rates higher than 0.28 h$^{-1}$. There is no obvious explanation for the discrepancy, since the same medium was used and since no catalase activity could be detected in either of the cultures. However, high growth rates under anaerobic conditions have been reported: Visser et al. (1990) report that S. cerevisiae CBS 1171 has an anaerobic $\mu_{\text{max}}$ of 0.40 h$^{-1}$ but do not state the aerobic $\mu_{\text{max}}$; Lagunas (1979, 1986) presents data from a shake-flask culture of S. cerevisiae S-13-Gal showing an anaerobic $\mu_{\text{max}}$ which is approximately 95% of the aerobic $\mu_{\text{max}}$. Lagunas (1979) concludes that aerobiosis as compared to anaerobiosis does not provide S. cerevisiae with an energetic advantage that allows it to grow faster under the former conditions. The present results support the conclusion that energy generation does not seem to limit growth of S. cerevisiae.

Formation of extracellular metabolites

The fact that glycerol formation is due to a redox imbalance has been recognized for a long time (Barron & Levine, 1952; Holzer et al., 1963), but it was only by the work of Nordström (1966, 1968) that it became apparent that biomass formation is the major cause of glycerol formation. The results in this work show that glycerol formation is higher under conditions where biomass synthesis is unrestricted and that limitation of biomass synthesis by nitrogen starvation leads to a decrease in glycerol formation. The experimental data also illustrate that the theoretical yield of ethanol on glucose ($Y_{\text{se}} = 0.51$ g g$^{-1}$) can only be approached if biomass formation and the concomitant glycerol formation are arrested, i.e. the function of the biomass should approach that of a catalyst. Since nitrogen starvation leads to decreasing yields of both biomass and glycerol and increasing yields of ethanol (Table 1), it may be tempting to conclude that such conditions are optimal for ethanol production. However, under nitrogen starvation, the rate of ethanol production will decrease with time due to catabolite inactivation of the glucose transporter (Fig. 4). Hence, long periods of nitrogen starvation should be avoided in the production of ethanol.

Each of the formed organic acids accounts for no more than 1% of the consumed glucose, but they have an important impact on the redox level in the cell (Fig. 3). Currently, the secretion of organic acids is not believed to have a direct function as, e.g. the formation of glycerol, and secretion is in most cases ascribed to leakage from the intracellular pool (Fraenkel, 1982; Gancedo & Serrano, 1989). Both succinic acid and pyruvic acid are produced throughout the cultivation, although at decreasing rates. The reason for the transient behaviour of the acetic acid concentration at the CER in phase III remains unexplainable at the moment.

Turnover of protein

The rate of protein turnover is traditionally estimated from the release of radioactive material from labelled protein (Betz, 1976). The obtained value is a minimum estimate, since reutilization of the labelled degradation products may occur. By application of this technique, the rate of protein turnover under nitrogen starvation has been estimated to be 2% h$^{-1}$ (López & Gancedo, 1979), 4% h$^{-1}$ (Johnston et al., 1977b) or 3% h$^{-1}$ (Betz, 1976) for S. cerevisiae and 4-7% h$^{-1}$ for Escherichia coli (Mandelstam, 1958). This confirms that turnover of protein does occur under conditions of nitrogen starvation. The rate of protein turnover of 6% h$^{-1}$ estimated in this study from the glycerol formation which cannot be ascribed to the secretion of organic acids is based on the assumption that no NAD$^+$ is formed in the degradation of protein. However, the estimated value seems reasonable, although a little high. Turnover of RNA also occurs, and although synthesis of RNA leads to formation of less NADH than the synthesis of protein [67 mmol NADH (mol RNA)$^{-1}$ versus 21.9 mmol NADH (mol protein)$^{-1}$], the turnover of e.g. mRNA is much faster than the turnover of protein. Hence, it cannot be excluded that the unaccounted for glycerol formation is connected at least partially with turnover of RNA.

The effect of ammonia on the EMP pathway

Based on in vitro experiments, it has been established that ammonia is an allosteric activator of phosphofructokinase (Ramaiah, 1974) and pyruvate kinase (Rhodes et al., 1986). Consequently, one may expect that depletion of ammonia will lead to a reduced activity of these two key enzymes and thus to a reduced flux through the EMP pathway (Fig. 2). Another explanation may be that the signalling pathway induced by fermentable growth medium is turned off due to depletion of ammonia (Thevelein, 1994). This pathway will be active as long as there is ammonia and excess glucose in the medium and will lead to an activation of enzymes through the cAMP-dependent protein kinase. Depletion of ammonia can be expected to have the opposite effect, i.e. causing deactivation of e.g. phosphofructokinase (Francois et al., 1984; Thevelein, 1994). It seems likely that depletion of ammonia triggers the abrupt decrease in the CER, but whether the mechanism is based on the direct allosteric action of ammonia or whether ammonia exerts action through a signal transduction pathway remains unclear.

Catabolite inactivation of the glucose transporter(s)

It seems likely that the continuous decrease in the CER (Fig. 2a) is due to a decrease in the uptake of glucose (Fig. 4), caused by catabolite inactivation of the glucose transporters (Lagunas et al., 1982; Busturia & Lagunas, 1986; Ribalba & Lagunas, 1994). This is supported by the fact that the decrease in the glucose-transport rate cannot nearly be accounted for by the effect the decreasing
extracellular glucose concentration will have on the glucose-transport rate, even if glucose was only transported by the low-affinity system with a $K_m$ of 20–25 mM. Both the high-affinity and the low-affinity carriers are affected by the inactivation, which is the result of endocytosis (Riballo & Lagunas, 1994). It is not the absence of ammonia per se that is the cause of inactivation but rather the arrest of protein synthesis (Busturia & Lagunas, 1986; Alonso & Konyk, 1978). The inactivation requires the presence of a fermentable substrate, since inactivation was not observed during carbon starvation but was induced when sugars were added to a carbon- and nitrogen-starved culture (Busturia & Lagunas, 1986). Addition of ammonia to ammonia-starved cells leads to reactivation of the sugar transporters, but the mechanism does not involve de novo synthesis of the sugar transporters but rather synthesis of a regulatory protein (Bely et al., 1994). Catabolite inactivation of the glucose carrier in the absence of ammonia is a great nuisance to oenology which is supported by results based on radioactively labelled RNA and protein (Johnston et al., 1977b). A large portion of the ribosomes is presumably inactive at low growth rates, as indicated by the results of Waldron et al. (1977), who showed that the fraction of ribosomes engaged in protein synthesis decreased from more than 80% at high growth rates to less than 50% in slowly growing cells. Nitrogen starvation virtually inhibits cells from reinitiating cell division but cells already engaged in cell division will complete the cell division cycle and eventually get arrested in the $G_1$ phase (Hartwell, 1974; Johnston et al., 1977b; Schulze, 1995).

$S. cerevisiae$ accumulates huge quantities of intracellular carbohydrates under conditions of limitation, e.g. by nitrogen in the presence of excess glucose (Lillie & Pringle, 1980; Slaughter & Nomura, 1992; Quain et al., 1981). Neither trehalose nor glycogen accumulate during exponential growth (Fig. 5a), but accumulation of both carbohydrates sets in right after depletion of the extracellular nitrogen source. Both carbohydrates continue to accumulate, as long as there is glucose present in the medium, although the rate of accumulation decreases towards the end of the cultivation. The majority of the sequestrated glucose ends up as glycogen, supporting the notion that glycogen serves as a storage carbohydrate (Lillie & Pringle, 1980; Panek, 1991). According to recent results, the functional role of trehalose is as a stress protectant rather than as an energy store (Wiernik, 1990; Eleuthero et al., 1993; van Dijk et al., 1995). This is in line with the fact that nitrogen starvation is usually considered a stress situation. Although the intracellular content of carbohydrates is very large, less than 2% of the metabolized glucose ends up as storage carbohydrates.

**Energetics**

The reported value of $Y_{ATP}$ for the exponential growth phase (80 mmol g$^{-1}$) is similar to values reported in the literature: 75–85 mmol g$^{-1}$ in a batch cultivation (defined medium) (Haukali & Lie, 1971) and 90 mmol g$^{-1}$ in a glucose-limited continuous culture (defined medium) at a dilution rate of 0.28 h$^{-1}$ (Verduyn et al., 1990a, b). Thus, the observation that $Y_{ATP}$ is significantly higher than the theoretically calculated value of $Y_{ATP}$ is well known and has also been observed for many other micro-organisms, e.g. $E. coli$ (Hempfling & Mainzer, 1975) and *Aerobacter aerogenes* (Stouthamer & Bettenhaussen, 1976). This discrepancy is usually ascribed to futile cycles (Auberson et al., 1989; Navas et al., 1993) or dissipation of ATP by the membrane ATPases (Stouthamer, 1979). Also it has been demonstrated that the presence of weak acids may lead to a significant dissipation of ATP due to an increased influx of protons and consequently to an increase in $Y_{ATP}$ (Verduyn et al., 1990b, 1992; Verduyn, 1992). However, since the concentration of weak acids is very low in phase I plus II, this specific mechanism does not seem to be a major cause of the observed difference. When the yeast becomes nitrogen starved, $Y_{ATP}$ increases significantly to more than 130 mmol g$^{-1}$. The increase in the extracellular concentration of weak acids in phase IV may partially account for this, but it seems likely that the general degree of energy dissipation increases. Results from anaerobically limited cultures seem to support this notion, e.g. Benthin et al. (1994) observed that nitrogen limitation leads to a significant increase in $Y_{ATP}$ for the lactic acid bacterium *Lactococcus cremoris* and similar results have been observed for *Klebsiella aerogenes* (Neijssel & Tempest, 1976; Neijssel et al., 1990). Thus, under conditions of nitrogen starvation or limitation, *S. cerevisiae* and other micro-organisms do not seem to regulate their energy consumption to the actual needs, which is manifested in a very high $Y_{ATP}$.

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**REFERENCES**


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