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Dual limitations: Kinetic and stoichiometric analysis[1]

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Abstract

Although a rigorous definition of growth-limiting substrate relies on a kinetic analysis, a simple stoichiometric analysis based on the calculation of the conversion is more applicable. Analysis of single and dual limitations is based on a simulated example for a substrate taken-up by facilitated diffusion (e.g. glucose) or by free diffusion (e.g. ethanol) and shows that a kinetic single or dual limitation can usually be predicted by comparing the conversion of two nutrients with the expected stoichiometry. Stoichiometric analysis of dual limitations is applied to experimental data for *Kluyveromyces marxianus* (for glucose and oxygen) and *Saccharomyces cerevisiae* (for glucose and ammonium). © 1998 Elsevier Science B.V.

Keywords: Decoupling; Free diffusion; Kinetic limitation; Limiting substrate; Multiple-limited growth; *Saccharomyces cerevisiae*; Stoichiometric limitation; Uptake kinetics

1. Introduction

What is limiting growth? When dealing with complex phenomena such as dual and multiple limitations, the very term "*limiting substrate*" ought to be clearly defined. The most easily understood concept concerns the growth-limiting substrate. In a batch culture, the growth-limiting substrate is the one that brings the growth to halt or significantly reduces it when it is completely exhausted. Growth will resume, if an additional amount of this substrate is added. The specific growth rate is a function of the limiting substrate in continuous culture. Adding an additional amount of this substrate in the form of a pulse or into the medium tank will increase the biomass formation rate, either transient or at steady state.

The counterpart of growth-limiting substrates are substrates whose residual concentration has absolutely no effect on the kinetics or growth stoichiometry of the growing culture. Adding more of a non-limiting substrate has no effect on the metabolic activity. The uptake rate of non-limiting nutrients is proportional to the limiting substrate uptake rate. An example is oxygen in chemostat cultures of *Saccharomyces cerevisiae* growing aerobically below the critical dilution rate. As long as the oxygen tension is kept >10%, varying it will have no effect on the culture [1]. The nature of the limiting substrate

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can be as follows: electron donor; electron acceptor; or biosynthetic nutrient. Typical examples are glucose, oxygen, and ammonium for the three respective categories. In the present paper, the limitation will not be discussed in terms of the nature of the substrate but in terms of detection of a potential limitation.

In chemostat cultures, dual growth limitations occur. If a continuous yeast culture growing on glucose is fed with an insufficient amount of oxygen for complete oxidative, i.e. respiratory metabolism, a mixed redox metabolism results and part of the glucose is fermented to ethanol [2]. Any increase in either glucose feeding rate or oxygen supply rate will result in an increase of the biomass formation rate and, consequently, both substrates must be regarded as kinetically growth limiting.

The affinity of the uptake system for the limiting substrate, or the permeability coefficient of solutes, is usually high and, consequently, the residual concentration of the limiting substrate is very low at moderate dilution rate. Thus, it may be difficult to determine the kinetic parameters accurately. In that case, the substrate uptake rate can be estimated from the supply rate. In this way, substrates which are practically completely consumed and which affect the metabolic activity are considered stoichiometrically limiting, and no attempt is made to determine the uptake kinetics. Occurrence of a limitation by a second nutrient can be assessed by stoichiometric analysis by comparing the relative supply of two nutrients to the yield observed under single-energy limitation. For example, in dual limitations by glucose and ammonium, ammonium limits biomass formation but an additional amount of glucose can be degraded by uncoupled catabolism. This simple approach is exemplified with simulations and with experimental data for *S. cerevisiae* and *Kluyveromyces marxianus* considering glucose and oxygen or glucose and ammonium as limiting substrates.

As explained in detail in the present paper, the uptake rate and the metabolic activity clearly depend on the residual concentration of the kinetically limiting substrate(s) in the culture medium. On the other hand, kinetically limiting substrates are not always completely consumed and, therefore, do not necessarily qualify as stoichiometrically limiting, for example at growth rate close to the washout.

2. Single limitation by a substrate taken-up by free diffusion

Let us consider growth-only limited by the energy source (denoted S1) as a reference case. For glucose, which is taken-up by facilitated diffusion, the uptake kinetics can be represented using the Monod equation:

$$q_G \equiv q_G^{max} = \frac{c_G}{c_G + K_G} \quad (1)$$

where q_G^{max} is the maximum specific uptake rate and K_G the affinity constant for glucose. The well-known profile of q_G as a function of c_G is depicted in Fig. 1 and illustrates that the glucose uptake rate is determined by the residual glucose concentration. For example, a pulse of glucose will result in a response of the microorganism. The consumption rate of any non-limiting nutrient S2 is proportional to the glucose uptake rate:

$$q_{S2} = q_G Y_{S2/G} \quad (2)$$

and is kinetically independent of the residual concentration of S2. A pulse of S2 will not affect the growth rate of the microorganisms and the additional amount of S2 added is entirely washed out. The residual concentration of S2 can be calculated by mass balance stating that conversion of S2 is proportional to the conversion of glucose:

$$c_{S2} = c_{S2,in} - (c_{G,in} - c_G) Y_{S2/G} \quad (3)$$

The estimation of the kinetic parameters of the Eq. (1) is often difficult. Although q_G^{max} can be determined in batch experiments or in continuous cultures growing

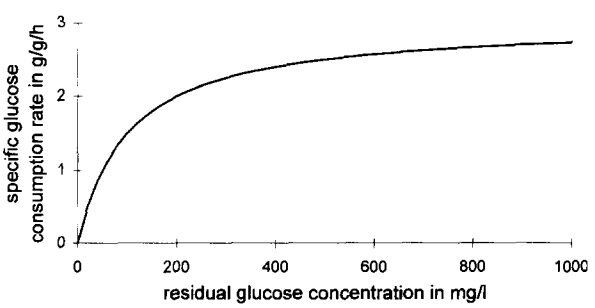

Fig. 1. Continuous culture limited by glucose according to Monod equation uptake (K_G=100 mg l^{-1}, q_G^{max} = 3 g g^{-1} h^{-1}). Relation between the residual concentration of glucose and the specific glucose uptake rate.

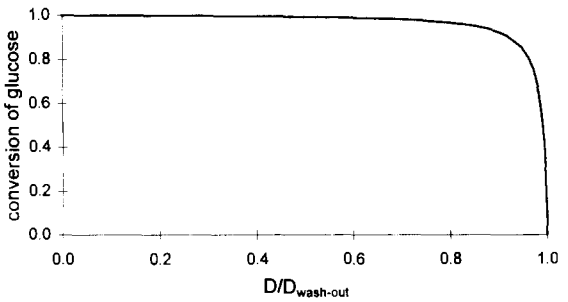

Fig. 2. Conversion of glucose as a function of $D/D_{washout}$ Conversion is higher than 95% for D lower than 84% of the washout dilution rate.

close to washout, the model is sensitive to K_G at low residual concentration of glucose and, therefore, the estimation of K_G is very sensitive to analytical errors. Although a limitation can only be due to a kinetic limitation at the cellular level, the residual concentration of the limiting substrates is usually low as compared to the incoming substrate concentration (when $c_{G,in}$ is much larger than the expected K_G, and for a dilution rate far below the washout dilution rate). For example, the conversion of glucose calculated from Fig. 1 for an inlet concentration of 10 g l^{-1} is depicted in Fig. 2 as a function of $D/D_{washout}$: the conversion of glucose is higher than 95% for a dilution rate below 84% of the washout dilution rate. Therefore, the conversion of a limiting substrate S1 can be considered as almost complete for $c_{S1,in} \gg K_{S1}$, and $D<0.8D_{washout}$. By comparison, the conversion of a nutrient supplied in excess is not complete even at very low dilution rates. This shows that a limitation can often be predicted from the inspection of the conversion of the nutrients. The advantage of this quantitative approach is its simplicity since it does not rely on the determination of kinetic parameters that may be difficult to estimate.

3. Uptake kinetics for free diffusion

The relation between the residual concentration of the nutrient and the specific uptake rate is somewhat different from Eq. (1) for nutrients taken up by free diffusion since the driving force depends on both the intracellular concentration and the extracellular concentration of substrate. Let us consider the free diffusion of ethanol defined by:

$$q_E = P_E \alpha (c_E - c_{E,intra}) \quad (4)$$

where P_E is the permeability coefficient of the cell membrane for ethanol (1.4×10^{-6} m s^{-1}, [3]), α the specific area of the cells (7.5 m^2 g^{-1}, assuming spherical cells of 4 μm diameter, a density of the cells of unity, and a relative water content of 80%), c_E the extracellular ethanol concentration and $c_{E,intra}$ the intracellular ethanol concentration. The diffusion rate of ethanol is depicted in Figs. 3(A) and 4(A) as a function of $c_E - c_{E,intra}$ on the left x-axis. The diffusion rate of ethanol (Eq. (4)) must equal the intracellular conversion rate (Eq. (5)) which can be described by a Monod saturation kinetic expression with respect to the intracellular ethanol concentration:

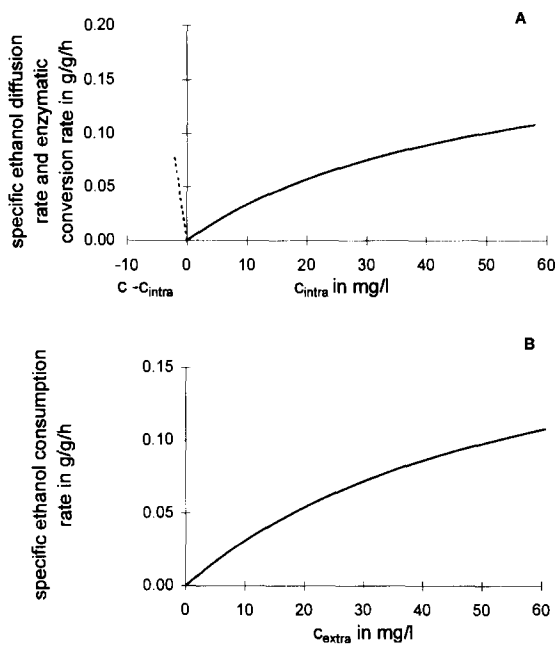

Fig. 3. Specific ethanol consumption rate as intracellular enzymatic conversion is the most limiting step (Φ_E =0.1). (A) Specific rate of enzymatic conversion (faint line) as a function of the intracellular concentration (right x-axis), and ethanol diffusion rate (dotted line) as a function of the ethanol concentration gradient (left x-axis). The extracellular ethanol concentration corresponds to the horizontal distance between the two curves and is mainly determined by the kinetics of intracellular conversion. (B) Plot of the specific ethanol consumption rate as a function of the residual ethanol concentration in the broth (calculated from Fig. 3(A)), which is very similar to a Monod profile (because $c_E \approx c_{E,intra}$).

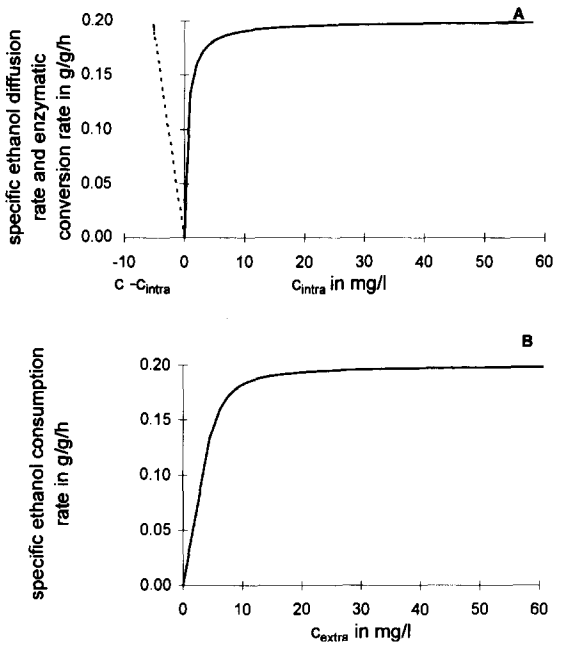

Fig. 4. Specific ethanol consumption rate as transfer by free diffusion is the most limiting step (Φ_E=10). (A) Specific rate of enzymatic conversion (faint line) as a function of the intracellular concentration, and ethanol diffusion rate (dotted line) as a function of the ethanol concentration gradient (see legend of Fig. 3). The extracellular ethanol concentration corresponds to the horizontal distance between the two curves and is mainly determined by the kinetics of free diffusion. (B) Plot of the specific ethanol consumption rate as a function of the residual ethanol concentration in the broth, which is very different to a Monod profile at low residual concentration (because $c_E \gg c_{E,intra}$).

$$q_E = q_E^{max} \frac{c_{E,intra}}{c_{E,intra} + K_E} \quad (5)$$

The specific enzymatic conversion rate of ethanol is plotted as a function of the intracellular ethanol concentration in Fig. 3(A) and 4(A) on the right x-axis. Considering Eqs. (4) and (5), one can calculate the specific ethanol conversion rate q_E as a function of the extracellular concentration of ethanol. On Fig. 3(A) and 4(A), the extracellular ethanol concentration is represented by the horizontal distance between the two curves at a given q_E since $c_E=(c_E-c_{E,intra})-c_{E,intra}$. The plot of the specific ethanol consumption rate as a function of the extracellular ethanol concentration is shown in Fig. 3(B) and 4(B) for different values of the parameter K_E (50 and 0.5 mg l^{-1}, respectively).

Growth is always limited by the enzymatic conversion of ethanol at high extracellular residual concentrations since the enzymatic machinery reaches a saturation limit. The relative importance of the reaction rate, as compared to the transfer rate at low residual ethanol concentrations, can be assessed by comparing the initial slopes of the two curves (for Eq. (4), q_E^{max}/K_E, and for Eq. (5), $P_E\ \alpha$) by analogy with the Thiele modulus calculation:

$$\Phi_E = \frac{q_E^{max}}{K_E P_E \alpha} \quad (6)$$

for $\Phi_E \ll 1$, ethanol uptake rate is mainly limited by the intracellular enzymatic conversion of ethanol, for $\Phi_E \gg 1$, the ethanol uptake rate is mainly limited by the transfer through the cell membrane. Fig. 3 shows a simulation for Φ_E=0.1 ($P_E\ \alpha=3.78\times10^{-2}$ l mg^{-1} h^{-1}, q_E^{max} = 0.2 g $g^{-1}h^{-1}$ and K_E=50 mg l^{-1}) and the ethanol consumption rate is mainly controlled by the rate of enzymatic conversion. In Fig. 4, Φ_E=10 (simulated with K_E=0.5 mg l^{-1}) and the transfer by free diffusion is the most limiting step at low residual ethanol concentrations.

4. Prediction of possible limitation from stoichiometric analysis

If the conversion of the energy source S1 is considered to be (almost) complete, the minimum supply rate of S2 required to fully convert S1 according to the original stoichiometry (S1-limitation) is estimated from Eq. (2). For a liquid substrate S2 (e.g. ammonium), the minimum inlet concentration, assuming a complete conversion of S1 and S2, is given as follows:

$$c_{S2,in,min} = c_{S1,in} Y_{S2/S1} \quad (7)$$

For a substrate supplied to the reactor in the gaseous phase and transferred to the liquid phase (oxygen for example), the minimum equilibrium concentration is,

$$c^*_{S2,min} = Y_{S2/S1} \frac{F c_{S1,in}}{k_{liq} a V} \quad (8)$$

where F is the liquid inlet flow rate, $k_{liq}a$ the transfer coefficient and V the volume of liquid phase [4,5].

Using this simple approach, it is relatively easy to assess if a second nutrient may be limiting. In practice, the residual concentration of S2 will not drop exactly

to zero, but certainly to a very low value corresponding to the kinetic limitation as will be discussed later.

The metabolic response of the microorganisms to a decreasing supply in oxygen or nitrogen source depends on the ability of the cells to use one substrate (glucose) independently of another (O_2, nitrogen source). If only one overall reaction is possible, growth can only be limited by one substrate at a time; if growth reaction can deviate from the original stoichiometry, a dual limitation is possible by kinetics and possibly by stoichiometry.

5. Change of kinetically limiting substrate

Let us consider the case where the consumption rates of S1 and S2 are always proportional, whatever the conditions. Such a case corresponds to growth limited by ethanol as energy source or oxygen as electron acceptor for *S. cerevisiae*, or aerobic growth on glucose for obligate aerobes. Due to the metabolism of *S. cerevisiae*, ethanol metabolism is absolutely dependent on oxygen consumption. For simplicity, let us assume that both uptake kinetics of S1 and S2 follow a Monod equation depending on the respective extracellular concentrations c_{S1} and c_{S2}.

The expression of the specific uptake rate of S1 and S2 are listed in Table 1 for the case where growth is limited by two complementary substrates S1 and S2. Growth stoichiometry remains the same in both cases. The uptake rate of the limiting nutrient follows a Monod kinetics, whereas the uptake rate of the non-limiting nutrient is determined by the growth stoichiometry.

In the case of two non-substitutable nutrients, growth is limited by the more stringent uptake rate [6]. If we assume that growth is limited by S1, the residual concentration of S2 can be calculated by mass balance using Eq. (3) as shown in Table 1 and is depicted in Fig. 5. The specific uptake rate of S2 (which is not growth-limiting) potentially allowed by the Monod uptake kinetics calculated with S2 is higher than the requirements to convert S1 calculated as $q_{S1}\ Y_{S2/S1}$.

The stoichiometric analysis based on the estimation of conversion rates predicted the minimum inlet con-

Table 1
Uptake kinetics for two complementary substrates S1 and S2 (e.g. ethanol and oxygen when transfer is not limiting)

Specific uptake rate	Growth limited by S1	Growth limited by S2
$q_{S1}=$	$q_{S1}^{max}\frac{c_{S1}}{c_{S1}+K_{S1}}$	$q_{S2}/Y_{S2/S1} < q_{S1}^{max}\frac{c_{S1}}{c_{S1}+K_{S1}}$
$q_{S2}=$	$q_{S1}Y_{S2/S1} < q_{S2}^{max}\frac{c_{S2}}{c_{S2}+K_{S2}}$	$q_{S2}^{max}\frac{c_{S2}}{c_{S2}+K_{S2}}$

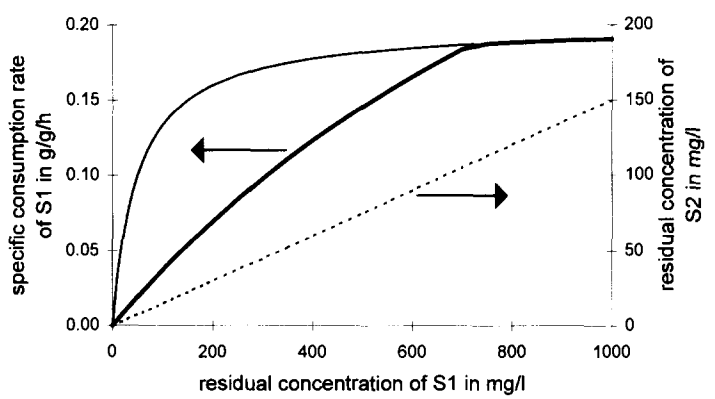

Fig. 5. Specific uptake rate of S1 according to the Monod kinetic (faint line) and residual S2 concentration (dotted line) for a kinetic limitation by S1 or S2 as a function of S1. The dotted line represents the residual concentration of S2 using Eq. (3) for the conversion of S1. Combined with Fig. 6, the culture switches from S2-limiting conditions to S1-limiting conditions at S1=650 mg l^{-1}. The bold line indicates the effective specific consumption rate of S1.

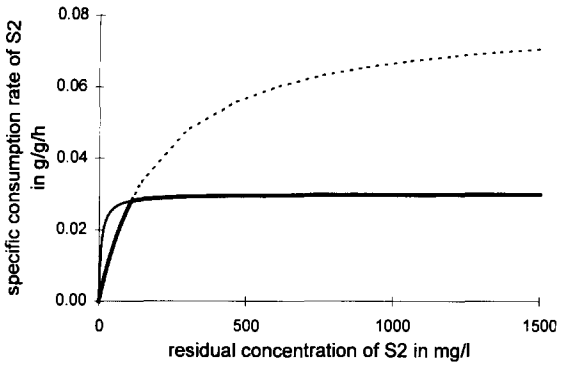

Fig. 6. Specific uptake rate of S2 according to a Monod kinetic (dotted line) and specific consumption rate of S2 required to convert the specific uptake rate of S1 according to Monod. The effective consumption rate of S2 (bold line) corresponds to the lowest of the two curves and shows the change from a single limitation by S2 to single limitation by S1.

centration corresponding to the full conversion of S2. However, uptake rate of S2 (according to Monod kinetic) may be determined by the low residual concentration of S2 when the supply of S2 is close to the minimum requirements to convert S1. The predicted stoichiometric limitation by S2 is now a kinetic limitation by S2. In that case, the specific consumption rate of S2 is kinetically determined by the residual concentration of S2, and S1 is no longer kinetically limiting (even if its residual concentration may be low as shown in Fig. 5). The specific consumption rate of S1 becomes independent of the residual concentration of S1 and is determined by the specific uptake rate of S2 as listed in Table 1. It is seen from Fig. 6 that for a residual concentration of S2 below ca. 97.5 mg l^{-1} (corresponding to S1 ca. 650 mg l^{-1}), the kinetics of uptake of S2 becomes more limiting than the possible uptake rate of S1. Thus, the culture changes from a limitation by the residual concentration of S1 to a limitation by the residual concentration of S2. The bold line in Figs. 5 and 6 correspond to the actual limitation by S2 at low substrate concentration and by S1 at higher residual concentrations.

In conclusion, growth is always single-limited by S1 or by S2, and the singular point corresponding to the change in limitation can be determined by solving $q_{S2}=q_{S1}\ Y_{S2/S1}$ with both q_{S1} and q_{S2} determined by the respective Monod kinetics (Table 1).

6. Dual limitation: stoichiometric analysis

If the growth stoichiometry can change depending upon the environmental conditions, the consumption rates of S1 and S2 are no longer in the proportion of the original stoichiometry and, consequently, the uptake rate of both S1 and S2 can be kinetically limited. This case has been extensively studied for glucose and oxygen with yeast, where the metabolism can be forced to change from purely oxidative to mixed oxido-reductive [2,5]. Another case concerns the growth of dual limitation by glucose and ammonium. In that case, the yield of nitrogen on glucose can decrease by biomass composition change, decoupling or ethanol formation.

Bae and Rittmann have proposed to model the specific growth rate with a double multiplicative Monod kinetic function of both S1 and S2 residual concentrations [7]:

$$q_X = q_X^{max} \frac{c_{S1}}{c_{S1} + K_{S1}} \frac{c_{S2}}{c_{S2} + K_{S2}} \tag{9}$$

Although difficult to verify experimentally, this model is not satisfactory since there is no unique solution for S1 and S2 at a given specific growth rate (the same q_X can be obtained for S1 high and S2 low or conversely).

Dual limitation by glucose and oxygen, or by glucose and ammonium, can be assessed by inspection of the conversion of the two substrates. Glucose residual concentration in continuous cultures of *K. marxianus* is below 10 mg l^{-1} as long as the dilution rate is not close to the washout dilution rate, and it is therefore tricky to determine accurately the kinetic parameters of the uptake system. As the oxygen supply to the continuous culture was decreased, the dissolved oxygen concentration decreased until it was no longer detectable [2]. Under these conditions, both glucose and oxygen conversions were almost equal to unity, and the culture could be considered as dually limited by glucose and oxygen. This conclusion can only be drawn from the stoichiometric analysis since residual concentration cannot be accurately determined to perform a kinetic analysis. Fig. 7 shows that the yield of O_2 on glucose is equal to the relative supply of oxygen on glucose as long as oxygen is limiting (O_2 transfer/glucose consumption < 0.39 mol per C-mol, corresponding to the yield of oxygen on

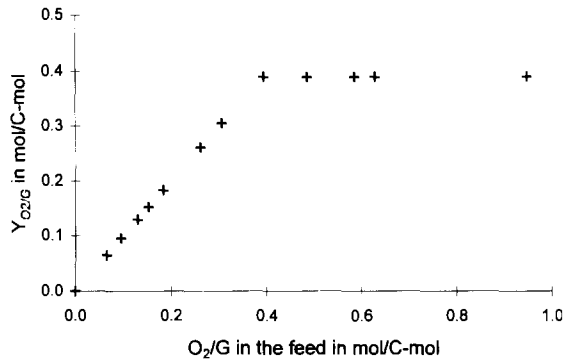

Fig. 7. Oxygen yield on glucose as a function of the oxygen transfer rate to glucose supply rate in the feed for a continuous culture of *K. marxianus* (D=0.20 h^{-1}) [2].

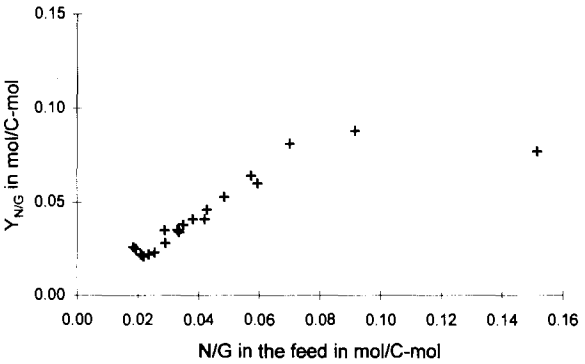

Fig. 9. Nitrogen yield on glucose as a function of the ammonium supply to glucose supply in the feed for a continuous culture of *S. cerevisiae* for various dilution rates [8].

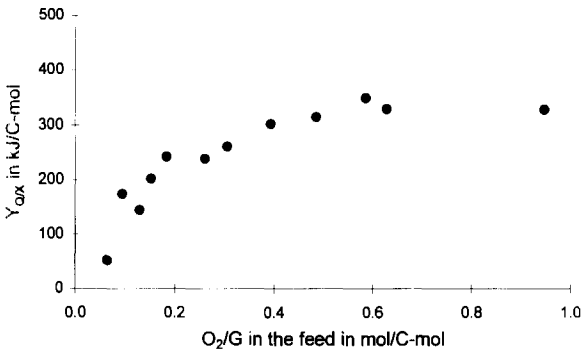

Fig. 8. Heat yield on biomass as a function of the oxygen transfer rate to glucose supply rate in the feed for a continuous culture of *K. marxianus* (D=0.20 h^{-1}) [2].

glucose under single limitation by glucose) since both glucose and oxygen conversion are total. A dual limitation is possible because the metabolism can gradually adapt from purely aerobic to purely anaerobic conditions. In addition, since the heat yield on biomass is significantly lower for the anaerobic metabolism than for the aerobic metabolism, the heat yield on biomass depicted on Fig. 8 reflects the change from aerobic to mixed oxido-reductive conditions as the oxygen supply is decreased at constant glucose supply.

Using a similar approach, the ammonium supply of a continuous cultures of *S. cerevisiae* grown on glucose can be decreased until it becomes limiting [8]. To asses the dual limitation, the nitrogen yield on glucose is compared to the ratio of the ammonium to glucose concentration in the feed as shown in Fig. 9. Three domains exist:

1. For a higher than 0.08 mol per C-mol ammonium-to-glucose ratio in the feed, the culture is single-limited by glucose. Residual ammonium concentration is significant, and the yield of nitrogen on glucose corresponds to the glucose-limited value.
2. For an ammonium-to-glucose ratio in the feed comprising between 0.08 and 0.02 mol per C-mol, the residual concentration of both glucose and ammonium are low or not detectable. The conversion of both nutrients is very close to one, and the culture can be considered as stoichiometrically dually limited. The measured molar yield of nitrogen on glucose was equal to the molar ratio of ammonium-to-glucose in the feed. The metabolism changed: the nitrogen content of biomass decreased [8] due to higher storage carbohydrates synthesis, catabolic decoupling occurs (higher glucose degradation by catabolism [9]), and ethanol may be produced.
3. For a lower than 0.02 mol per C-mol ammonium-to-glucose ratio in the feed, glucose accumulated in the reactor so that glucose conversion was below one. However, the culture was not single-limited by ammonium since, at a given ammonium uptake rate, the uptake rate of glucose was influenced by the residual concentration of glucose, which defined the kinetic dependence of q_G on c_G. Biomass formation was limited by ammonium, whereas catabolism was limited by both ammo-

nium and glucose [10]. Although the residual concentration does not correspond to the level expected from a Monod kinetic, the growth reaction was kinetically limited by both glucose and ammonium. This shows that, under severe ammonium limitation, glucose uptake depends upon the residual glucose concentration but no longer follows a Monod kinetics.

7. Conclusions

Growth limiting conditions are defined by a kinetic dependence of the specific uptake rate of a nutrient on the residual concentration of this species. However, this relation may be difficult to establish due to the usually low residual concentration of a limiting substrate. Therefore, a stoichiometric analysis based on the calculation of conversion rates (up to moderate dilution rate and for an inlet concentration higher than the expected saturation constant) is easier and gives a very good indication of the possible occurrence of a kinetic limitation [10]. In practice, a substrate can be considered as kinetically limiting if its conversion is very close to unity (for the following conditions: $D < 0.8 D_{\text{washout}}$ and for an inlet concentration higher than the expected saturation constant) and if its consumption rate is not influenced when the residual concentration is increased (for example, by a pulse, or by changing the feed medium composition). Conversely, even if the residual concentration of a nutrient is high (and, thus, its conversion lower than one), it must be checked that the uptake rate of this nutrient is not affected by a change of the inlet concentration when the dilution rate and the supply rate of other nutrients are kept constant.

8. Nomenclature

α	specific area of the cells, m^2
Φ_E	ratio between enzymatic conversion and free diffusion at low ethanol residual concentration
c_i	concentration of species i, $g\,l^{-1}$
D	dilution rate, h^{-1}
D_{washout}	dilution rate at washout, h^{-1}
F	liquid inlet flow rate, $l\,h^{-1}$
K_i	affinity constant for species i, $g\,l^{-1}$
$k_{\text{liq}}a$	transfer coefficient, h^{-1}
P_E	permeability coefficient of the cell membrane for ethanol, $m\,s^{-1}$
q_i	specific uptake rate of i, $g\,g^{-1}\,h^{-1}$
$q_i^{\max}$	maximum specific uptake rate of i, $g\,g^{-1}\,h^{-1}$
V	volume of liquid phase, l
$Y_{j/i}$	molar yield of j on i, (C)-mol (C)-mol^{-1}

9. Subscripts

in	inlet
extra	extracellular
intra	intracellular
min	minimum

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