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Energetics and product formation by *Saccharomyces cerevisiae* grown in anaerobic chemostats under nitrogen limitation

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Abstract Anaerobic fermentation of glucose (20 g/l) by *Saccharomyces cerevisiae* CBS 8066 was studied in a chemostat (dilution rate = 0.05–0.25 h^{-1}) at different concentrations of the nitrogen source (5.00 g/l or 0.36 g/l ammonium sulphate). The ethanol yield (g ethanol produced/g glucose consumed) was found to be higher and the glycerol yield (g glycerol formed/g glucose consumed) lower during nitrogen limitation than under carbon limitation. The biomass yield on ATP (g dry weight biomass produced/mol ATP consumed), was consequently found to be lower during nitrogen-limited conditions.

Introduction

A fundamental question in cell energetics is whether Y_{ATP}, the amount of biomass formed per mole of ATP, is different under different environmental conditions, or if it is a biological constant (Bauchop and Elsden 1960). During anaerobic conditions the ATP formed in yeast catabolism can be exactly calculated from the amounts of ethanol, acetic acid and glycerol formed, whereas during aerobic conditions the P/O ratio must be known. *Saccharomyces cerevisiae* is one of the few yeast species known to be capable of anaerobic growth (Visser et al. 1990) and is therefore well suited for the study of cell energetics. For industrial production of ethanol, anaerobic fermentation of hexoses by *S. cerevisiae* is of great importance. During anaerobic fermentation, considerable amounts of glycerol are formed as an unwanted by-product. As much as 10% of the glucose can be converted to glycerol instead of ethanol during anaerobic fermentation of glucose (Schatzmann 1975). A change of Y_{ATP} will influence both the cell yield (g biomass formed/g substrate consumed) and the glycerol yield (g glycerol formed/g substrate consumed), since the glycerol production is linked to the biomass formation (Bruinenberg et al. 1983; Lagunas and Gancedo 1973; Oura 1977).

It has been found for bacteria that the uptake rate of glucose during limitation of potassium or nitrogen is higher than under carbon-limited conditions (Neijssel and Tempest 1987). The uptake rate is thus not always balanced by anabolic requirements. Also *S. cerevisiae* shows an incomplete coupling between the sugar uptake and the anabolic requirements. It has been found that glucose in excess of what is required for anabolic purposes is taken up if a glucose pulse is added to an aerobic chemostat, and that this causes ethanol formation (Van Urk et al. 1988). Larsson *et al.* (1993) studied carbon- and nitrogen-limited growth of *S. cerevisiae* under aerobic conditions in a chemostat and found evidence of an incomplete coupling between anabolism and catabolism. The critical dilution rate at which growth changes from purely respiratory to respiro-fermentative, was found to depend on the nitrogen concentration of the medium.

The previous studies mentioned were carried out under aerobic conditions, which makes an analysis of the cell energetics difficult, since the exact value of the P/O ratio is unknown and may be changing. In the present work, *S. cerevisiae* was grown in anaerobic chemostats during carbon or nitrogen limitation. The aim was to compare nitrogen-limited and carbon-limited growth with respect to cell energetics and the formation of products such as biomass, ethanol and glycerol.

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Materials and methods

Strain and medium

The yeast strain *Saccharomyces cerevisiae* (CBS 8066) obtained from Centraalbureau voor Schimmelcultures, Delft, The Netherlands, was maintained on agar plates made from yeast extract 10 g/l, peptone from soy 20 g/l (Serva, Heidelberg, Germany) and D-glucose, 20 g/l (puriss, Merck, Darmstadt, Germany) at 4° C. Inoculum cultures were grown in 250-ml conical flasks (liquid volume 150 ml) fitted with cotton plugs on a rotary shaker at 30° C for 70–74 h at a shaker speed of 140 rpm. The inoculum medium consisted of 6.7 g/l yeast nitrogen base (YNB, Difco, Detroit, Mi.) and 10 g/l D-glucose. The medium used during aerobic batch growth in the fermentor consisted of 1.7 g/l YNB without amino acids and ammonium sulphate (Difco), 10 g/l D-glucose and $(NH_4)_2SO_4$ (Serva), either 5.0 g/l before the start of carbon-limited chemostats or 1.25 g/l before the start of nitrogen-limited chemostats. During the anaerobic chemostat experiments the concentration of D-glucose was 20 g/l, YNB without amino acids and ammonium sulphate was 1.7 g/l, and the medium was supplemented with 10 mg/l ergosterol (United States Biochemical Corp., Cleveland, Ohio) and 420 mg/l Tween 80 (unsaturated fatty acids, Merck). The concentration of $(NH_4)_2SO_4$ was 5.0 g/l in the carbon-limited experiments and 0.356 g/l in the nitrogen-limited experiments. Ergosterol and Tween 80 were dissolved in a few millilitres of ethanol and heated to 100° C for 10 min. The sugar was sterilized separately from the other medium components. Less than 10 mg/l antifoam (Antifoam 289, Sigma Chemicals, St. Louis, Mo.) was used in the fermentor medium.

Metabolites and cell concentration

Cell concentration was determined from duplicate samples (2×10 ml), which were cooled in an ice-bath, centrifuged at 5000 rpm for 5 min and washed twice with distilled water, after which the samples were dried for 24 h at 105° C. The standard deviation was less than 1.3% for the dry-weight determinations. For analysis of the metabolites, cells were separated by centrifugation and the samples were stored in a freezer at − 20° C. Glucose, ethanol and glycerol were determined by HPLC analysis. A FAM-pak column (Waters, Milford, Mass.) kept at 56° C was used together with a refractive-index detector (model 410, Waters). The eluent used was 1.5 mM H_3PO_4 with a flow rate of 1 ml/min. Repeated analyses gave the following standard errors of analysis for the different compounds: glucose < 1.5% of the measured value for concentrations above 1.5 g/l and < 2.7% otherwise; ethanol < 3.8% for concentrations above 5 g/l and 5% otherwise; glycerol < 5% for concentrations above 1.3 g/l.

Acetic acid and succinic acid concentrations were determined by UV absorbance methods using enzymatic kits (test combination catalogue no. 176 281 for succinic acid and no. 148 261 for acetic acid; Boehringer-Mannheim, Mannheim, Germany). Ammonium was determined by a UV absorbance method with an enzymatic kit (test combination no. 1112 732; Boehringer-Mannheim).

Continuous culture conditions

A 2.5 l laboratory-scale fermenter (Bioflo III, New Brunswick Scientific, Edison, N.J.) with a working volume of 1.5 l was used in the experiments.The fermentor was equipped with a condensor, a polarographic oxygen electrode (Phoenix electrode company, Houston, Tex.) and a pH electrode (Ingold, Switzerland). The pH was controlled at 4.5 by the addition of 1 M NaOH, and the temperature was controlled at 30° C. The fermentor was inoculated with 50 ml inoculum culture, bringing the total volume to 1.5 l and aerobic conditions (1 vvm airflow, 500 rpm) were maintained until the characteristic diauxic growth (Fiechter et al. 1981) was finished. After the batch growth phase, the operation was changed to continuous mode and anaerobic conditions. Nitrogen, certified less than 5 ppm O_2 [ADR class 2, 1(a), AGA, Lidingö, Sweden] was sparged through the fermentor at a flow rate of 0.2 l/min. Nitrogen was also sparged through the medium bottle at a lower flow rate. The gas flowing from the condensor was bubbled through a beaker of water to provide an extra barrier to oxygen diffusion. The oxygen diffusion into the system has previously been determined to be approximately 12 $\mu g\,l^{-1}\,h^{-1}$ (Lidén et al. 1993). Samples were taken directly from the fermentor after three to five residence times, except for the first dilution rate where samples were not taken until after a minimum of five residence times. This has previously been shown to give steady-state conditions (Oelz et al. 1993). Confirmation of the steady-state conditions at the lowest dilution rate was also obtained by returning to the lowest dilution rate a second time, after the highest dilution rate had been examined. Duplicate chemostat experiments showed good reproducibility.

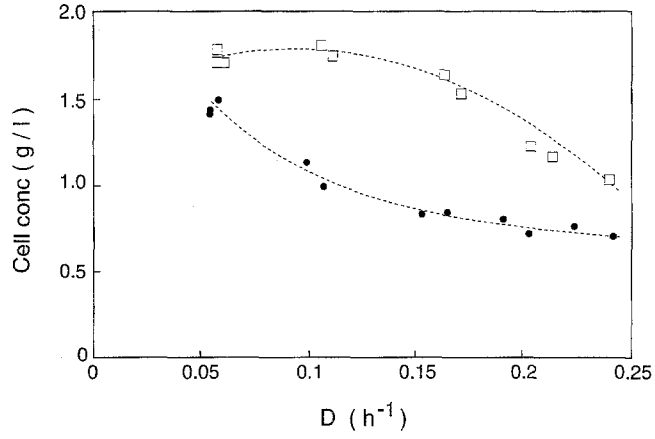


Fig. 1 Cell concentrations obtained in anaerobic chemostat cultures of *S. cerevisiae* grown in a synthetic medium with glucose (20 g/l) as a carbon and energy source. □ Carbon-limited chemostats, medium concentration of $(NH_4)_2SO_4$ = 5.0 g/l; ● nitrogen limited chemostats, medium concentration of $(NH_4)_2SO_4$ = 0.36 g/l. *D* dilution rate

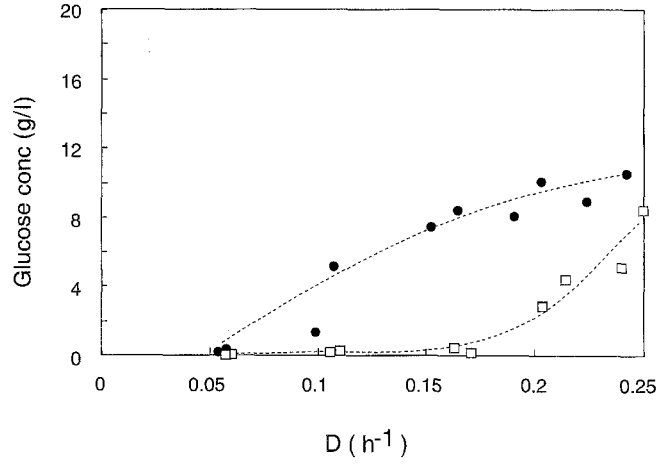
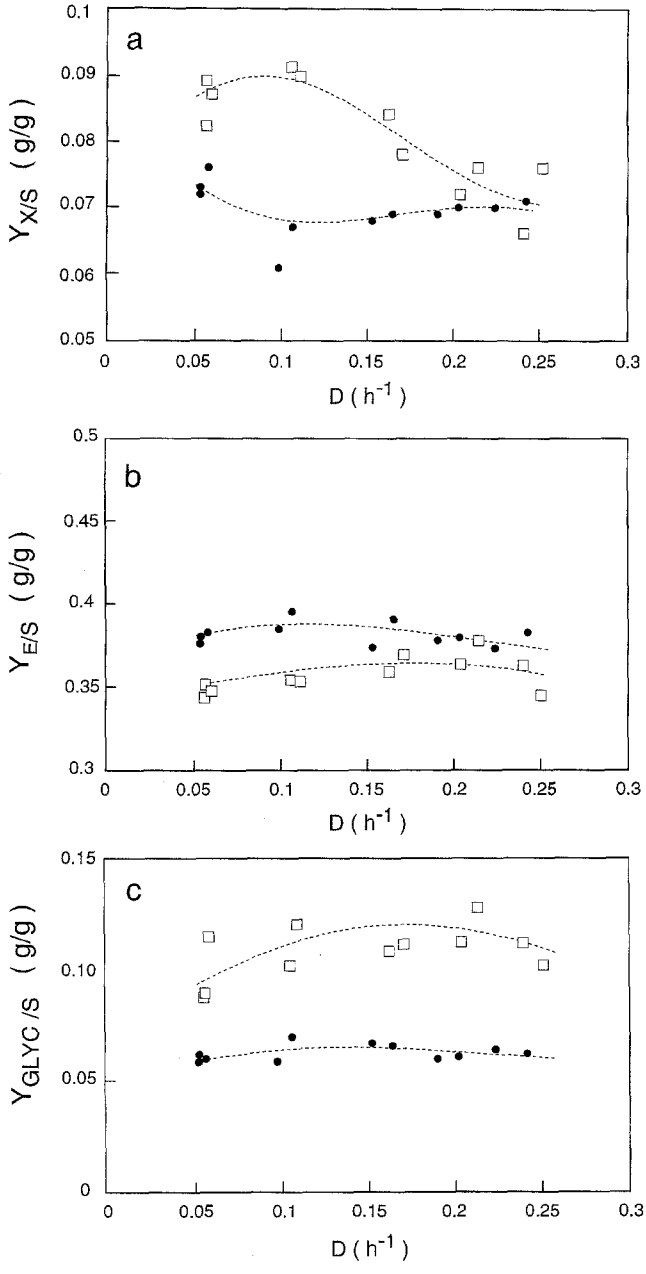


Fig. 2 Residual glucose concentration in the chemostats. □ Carbon-limited chemostats, ● nitrogen-limited chemostats



Figs. 3a–c Yields of biomass, ethanol and glycerol in anaerobic chemostat cultures of *S. cerevisiae* grown in a synthetic medium with glucose (20 g/l) as a carbon and energy source. □ Carbon-limited chemostats, ● nitrogen-limited chemostats

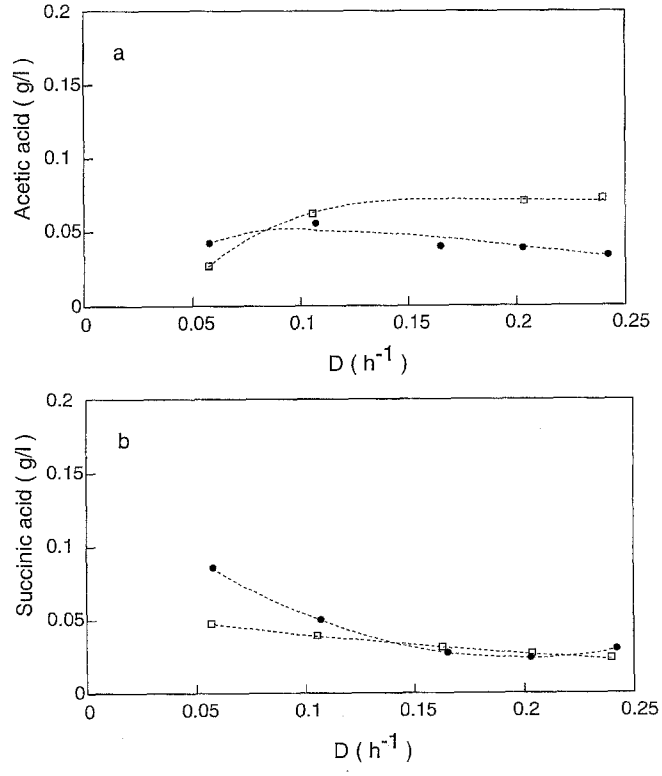
Results

Product yields

The biomass concentrations obtained in the anaerobic chemostat experiments are shown in Fig. 1, and the residual glucose concentrations in the medium are shown in Fig. 2. For the carbon-limited conditions, the maximum biomass concentration was found for a dilution rate of 0.10 h^{-1}, whereas for the nitrogen-limited conditions the maximum cell concentration was obtained at the lowest dilution rate studied. The yields of biomass, $Y_{X/S}$ (g/g), ethanol, $Y_{E/S}$ (g/g), and glycerol, $Y_{GLYC/S}$ (g/g), are shown in Figs. 3a–c. Small amounts of acetic acid and succinic acid were also formed (Fig. 4a, b).

A statistical analysis showed that, on average, $Y_{E/S}$ did not significantly depend on the dilution rate, neither for the carbon-limited cultivations (observed t value = 1.85), nor for the nitrogen-limited cultivation ($t = -0.29$). Furthermore, the average value of $Y_{E/S}$ for nitrogen-limited conditions, 0.381 g/g, was significantly higher than that for carbon-limited conditions, 0.357 g/g ($t = 6.24$), whereas the average glycerol yield was significantly lower for the nitrogen-limited, 0.063 g/g, than for the carbon-limited conditions, 0.11 g/g, ($t = -11.7$).

Practically all the nitrogen present in the medium was used by the cells (the residual ammonium concentration in the broth was less than 0.1 mM) in the nitrogen-limited chemostats. During carbon-limited conditions the residual concentration of ammonium was larger than 55 mM for all dilution rates. Calculations of the amount of ammonium consumed per amount of biomass produced showed that less ammonium is consumed per unit of biomass formed



Figs. 4a–b Concentrations of acetic acid and succinic acid obtained in anaerobic chemostat cultures of *S. cerevisiae* grown in a synthetic medium with glucose (20 g/l) as a carbon and energy source. □ Carbon-limited chemostats, ● nitrogen-limited chemostats

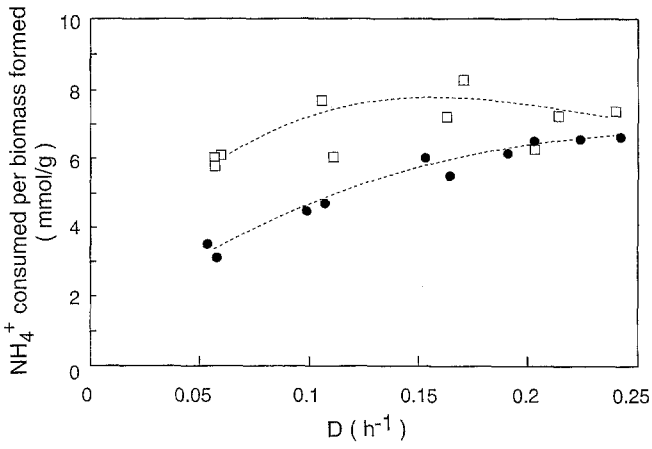


Fig. 5 Amount of ammonium consumed per biomass formed in anaerobic chemostat cultures of *S. cerevisiae* grown in a synthetic medium with glucose (20 g/l) as a carbon and energy source. □ Carbon-limited chemostats, ● nitrogen-limited chemostats

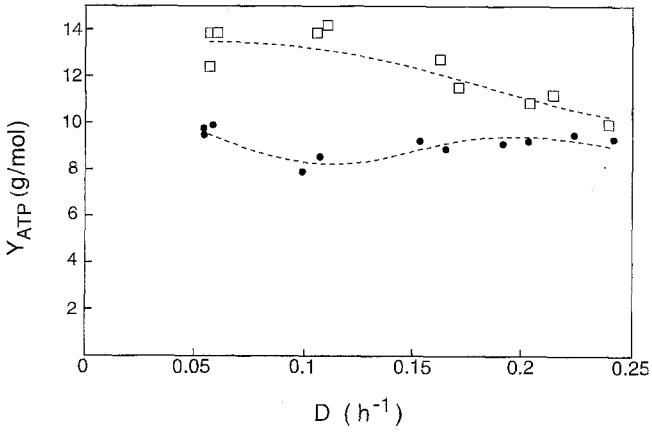


Fig. 6 Calculated biomass yields on ATP for chemostat cultures of *S. cerevisiae* grown in a synthetic medium with glucose (20 g/l) as a carbon and energy source. □ Carbon-limited chemostats, ● nitrogen-limited chemostats

during nitrogen limitation than during carbon limitation, especially at low dilution rates (Fig. 5).

ATP yield

From the data in Fig. 3a–c a calculation of the ATP yield (g/mol ATP), denoted Y_{ATP}, was made according to

$$Y_{ATP} = \frac{Y_{X/S}}{\alpha_E Y_{E/S}/M_E + \alpha_{GLYC/S} Y_{GLYC/S}/M_{GLYC/S}}$$

where M_E and $M_{GLYC/S}$ are the molar masses (g/mol) of ethanol and glycerol. The coefficients, relating ATP formation to product formation are: $\alpha_E = 1$ and $\alpha_{GLYC/S} = -1$ (mol ATP formed/mol product formed) for ethanol and glycerol respectively. Other products, such as acetic acid and succinic acid, are present in very low amounts (Fig. 4a, b) and therefore only marginally affect the calculation of the ATP yield. The results of the ATP calculations are shown in Fig. 6. For the carbon-limited cultures, the ATP yield showed a significant negative slope ($t = -4.1$) when plotted against dilution rate, which indicates an increased ATP demand for growth at high dilution rates. However, for nitrogen-limited growth the ATP demand was found not to depend on the dilution rate ($t = -0.2$).

Discussion

Energetics

An important result of this study is that the biomass yield on ATP for the nitrogen-limited cultures is lower than for carbon-limited cultures, in particular at low dilution rates. The cells grown under nitrogen limitation have a lower nitrogen content at low dilution rates (cf. Fig. 5). The synthesis of 1 g protein has been estimated to require 0.391 mol ATP, whereas the synthesis of 1 g polysaccharide has been estimated to require only 0.123 mol ATP (Stouthamer 1979). If only the biosynthetic ATP requirements are considered one could therefore expect the ATP demand to be higher for the cells with a high nitrogen content, which is qualitatively the opposite of what is found in this study. One possible explanation could be that the ATP requirements for ammonium metabolism increases at very low concentrations of ammonium. This could be caused by a higher ATP cost of transport of ammonium, for example, or a higher ATP cost for incorporation of ammonium into amino acids. In addition, an increased maintenance energy requirement may have to be considered. However, the ATP requirements for maintaining cell homeostasis can not explain the increased ATP consumption during nitrogen-limiting conditions (Lagunas and Ruiz 1988; Nilsson *et al.* 1995). Another explanation could be that the extra ATP requirement is caused mainly by an uncoupling of ATP used in anabolism and ATP generated in catabolism (Larsson et al. 1993). This uncoupling may be caused by some kind of energy-spilling cycle. Several different possible ATP-demanding cycles have been suggested, in particular for bacteria (Neijssel and Tempest 1990; Stouthamer 1979).

Glycerol formation

The formation of glycerol by *S. cerevisiae* during anaerobic fermentation has mainly been linked to the redox balance (Oura 1977), although also the role of glycerol as an osmoregulator is well established (Blomberg and

Adler 1992). The surplus of NADH causing glycerol formation comes from three principal sources: from biosynthesis, from the formation of acetic acid and from the formation of succinate. The calculated surplus from anabolic reactions depends mainly on the protein contents of the cells, but also on the RNA and the lipid contents. During anaerobic conditions, it has been suggested that no NADH is produced from lipid biosynthesis if fatty acids are provided in the medium (Verduyn et al. 1990). Since the nitrogen content, and thereby most likely also the protein content, is lower in the cells grown under nitrogen limitation, it is not surprising that the glycerol yield is also lower.

In conclusion, the results presented here show that nitrogen limitation has a profound effect on cell energetics of *S. cerevisiae*. A consequence of the changed cell energetics is that the ethanol yield, $Y_{E/S}$, is higher for nitrogen-limited conditions than for carbon-limited conditions. Furthermore, the glycerol formation is strongly influenced by nitrogen limitation. Both these effects are important for the optimization of hexose fermentation by *S. cerevisiae*.

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