

Influence of the Carbon:Nitrogen Ratio of the Growth Medium on the Cellular Composition and the Ability of the Methylophilic Yeast *Hansenula polymorpha* to Utilize Mixed Carbon Sources

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The methylophilic yeast *Hansenula polymorpha* was grown in a chemostat with a medium containing a mixture of glucose (C₆) and methanol (C₁) (87.8% C₆: 12.2% C₁, w/w) as sole carbon source and NH₄⁺ as sole nitrogen source. At a constant growth rate (D 0.10 h⁻¹) the influence of the carbon:nitrogen ratio (C:N) of the inflowing medium on the cellular and enzymic composition of the cells was studied. Three distinct growth regimes were recognized. A medium with a C:N ratio < 12 resulted in carbon-limited growth (high cellular protein content, low carbohydrate content) and under these conditions glucose and methanol were utilized simultaneously. A medium with a C:N ratio > 31 resulted in nitrogen-limited growth (low protein but high carbohydrate content of the cells) and the cells metabolized only glucose. A transition growth regime was observed during growth on media with intermediate C:N ratios (12 < C:N < 31). When assessed from both substrate consumption and cellular composition, growth was double-substrate (carbon and nitrogen)-limited. In this transition growth regime, changes in carbon metabolism and the cellular and enzymic composition of the cells were found. With increasing C:N ratios in the growth medium a gradual repression of the synthesis of methanol-assimilating and dissimilating enzymes was found. This effect was most pronounced for alcohol oxidase, and as a consequence the cells switched from the utilization of the carbon substrate mixture to growth on glucose alone. The data presented suggest that the range within which double-substrate-limited growth can be expected is predictable from the composition of cells grown under single substrate limitation.

INTRODUCTION

In all ecosystems one of the key factors controlling the rate of cell growth is the availability of nutrients. In contrast to multicellular organisms, microbes can adapt relatively quickly to various nutrient limitations by changing their cellular composition. Single nutrient limitations such as carbon, nitrogen or inorganic ions have been much studied but little attention has been directed to the fact that growth, as it occurs in nature, is not necessarily limited by a single nutrient. Microbes are unlikely to grow over an extended period of time under 'steady-state' conditions but rather under conditions where the supply of nutrients fluctuates considerably. Only recently have microbiologists started to investigate the physiology and growth behaviour of microbes under multi-substrate-limited, gradient or transient growth conditions (e.g. Dijkhuizen, 1979; Gottschal, 1980; Wimpenny *et al.*, 1983; Leegwater, 1983).

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Abbreviations: C₁, methanol; C₆, glucose; c₀, n₀, concentration of carbon or nitrogen, respectively, in the inflowing medium (g l⁻¹); c, n, concentration of carbon or nitrogen, respectively, in the culture liquid (g l⁻¹; mg l⁻¹); D, dilution rate (h⁻¹); q_C, q_{C₆}, q_{C₁}, q_N, specific rate of carbon, glucose, methanol or nitrogen consumption, respectively [g substrate (g biomass)⁻¹ h⁻¹]; x, biomass concentration (g l⁻¹); Y_{X/C}, Y_{X/N}, growth yield with respect to carbon or nitrogen, respectively [g biomass (g substrate)⁻¹]; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase.

During double-substrate-limited growth of methylotrophic yeasts with glucose and methanol (Egli *et al.*, 1980, 1982) the simultaneous utilization of glucose and methanol in chemostat cultures was strongly dependent on the nature of the growth limitation. During carbon-limited growth the cells were able to utilize both substrates simultaneously and completely, whereas under nitrogen limitation the synthesis of enzymes required for methanol utilization was repressed, and therefore glucose was the only source of carbon metabolized by the cells. The kinetics of this repression have also been studied (Egli, 1982). In an experiment where the ratio of C:N in the feed to the chemostat was gradually raised to achieve nitrogen-limited growth, the cells were for some time under conditions where neither residual carbon (glucose and/or methanol) nor nitrogen (NH_4^+) could be detected in the culture. This indicates that between carbon limitation (nitrogen in excess) and nitrogen limitation (carbon source in excess) an additional growth regime exists where growth is limited by both nutrients. As the growth of microbes under such transition conditions has only been reported twice (Huetting & Tempest, 1979; Gräzer, 1985) we decided to study the phenomenon in more detail and to investigate the influence of the C:N ratio in the inflowing medium on the physiology of a methylotrophic yeast, particularly its ability to utilize mixtures of glucose and methanol simultaneously.

METHODS

Organism and cultivation. *Hansenula polymorpha* (CBS 4732) was grown in continuous culture (37 °C, pH 5.0) on a synthetic medium (Egli & Fiechter, 1981). A mixture of glucose (C_6) and methanol (C_1) of constant composition (87.8% C_6 :12.2% C_1 , w/w) was used as the sole source of carbon and energy; NH_4^+ was the only source of nitrogen. The C:N ratio in the inflowing medium was varied by changing the ratio C_6/C_1 -mixture: NH_4^+ . The C:N ratio of the medium indicated on the abscissa in the Figures was always calculated on the basis of total carbon from both glucose and methanol, whether utilized or not, and of nitrogen from NH_4^+ . To avoid wall growth due to methanol accumulation in the fermenter, the concentration of this substrate in the feed was always kept below 1.2 g l⁻¹. Dissolved oxygen tension was kept above 50% air saturation and the concentration of dry biomass in the fermenter was always lower than 5.0 g l⁻¹ to ensure that growth of the cells was always limited by either carbon or nitrogen.

Substrate concentrations. Glucose was measured enzymically (GOD-Perid test, Boehringer) and methanol was analysed by gas chromatography as reported by Egli *et al.* (1983). The detection limits for glucose and methanol were 2.0 mg l⁻¹ and 1.0 mg l⁻¹, respectively. NH_4^+ was measured spectrophotometrically using the indophenol method of Scheiner (1976). The reproducibility of the measurement of residual NH_4^+ -N was < ± 5% at concentrations > 0.10 g l⁻¹; between 0.10 and 0.0005 g l⁻¹ it was < ± 20% and reproducible measurements were not possible at concentrations lower than 0.0005 g NH_4^+ l⁻¹. To avoid substrate losses due to substrate consumption by the cells after sampling, the residual concentrations of glucose, methanol and NH_4^+ in the culture medium were assayed by the filtration technique described by Egli *et al.* (1983). With this technique the cells could be separated from the medium in less than 0.5 s.

Cellular components. For dry weight measurements the cells were collected by centrifugation, washed twice with distilled water and dried to constant weight at 105 °C. Parallel samples varied by < ± 1%. For the determination of cellular components, the cells were collected from the chemostat outlet and immediately cooled to 0 °C; they were then harvested by centrifugation at 4 °C, washed twice with sodium phosphate buffer (50 mM, pH 7.0), freeze-dried and stored. Before the individual analyses, the freeze-dried cells were dried again over phosphorus pentoxide.

The content of carbon, hydrogen and nitrogen in freeze-dried cells was measured with a C, H, N-Analyzer model 185 (F & M Scientific). All values reported are the mean of at least three determinations and the deviations for C, H and N for separate samples were always < ± 3.5%, ± 11.9% and ± 3.7%, respectively, of the absolute value.

The biuret method, as described by Herbert *et al.* (1971), was used for the determination of the protein content of freeze-dried cells.

Glycogen was measured by the method of Becker (1978).

The content of total carbohydrates was determined by the phenol method described by Herbert *et al.* (1971).

Enzyme assays. Cell-free extracts were prepared as described by Egli *et al.* (1983). All enzyme assays were done at 37 °C. The following enzymes were measured according to previously published methods: alcohol oxidase (EC 1.1.3.13; van Dijken *et al.*, 1976), dihydroxyacetone kinase (van Dijken *et al.*, 1978), formaldehyde dehydrogenase (EC 1.2.1.1; Egli *et al.*, 1980), formate dehydrogenase (EC 1.2.1.2; Egli *et al.*, 1980), glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Kornberg & Horecker, 1955), glucose-6-phosphate isomerase (EC 5.3.1.9; Egli *et al.*, 1983), 6-phosphogluconate dehydrogenase (EC 1.1.1.44; Egli *et al.*, 1982), ribose-5-phosphate isomerase (EC 5.3.1.6; Wood, 1970), ribulose-5-phosphate 3-epimerase (EC 5.1.3.1; Wood, 1970),

transaldolase (EC 2.2.1.2; Tchola & Horecker, 1966), transketolase (EC 2.2.1.1; Waites & Quayle, 1981). The assay for NADP⁺-dependent glutamate dehydrogenase (EC 1.4.1.2) contained, per ml: potassium phosphate buffer pH 7.8, 100 µmol; 2-oxoglutarate, 3.3 µmol; NH₄Cl, 33.3 µmol; NADPH, 0.50 µmol; and cell-free extract. The reaction was started by adding 2-oxoglutarate. The Lowry method was used to determine the protein concentrations in cell-free extracts. Bovine serum albumin (Armour) was used as the standard.

Enzyme units. The specific activities of all enzymes are expressed as the rate of substrate consumption (or product formation) per unit of protein [µmol min⁻¹ (mg protein)⁻¹].

RESULTS

H. polymorpha was grown in chemostat culture (D 0.10 h⁻¹) on a glucose-methanol mixture of constant composition, and with NH₄⁺ as sole nitrogen source, as described in Methods. The experiment was started by growing cells on the medium with the lowest C:N ratio used in this study. After analysis of the resulting steady-state the ratio was increased stepwise. The C:N ratios, the composition of the media used and the exact residual concentrations of the substrates of interest are given in Table 1. On the basis of substrate utilization by the cells, and their cellular and enzymic composition, three different growth regimes could be recognized as the C:N ratio of the supplied medium was changed. The most important characteristics of these regimes are summarized in Table 2. In all the figures, the transition growth regime is indicated by a shaded area.

Table 1. *Substrate and dry biomass concentrations during cultivation of H. polymorpha*

H. polymorpha was grown in a chemostat at a constant dilution rate of 0.10 h⁻¹ with media of different C:N ratios. All concentrations are given in g l⁻¹.

C:N	Substrate concentrations in the inflowing medium			Dry cell weight formed and residual substrate concentrations			
	C ₆	C ₁	NH ₄ ⁺ -N	Dry wt	C ₆	C ₁	NH ₄ ⁺ -N
1.00	4.56	0.67	2.088	2.71	—	—	1.837
2.79	4.56	0.67	0.746	2.86	—	—	0.509
6.63	4.28	0.64	0.294	2.70	—	—	0.1233
11.32	4.15	0.61	0.167	2.40	—	—	0.0161
14.46	4.44	0.63	0.139	2.44	—	—	0.0035
15.89	4.34	0.62	0.124	2.61	—	—	0.0005
18.40	4.61	0.64	0.113	2.69	—	—	—
21.95	5.53	0.72	0.113	3.20	—	0.007	—
26.73	6.69	0.91	0.113	3.79	—	0.78	—
35.04	8.85	1.13	0.113	4.74	0.0038	1.04	—
40.13	6.99	0.90	0.078	3.08	0.0094	0.84	—
59.11	5.89	0.82	0.045	1.42	2.07	0.78	—

—, Not detected (C₆ < 2 mg l⁻¹; C₁ < 1 mg l⁻¹; NH₄⁺-N < 0.5 mg l⁻¹).

Table 2. *Characterization of different growth regimes observed during the cultivation of H. polymorpha*

H. polymorpha was grown in a chemostat at a constant dilution rate of 0.10 h⁻¹ with media of different C:N ratios. All concentrations are given in mg l⁻¹.

Growth regime	C:N ratio range of media	Residual substrate in the culture			Cell protein content	Cell carbohydrate content	Alcohol oxidase (specific activity)
		C ₆	C ₁	NH ₄ ⁺			
Carbon limitation	≤ 11.32	< 2	< 1	> 3.5	High	Low	High
Transition	11.32 < C:N ≤ 18.40	< 2	< 1	≤ 3.5	Reduced	Enhanced	Reduced
	18.40 < C:N ≤ 26.73	< 2	> 1	< 3.5	Reduced	Enhanced	Low
Nitrogen limitation	≥ 35.04	> 2	> 1	< 3.5	Low	High	Low

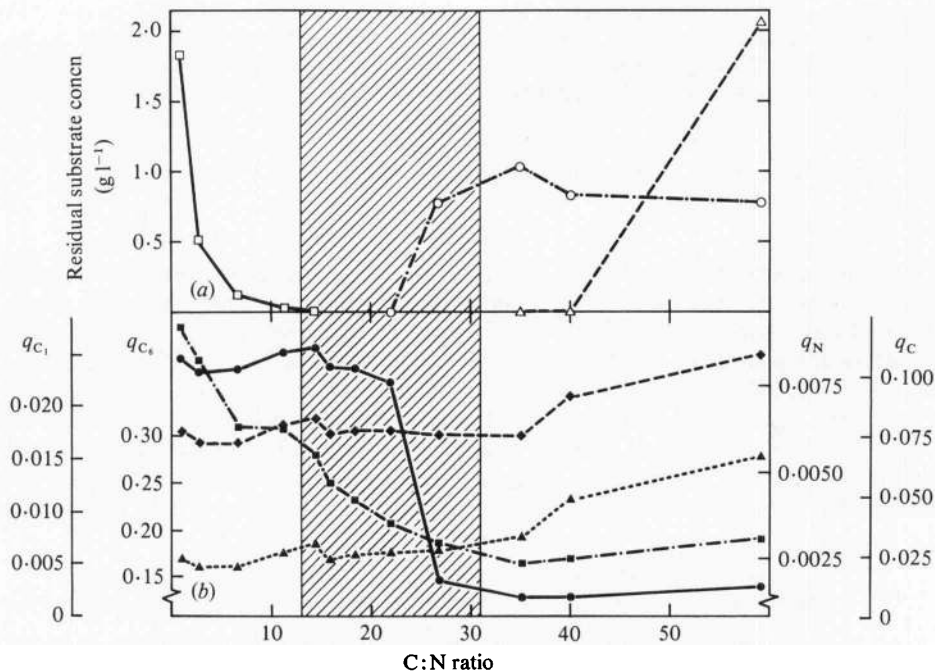


Fig. 1. Influence of the C:N ratio in the inflowing medium on the utilization of a mixture of glucose and methanol (87.8% C₆:12.2% C₁, w/w) by *H. polymorpha* growing in a chemostat at a dilution rate of 0.10 h⁻¹. (a) Residual concentration of NH₄⁺-N (□), glucose (△) and methanol (○). (b) Specific substrate consumption rates [g (g dry wt h)⁻¹] for glucose (q_{C₆}, ▲), methanol (q_{C₁}, ●), NH₄⁺-N (q_N, ■) and total carbon (q_C, ◆). The shaded area indicates the transition growth regime.

Utilization of carbon and nitrogen sources

The residual concentrations of glucose, methanol and NH₄⁺, and the specific rates of substrate consumption, are shown in Fig. 1 as a function of the C:N ratio of the inflowing medium. As can be seen from the residual substrate concentrations in the culture (Fig. 1a, Table 1) there were four different stable physiological states. (1) At low C:N ratios (≤11.32) growth was clearly carbon-limited, i.e., both carbon sources were simultaneously utilized to completion, whereas NH₄⁺ was present in excess. (2) Growth with a medium of 11.32 < C:N ≤ 18.40 resulted in complete utilization of the two carbon sources as well as the nitrogen source. (3) With a further increase of the C:N ratio (18.40 < C:N ≤ 26.73) repression of C₁-metabolism occurred and the cells switched from the utilization of C₁/C₆-mixtures to growth on glucose only. As a result methanol accumulated, whereas the residual concentrations of glucose and NH₄⁺-N were below 2.0 and 0.5 mg l⁻¹, respectively. (4) C:N ratios ≥ 35.04 resulted in classic nitrogen-limited growth. Although more than 99.5% of the glucose supplied was metabolized by the cells at C:N ratios of 35.04 and 40.13 the residual glucose concentrations were significant (3.8 and 9.4 mg l⁻¹, respectively).

The above pattern of substrate utilization is reflected in the specific rates of substrate consumption (Fig. 1b). As long as both carbon sources were utilized to completion, their specific rates of consumption were constant and varied by < ± 5% (q_{C₁} 0.024 ± 0.001 h⁻¹; q_{C₆} 0.170 ± 0.008 h⁻¹; q_C 0.077 ± 0.003 h⁻¹). At a C:N ratio ≥ 40.13 the specific rate of glucose consumption was enhanced by 40–50%, which indicates a change in the physiological state of the cells. At the same time a drop in growth yield from Y_{X/C} = 1.30 ± 0.06 to Y_{X/C} = 0.92 was observed. Whether more carbon was channelled into product forming pathways or into dissimilation was not investigated.

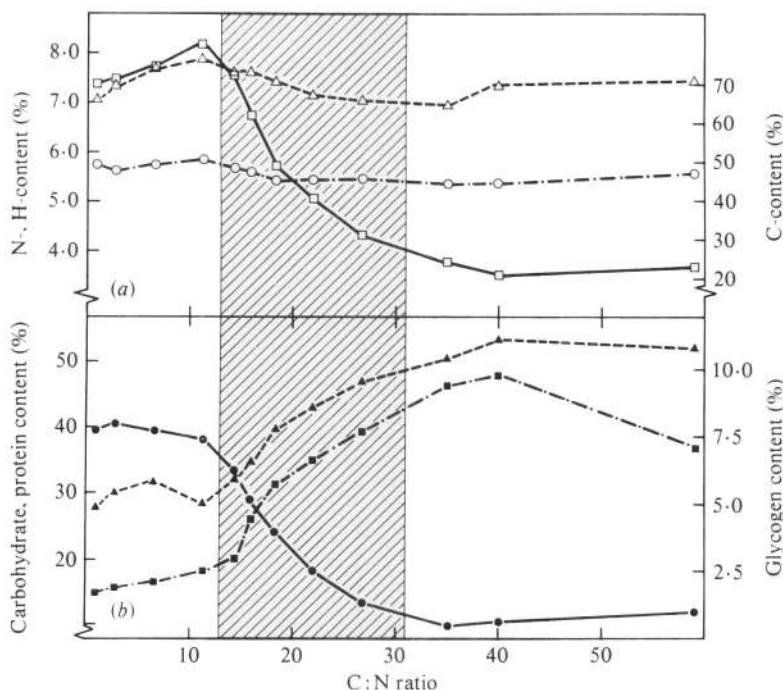


Fig. 2. Elemental and cellular composition of *H. polymorpha* as a function of the C:N ratio in the inflowing medium. Culture conditions were as specified in Fig. 1. (a) Cellular content of carbon (O), hydrogen (Δ) and nitrogen (\square). (b) Cellular content of protein (\bullet), glycogen (\blacksquare) and total carbohydrate (\blacktriangle). The shaded area indicates the transition growth regime.

The specific rate of nitrogen consumption was constant ($6.31 \times 10^{-3} \pm 0.03 \times 10^{-3} \text{ h}^{-1}$) both as long as the nitrogen source was present in excess, and during nitrogen-limited growth with C:N ratios ≤ 35.04 ($q_N 2.75 \times 10^{-3} \pm 0.35 \times 10^{-3} \text{ h}^{-1}$). In between carbon-limitation and nitrogen-limitation, q_N reflected the supply of nitrogen to the cells and decreased as the C:N ratio of the inflowing medium was increased.

Cellular composition

Cells of *H. polymorpha* grown under the conditions described were analysed to determine their elemental composition (C, N, H) and their protein, total carbohydrate and glycogen content (Fig. 2). The hydrogen content showed little variation. The cellular carbon content was slightly higher in cells grown under carbon-limited conditions ($49.1 \pm 1.2\%$) than in cells grown under nitrogen-limited conditions ($45.7 \pm 0.9\%$). The cellular nitrogen content was high (7.5–8.0%) as long as the cells were supplied with a medium with a C:N ratio ≤ 14.46 and decreased gradually with increasing C:N ratios. Growth under nitrogen-limited conditions (C:N ≥ 35.04) resulted in cells with a nitrogen content of approximately 3.7%.

The total carbohydrate, glycogen and protein content (Fig. 2b) followed the expected pattern: low carbohydrate/high protein content during carbon-limited growth and high carbohydrate/low protein content during nitrogen-limited growth. Transition from carbon-limited cell composition to nitrogen-limited cell composition was a gradual process; within the range of $\sim 13 < \text{C:N} < \sim 30$ the cell was able to adjust its composition in response to pertaining environmental conditions. It should be noted that the carbohydrate content assay includes contributions from both DNA and RNA.

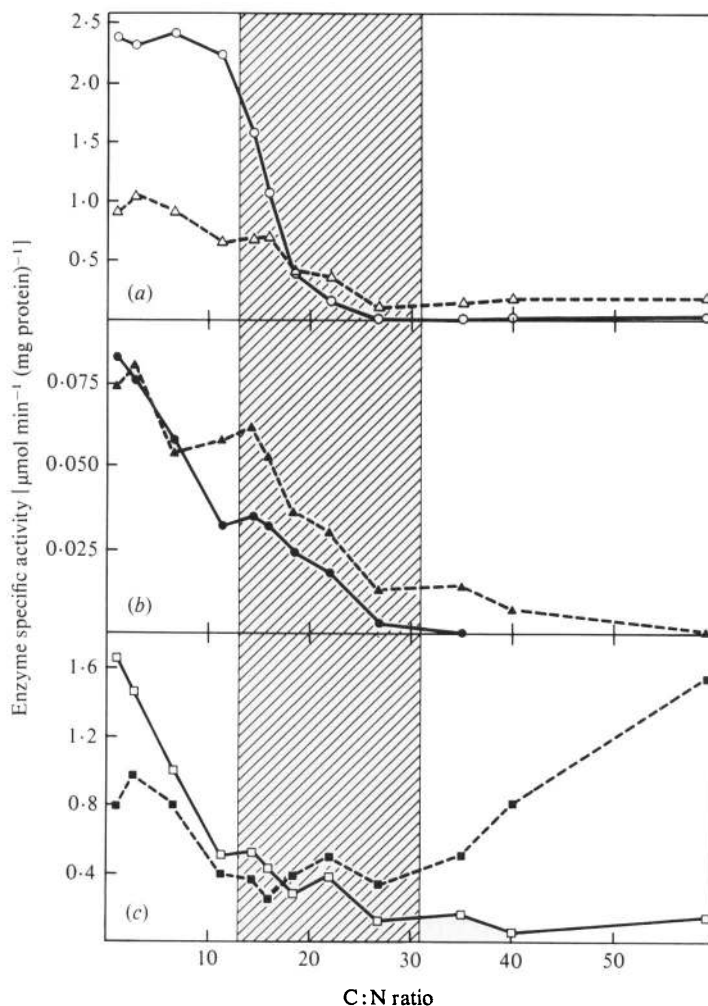


Fig. 3. Enzyme specific activities in cell-free extracts of *H. polymorpha* as a function of the C:N ratio in the inflowing medium. Culture conditions were as specified in Fig. 1. (a) Alcohol oxidase (O), formaldehyde dehydrogenase (Δ); (b) formate dehydrogenase (\bullet), dihydroxyacetone kinase (\blacktriangle); (c) ribulose-5-phosphate epimerase (\blacksquare), ribose-5-phosphate isomerase (\square). The shaded area indicates the transition growth regime.

Specific activities of enzymes

The specific activities of enzymes involved in the metabolism of glucose and methanol and in the assimilation of NH_4^+ are shown in Figs 3 and 4. As expected from the substrate utilization pattern (Fig. 1a) enzymes involved in the metabolism of methanol were synthesized under carbon-limited growth conditions and their synthesis was repressed when nitrogen was the growth-limiting substrate. However, two different regulatory patterns were observed. The peroxisomal enzyme alcohol oxidase (Fig. 3a) was synthesized at a reduced rate when the residual nitrogen concentration in the culture was between 16.1 and 3.5 mg l^{-1} ($11.32 < \text{C:N} < 14.46$). Repression was complete when the C:N ratio in the feed was > 21.95 . This clear onset of repression was not observed for the two methanol dissimilating enzymes formaldehyde and formate dehydrogenase, nor for the C_1 -assimilating enzyme dihydroxyacetone kinase (Fig. 3a, b).

Ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transaldolase and transketolase take part in both the assimilation of C_1 -units from methanol (dihydroxyacetone pathway) and the metabolism of glucose. Except for ribose-5-phosphate isomerase, their specific activity

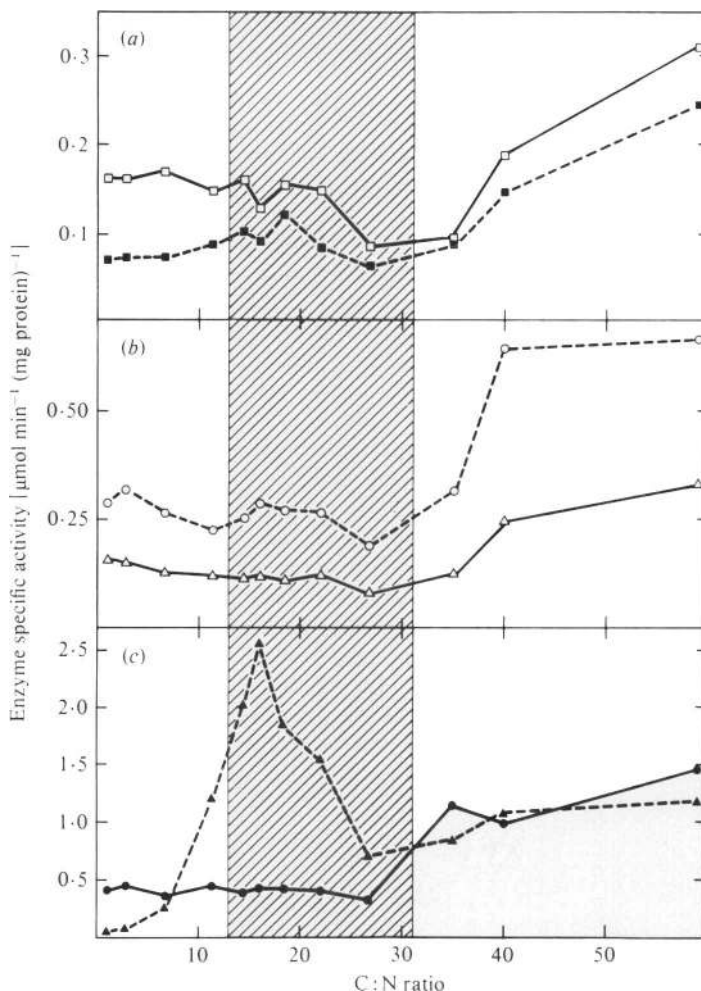


Fig. 4. Enzyme specific activities in cell-free extracts of *H. polymorpha* as a function of the C:N ratio in the inflowing medium. Culture conditions were as specified in Fig. 1. (a) Transaldolase (\square), transketolase (\blacksquare); (b) glucose-6-phosphate dehydrogenase (\circ), 6-phosphogluconate dehydrogenase (\triangle); (c) glucose-6-phosphate isomerase (\bullet), glutamate dehydrogenase (\blacktriangle). The shaded area indicates the transition growth regime.

was either approximately constant or decreased slightly with increasing C:N ratios as long as no residual glucose was detected in the culture (Figs 3c, 4a). When residual glucose was present their specific activity was enhanced two to threefold. Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Fig. 4b) and glucose-6-phosphate isomerase (Fig. 4c), three enzymes which are primarily involved in glucose metabolism, responded similarly. The specific activity of ribose-5-phosphate isomerase showed a very similar pattern to those enzymes involved in methanol metabolism. This indicates the important role of this enzyme in the rearrangement reactions of the dihydroxyacetone cycle of formaldehyde fixation.

In cell-free extracts of *H. polymorpha* the specific activity of glutamate dehydrogenase (GDH), the key enzyme in the assimilation of NH_4^+ in yeasts, increased approximately 50-fold from $0.057 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ to a maximum of $2.575 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ as the C:N ratio of the medium was increased from 1.00 to 15.89. A further increase in the C:N ratio to 30 resulted in a reduction of the specific activity of GDH to approximately $1 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$; growth with C:N ratios > 30 had little further effect on the specific activity.

DISCUSSION

Modes of growth limitation

In our experiment, the classic concept of growth limitation, namely that a linear relationship exists between biomass produced and substrate concentration (compare e.g. Stephenson, 1949; Tempest, 1969), is valid as long as either the nitrogen or the carbon source is clearly present in excess. The cellular composition with respect to total protein and carbohydrates is then not influenced by changes of the medium C:N ratio. However, when media with $12 < C:N < 45$ are fed to the cells, and both the carbon and the nitrogen sources are completely metabolized, the concept of single substrate limitation no longer applies. Within this C:N range, the organism responds to a change in the ratio of the two limiting substrates by altering its cellular composition to match the nutrient intake. However, if it is assumed that, instead of a single substrate, the combination of carbon and nitrogen source, at a fixed C:N ratio, is growth-limiting, the concept of growth limitation within the transition growth regime can still be applied for a fixed growth rate.

Only two examples of double-substrate-limited growth of aerobic microbes have been reported. Hueting & Tempest (1979) studied the pattern of overflow metabolite production by *Klebsiella aerogenes* during the transition from carbon to either potassium or ammonia limitation. Double substrate-limitation was also observed for a culture of *Hyphomicrobium* ZV620 growing with methanol and ammonia as the source of carbon and nitrogen (Gräzer, 1985). The phenomenon is probably widespread, occurring whenever organisms are grown under conditions of substrate limitation in media of different ratios of two substrates. The effect may be predicted from the known growth yields for the individual substrates as our own data illustrate. The concentration of biomass in the culture (x) can be calculated from equation (1), where c_0 and n_0 are the concentrations of carbon and nitrogen in the feed, c and n are the residual concentrations of carbon and nitrogen in the culture, and $Y_{X/C}$ and $Y_{X/N}$ are the growth yields for carbon and nitrogen, respectively, measured under either carbon-limited or nitrogen-limited growth conditions.

$$x = (c_0 - c) Y_{X/C} = (n_0 - n) Y_{X/N} \quad (1)$$

$$c_0/n_0 = Y_{X/N}/Y_{X/C} \quad (2)$$

Because both c and n are zero under double-substrate-limitation, equation (1) can be rearranged to yield the C:N ratio of the inflowing medium (equation 2). In order to estimate the minimum C:N ratio at which the cells switch from carbon-limited to carbon/nitrogen-limited growth $Y_{X/C}$ and $Y_{X/N}$ measured under carbon-limited growth conditions (1.30 and 16.0, respectively) are inserted in equation 2. The critical C:N ratio of 12.3 calculated in this way corresponds closely with the experimentally observed value (Fig. 1a). Using $Y_{X/C}$ and $Y_{X/N}$ measured for a nitrogen-limited culture the same procedure would yield the critical medium C:N ratio for the maximum C:N ratio. Equation (1) indicates that in order to obtain double-substrate-limited growth with substrates S_1 and S_2 , the growth yields Y_{X/S_1} and/or Y_{X/S_2} have to be different under S_1 -limited and S_2 -limited growth conditions. Therefore, the wider the range within which a cell component affected by the substrate(s) studied can vary, the more extended the double-substrate-limited growth regime will be.

Since the cellular composition does also vary with growth rate, and differences are more pronounced in slow growing cells (Herbert, 1976), one can expect the extension of the transition growth regime to become narrower with increasing growth rates.

Cellular and enzymic composition

The generally observed phenotypic response of microbes brought about by the limited availability of the two primary nutrients carbon and nitrogen may be summarized as follows (for reviews see Harder & Dijkhuizen, 1983; Tempest *et al.*, 1983; Harder *et al.*, 1984; Tempest & Neijssel, 1978). Carbon-limited growth results in cells with low carbohydrate and storage polymer and high protein contents. Such cells are usually able to use mixtures of carbon substrates simultaneously due to derepression of many catabolic enzymes. Cells grown under

nitrogen-limitation have a reduced protein content and high carbohydrate and storage polymer contents; surplus carbon is frequently excreted into the medium in the form of an intermediary metabolite.

Two studies with *H. polymorpha* and *Hyphomicrobium* ZV620 (Gräzer, 1985) clearly document the different regulation patterns which can be observed for the individual enzymes as the C:N ratio in the growth medium is gradually increased. In *Hyphomicrobium* ZV620, where NH_4^+ is assimilated either via GDH at high residual NH_4^+ concentrations, or the high affinity enzyme system of glutamine synthetase (GS)/glutamate synthase (GOGAT), an 'all-or-nothing' type of response was observed for the specific activity of GDH. In this bacterium GDH was the main enzyme responsible for the assimilation of NH_4^+ during growth with C:N ratios ≤ 7.1 . However, growth with a C:N ratio < 7.1 resulted in a severe repression of the synthesis of GDH and its specific activity in cell-free extracts was reduced from 0.125 (C:N 7.1) to 0.02 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ (C:N 7.7). For *H. polymorpha* no similar regulatory pattern was observed for any of the enzymes assayed. It was only alcohol oxidase for which a clear onset and relatively stringent repression was found when the residual concentration of NH_4^+ dropped below 16.1 mg l $^{-1}$.

The specific activity pattern observed for GDH in *H. polymorpha* again differed markedly from all the other enzyme patterns. This enzyme is exclusively responsible for the assimilation of NH_4^+ in yeasts (Brown, 1976, 1980). Since yeast GDH has a low affinity for NH_4^+ the cells have to ensure the supply of nitrogen at low environmental NH_4^+ concentrations by increasing the amount of enzyme. *H. polymorpha* also responds to low external concentrations of NH_4^+ by the increased synthesis of this enzyme (Fig. 4c). It is notable that not only at low but also at relatively high NH_4^+ concentrations, at which this substrate is considered to be present in excess, the specific activity of NADP-dependent GDH inversely reflects the NH_4^+ concentration in the culture medium.

In methylotrophic yeasts the synthesis of the peroxisomal enzyme alcohol oxidase is repressed by high concentrations of glucose and various other carbon sources (Veenhuis *et al.*, 1983). Alcohol oxidase activity was lost immediately when a culture of *H. polymorpha* was shifted from carbon-limited to nitrogen-limited growth conditions, although the glucose concentration in the culture was still undetectable (< 2 mg l $^{-1}$) (Egli, 1982). This indicated that the reduced availability of the nitrogen source might also regulate the synthesis of this enzyme. There is strong evidence from the data reported in this paper that nitrogen availability is indeed an important parameter in the regulatory control of alcohol oxidase. Repression started when the extracellular concentration of $\text{NH}_4^+\text{-N}$ was in the range 16.1–3.5 mg l $^{-1}$ and was co-ordinated with a reduction in the protein content of the cells. Increased synthesis of glycogen, on the other hand, started at $\text{NH}_4^+\text{-N}$ concentrations of 3.5–0.5 mg l $^{-1}$ and residual glucose was only detectable at C:N ratios > 35 . Regulation of the synthesis of alcohol oxidase by glucose catabolite repression and by nitrogen would not be surprising because under certain growth conditions this enzyme can account for up to 20% of the total cell protein (van Dijken *et al.*, 1976).

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REFERENCES

- BECKER, J. U. (1978). A method of glycogen determination in whole yeast cells. *Analytical Biochemistry* **86**, 56–64.
- BROWN, C. M. (1976). Nitrogen metabolism in bacteria and fungi. In *Continuous Culture 6: Applications and New Fields*, pp. 170–183. Edited by A. C. R. Dean, D. C. Ellwood, C. G. T. Evans & J. Melling. Chichester: Ellis Horwood.
- BROWN, C. M. (1980). Ammonia assimilation and utilization in bacteria and fungi. In *Microorganisms and Nitrogen Sources*, pp. 511–535. Edited by J. W. Payne. Chichester: John Wiley.
- VAN DIJKEN, J. P., OTTO, R. & HARDER, W. (1976). Growth of *Hansenula polymorpha* in a methanol-limited chemostat. *Archives of Microbiology* **111**, 137–144.

- VAN DIJKEN, J. P., HARDER, W., BEARDSMORE, A. J. & QUAYLE, J. R. (1978). Dihydroxyacetone: an intermediate in the assimilation of methanol by yeasts? *FEMS Microbiology Letters* **4**, 97-102.
- DIJKHUIZEN, L. (1979). *Regulation of autotrophic and heterotrophic metabolism in Pseudomonas oxalaticus OX.1*. PhD thesis, University of Groningen, Holland.
- EGLI, TH. (1982). Regulation of protein synthesis in methylotrophic yeasts: repression of methanol dissimilating enzymes by nitrogen limitation. *Archives of Microbiology* **131**, 95-101.
- EGLI, TH. & FIECHTER, A. (1981). Theoretical analysis of media used in the growth of yeast on methanol. *Journal of General Microbiology* **123**, 365-369.
- EGLI, TH., VAN DIJKEN, J. P., VEENHUIS, M., HARDER, W. & FIECHTER, A. (1980). Methanol metabolism in yeasts: regulation of the synthesis of catabolic enzymes. *Archives of Microbiology* **124**, 115-121.
- EGLI, TH., KÄPPELI, O. & FIECHTER, A. (1982). Regulatory flexibility of methylotrophic yeasts in chemostat cultures: simultaneous assimilation of glucose and methanol at a fixed dilution rate. *Archives of Microbiology* **131**, 1-7.
- EGLI, TH., LINDLEY, N. D. & QUAYLE, J. R. (1983). Regulation of enzyme synthesis and variation of residual methanol concentration during carbon-limited growth of *Kloeckera* sp. 2201 on mixtures of methanol and glucose. *Journal of General Microbiology* **129**, 1269-1281.
- GOTTSCHAL, J. C. (1980). *Mixotrophic growth of Thiobacillus A 2 and its ecological significance*. PhD thesis, University of Groningen, Holland.
- GRÄZER, S. (1985). *Chemostatstudie über die Regulation der ammoniumassimilierenden Enzyme in Hyphomicrobium ZV 620*. Dissertation no. 7790, ETH, Zürich, Switzerland.
- HARDER, W. & DIJKHUIZEN, L. (1983). Physiological responses to nutrient limitation. *Annual Review of Microbiology* **37**, 1-23.
- HARDER, W., DIJKHUIZEN, L. & VELDKAMP, H. (1984). Environmental regulation of microbial metabolism. *Symposia of the Society for General Microbiology* **36**, 51-95.
- HERBERT, D., PHIPPS, P. J. & STRANGE, R. E. (1971). Chemical analysis of microbial cells. *Methods in Microbiology* **5B**, 209-344.
- HERBERT, D. (1976). Stoichiometric aspects of microbial growth. In *Continuous Culture 6: Applications and New Fields*, pp. 1-30. Edited by A. C. R. Dean, D. C. Ellwood, C. G. T. Evans & J. Melling. Chichester: Ellis Horwood.
- HUETING, S. & TEMPEST, D. W. (1979). Influence of the glucose input concentration on the kinetics of metabolite production by *Klebsiella aerogenes* NCTC 418, growing in chemostat culture in potassium- or ammonium-limited environments. *Archives of Microbiology* **123**, 189-194.
- KORNBERG, A. & HORECKER, B. L. (1955). Glucose-6-phosphate dehydrogenase. *Methods in Enzymology* **1**, 323-327.
- LEEGWATER, M. P. M. (1983). *Microbial reactivity: its relevance to growth in natural and artificial environments*. PhD thesis, University of Amsterdam, Holland.
- SCHNEIDER, D. (1976). Determination of ammonia and Kjeldahl nitrogen by indophenol method. *Water Research* **10**, 31-36.
- STEPHENSON, M. (1949). Growth and nutrition. In *Bacterial Metabolism*, 3rd edn, pp. 159-178. London: Longman, Green.
- TCHOLA, O. & HORECKER, B. L. (1966). Transaldolase. *Methods in Enzymology* **9**, 499-505.
- TEMPEST, D. W. (1969). Quantitative relationships between inorganic cations and anionic polymers in growing bacteria. *Symposia of the Society for General Microbiology* **19**, 87-111.
- TEMPEST, D. W. & NEIJSSSEL, O. M. (1978). Ecophysiological aspects of microbial growth in aerobic nutrient-limited environments. *Advances in Microbial Ecology* **2**, 105-153.
- TEMPEST, D. W., NEIJSSSEL, O. M. & ZEVENBOOM, W. (1983). Properties and performance of microorganisms in laboratory culture: their relevance to growth in natural ecosystems. *Symposia of the Society for General Microbiology* **34**, 119-152.
- VEENHUIS, M., VAN DIJKEN, J. P. & HARDER, W. (1983). The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. *Advances in Microbial Physiology* **24**, 1-82.
- WAITES, M. J. & QUAYLE, J. R. (1981). The interrelation between transketolase and dihydroxyacetone synthase activities in the methylotrophic yeast *Candida boidinii*. *Journal of General Microbiology* **124**, 309-316.
- WIMPENNY, J. W. T., LOVITT, R. W. & COOMBS, J. P. (1983). Laboratory model systems for the investigation of spatially and temporally organised microbial ecosystems. *Symposia of the Society for General Microbiology* **34**, 67-117.
- WOOD, T. (1970). Spectrophotometric assay for D-ribose-5-phosphate ketol-isomerase and for D-ribose-5-phosphate 3-epimerase. *Analytical Biochemistry* **33**, 297-306.