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Calorimetry of dual limitations in yeast cultures ☆

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Abstract

Chemostat cultures of one aerobic fermenting yeast (*Saccharomyces cerevisiae*) and one aerobic respiring yeast (*Kluyveromyces fragilis*) have been grown under dual C + N limitation in an isothermal reaction calorimeter. The dual limitations resulted in uncoupled oxidation of part of the glucose, which enabled the culture to adapt the ratio of nitrogen to carbon consumption rates precisely to the N/C ratio that was fed into the calorimeter. This conclusion has been reached based on the calorimetric measurements, which reflect uncoupled respiration conspicuously as abnormal heat yields or ratios of heat release per biomass grown.

Keywords: Carbon limitation; Metabolism; Nitrogen limitation; Yeast

List of symbols

C_N	concentration of nitrogen source ($(NH_4)_2SO_4$)/mol l^{-1}
C_{N0}	initial concentration of nitrogen source in the combined feeds/mol $^{-1}$
q_s	specific glucose uptake rate/Cmol h^{-1} $Cmol^{-1}$
q_N	specific nitrogen uptake rate/mol h^{-1} $Cmol^{-1}$

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q_O specific oxygen uptake rate/mmol h^{-1} g^{-1}
S glucose concentration/Cmol l^{-1}
S_0 initial glucose concentration in the combined feeds/Cmol $^{-1}$
x_3 atomic coefficient for nitrogen in the Cmolar elemental formula of biomass ($CH_{x_1}O_{x_2}N_{x_3}$)
$Y'^{ox}_{N/S}$ stoichiometric coefficient for nitrogen uptake (ratio of nitrogen source to glucose uptake rates) for fully oxidative metabolism/mol $Cmol^{-1}$
$Y'_{N/S}$ stoichiometric coefficient for nitrogen uptake for any metabolism/mol $Cmol^{-1}$
$Y'_{P/S}$ stoichiometric coefficient of ethanol production, or Cmolar ethanol yield/Cmol $Cmol^{-1}$
$Y'_{X/S}$ stoichiometric coefficient for growth or Cmolar biomass yield/Cmol $Cmol^{-1}$
$Y'_{Q/X}$ heat yield, or experimental enthalpy change of growth reaction per Cmol of biomass grown/kJ $Cmol^{-1}$

1. Introduction

Studying limitations in microbial culture is of high pertinence to biotechnology because limitation is one of the most powerful tools to force such cultures to perform in a predetermined way. As shown in Fig. 1, bioreactors are normally used as a converter. A certain mix of substrates or starting materials is fed to the culture which converts them into a number of products, including the actual target product. The microbial culture will transform the input according to particular kinetics and stoichiometry, determined by its metabolism. The latter depends in turn on the limitation under which the culture is grown. It is therefore often possible to shift the metabolism into the optimal state for synthesis of the target product by applying appropriate limitations.

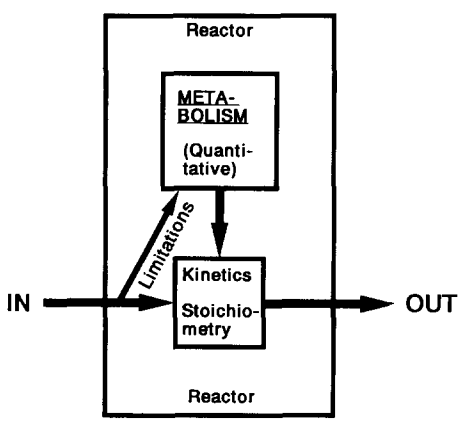

Fig. 1. Schematic representation of influence of limitations on metabolism, and on conversion stoichiometry and kinetics in microbial cultures.

In studying the effect of limitations on metabolism and on conversion kinetics it is of prime importance to make use of tools enabling detection of shifts in metabolism from outside the bioreactor. Calorimetry has been shown to be a very useful method in this respect because it yields a continuous quantitative reading of metabolic activity [1]. This has already been shown to be true for the detection and assessment of dual carbon and oxygen limitations [2,3] together with those limitations due to restricted respiratory capacity [4]. The purpose of this paper is to demonstrate the use of calorimetry in studying C + N limitations in yeasts.

As in earlier studies, this work will pertain to one example of a so called "aerobic respiring" yeast (in the notation of Alexander and Jeffries [5]) and one example of an "aerobic fermenting" yeast. They differ by the lack and the presence, respectively, of a respiratory bottleneck and are therefore expected to respond differently to dual carbon and nitrogen limitations.

The reason why dual limitations are examined lies in the fact that such studies are usually carried out in chemostat cultures. These are always limited by a least one factor, usually the carbon and energy substrate (glucose in our case). The aim of this paper is therefore to investigate whether such yeast cultures can also undergo dual limitations, and if this is the case, how they affect the metabolism, and how the resulting shift in metabolism manifest itself in the heat release rate.

2. Material and method

Kluyveromyces fragilis NRRL 1109 and *Saccharomyces cerevisiae* CBS 426 were grown in a standard medium containing 20 g l^{-1} glucose. The other medium components are described in detail elsewhere [6,7].

The nitrogen source to carbon source feed ratio was varied in the chemostat experiments by a set-up depicted in Fig. 2. The growth medium in the main feed

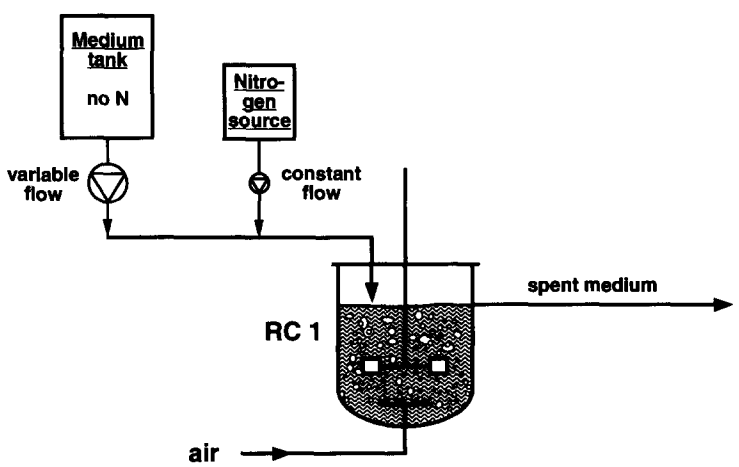

Fig. 2. Experimental set-up used to create dual N + C limitations in continuous cultures.

tank contained no nitrogen and was fed into the continuous fermenter at a variable flow rate. The auxiliary feed tank contained a $(NH_4)_2SO_4$ solution which was pumped into the bioreactor at a constant rate. Increasing the feed rate from the main medium tank therefore increased the overall effective dilution rate but diminished the ratio of nitrogen to carbon feed rates. For both yeasts the whole range of dilution rates was scanned twice with $(NH_4)_2SO_4$ concentrations of 13.4 g l^{-1} and of 4.6 g l^{-1}, respectively, in the auxiliary feed tank.

The continuous bioreactor consisted of a 2 l isothermal reaction calorimeter (RC-1, Mettler–Toledo, Greifensee, Switzerland) modified for biological work [8]. Temperature was maintained constant at 30°C, by the calorimeter, and pH at 4 by automatic addition of 2 M NaOH.

The RC-1 permitted a continuous readout of the heat released by the culture in Watts. Details of the experimental set-up and the calorimetric measuring technique have been reported elsewhere [2,6,7].

3. The nature of dual limitations in continuous cultures

If a yeast culture grows fully aerobically in continuous culture with no other limitation than by glucose, it does so according to a defined stoichiometry reflecting fully respiratory (or oxidative) metabolism. Therefore the specific or volumetric nitrogen uptake rate should be tightly coupled to the glucose uptake rate. If these are measured as a function of dilution rate and plotted against one another a straight line should result, as shown in Fig. 3(a). The slope of this line is equal to the Cmolar stoichiometric coefficient of nitrogen consumption with respect to glucose consumption ($Y'^{ox}_{N/S}$).

In chemostats the glucose uptake rate (q_s) can be controlled by the dilution rate. Because chemostat cultures are limited by glucose, virtually all of this substrate fed to the culture will be consumed. Therefore the value of the consumed q_s on the x axis of Fig. 3(a) will be equal to the one fed into the culture.

At low dilution rates (supply A of glucose), the uptake rate of the nitrogen source, determined by the straight line of the slope $Y'^{ox}_{N/S}$, will be smaller than the constant and non-limiting nitrogen supply rate, indicated by the horizontal line on the y axis. The solid dot indicating the consumption rate is therefore lower than the open circle indicating the supply rate (point A on Fig. 3(a)). Thus a certain amount of the nitrogen source will remain unused and can be detected as a positive ammonium concentration in the culture broth. We will refer to this situation as growth under a single, i.e. carbon, limitation.

As the dilution rate is increased both the consumption rates of glucose and nitrogen will go up until the latter reaches the rate at which the nitrogen source is supplied to the culture (point B on Fig. 3(a)). A free nitrogen source will no longer be left in the culture and the nitrogen source itself becomes a limiting compound. If we continue to increase the dilution rate and therefore the carbon substrate supply rate, either of two limiting behaviours could result.

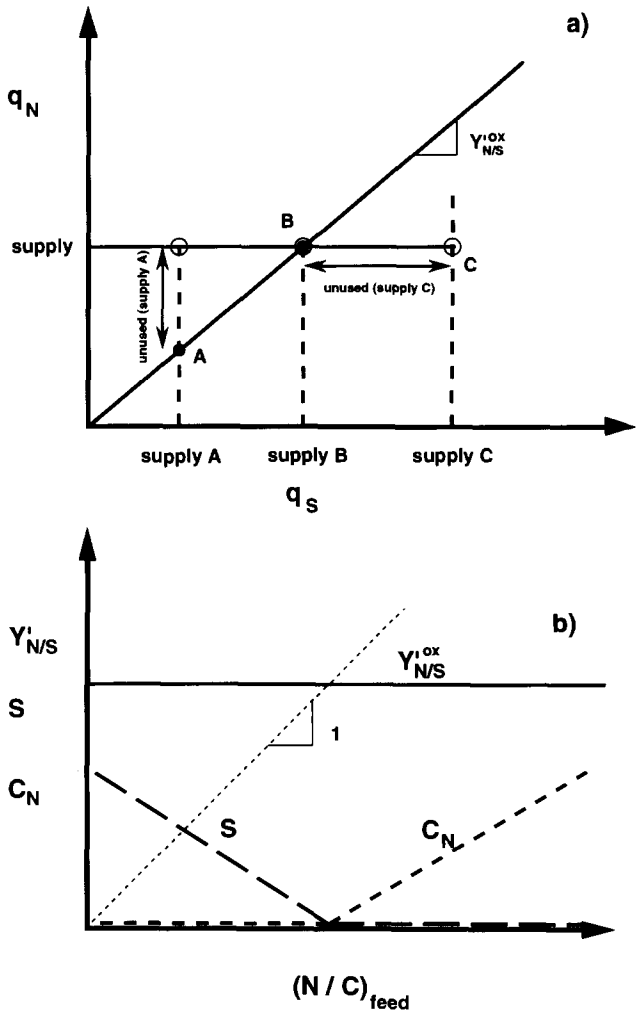

Fig. 3. Consumption kinetics of the carbon and the nitrogen source for a hypothetical culture with a strictly constant metabolism: (a) nitrogen consumption rate as a function of glucose consumption rate where points inidicate specific consumption rates (●) as compared to specific supply rates (○) for low (A), medium (B), and high (C) glucose supply rates; (b) ratio of nitrogen to glucose consumption rate $Y'_{N/S}$ and residual nitrogen and glucose concentration as a function of nitrogen to glucose feed ratio; for further explanations see text.

In a first scenario, the metabolism stays strictly the same, which means that the ratio of N to C uptake (i.e. $Y'^{ox}_{N/S}$) remains unchanged. Because however, the nitrogen uptake rate cannot exceed the limiting supply rate, the point indicating the two consumption rates (solid dot) will remain at the same location. The point indicating the respective supplies (open circle) moves to the right with increasing dilution rate (point C). This clearly shows that part of the glucose supplied to the

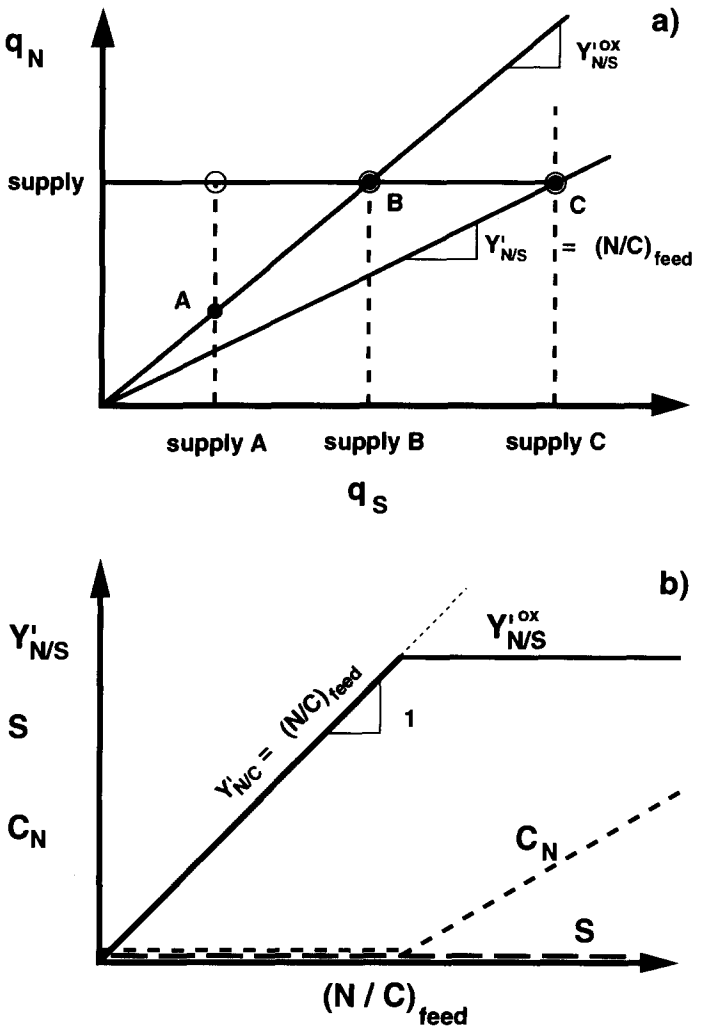

Fig. 4. Consumption kinetics of the carbon and nitrogen source for a culture exhibiting dual C + N limitations: (a) and (b) see legend to Fig. 3.

culture can no longer be taken up: the nitrogen is now the limiting compound. If the data is replotted as a function of the ratio of carbon to nitrogen supply rates it would look as shown in Fig. 3(b). Increasing the dilution rate decreases the N/C feed ratio which therefore means moving from the right to the left on such a plot.

Figure 4 shows another possible extreme behaviour. If the culture can change its metabolism in such a way that the excess glucose is also completely consumed then the point indicating the consumption rates could move to the right and coincide with the point indicating the feed rates (Fig. 4(a)). Both nitrogen source and

glucose would be consumed completely and they would have to be considered as limiting compounds at same time. This situation is described as dual C + N limitation. It is obvious that the ratio of nitrogen to glucose consumption rates (noted $Y'_{N/S}$) must now be lower because the culture cannot take up more nitrogen than the amount indicated by the horizontal line, but degrades an amount of glucose that is considerably higher than before.

Replotted as a function of N/C feed ratio, this case would look as depicted in Fig. 4(b). Increasing the dilution rates will mean decreasing the N/C feed ratio and therefore also decreasing the unused nitrogen concentration. In the right hand side of the diagram, however, the nitrogen is still non-limiting and the stoichiometric coefficient $Y'_{N/S}$ will remain constant at $Y'^{ox}_{N/S}$. As soon as the N/C feed ratio has been lowered to a value equal to $Y'^{ox}_{N/S}$, the nitrogen is completely consumed and becomes limiting. As the feed ratio is further decreased, both nitrogen source and glucose are used up completely and the ratio of uptake rates, i.e. $Y'_{N/S}$, has to adapt to whatever is fed to the culture. This means that it will decrease on a 45° line with decreasing N/C feed ratios.

4. Dual N + C limitation in *S. cerevisiae*

Figure 5 shows some of the data reported by Larsson et al. [7] replotted in the form of Figs. 3(b) and 4(b). There is indeed a window between N/C feed ratios of 0.02 and 0.08 in which both the carbon and the nitrogen source are completely consumed and dual limitation prevails. The measured stoichiometry coefficient $Y'_{N/S}$ behaves almost exactly as predicted in Fig. 4(b), being constant at about 0.08 at N/C feed ratios above this value and decreasing along the 45° line (solid thick line) below it.

Reducing the nitrogen consumption per Cmol of glucose catabolized, (i.e. degrading additional glucose without increasing nitrogen consumption), must also entail a reduction of the biomass yield per glucose $Y'_{X/S}$. The expected biomass yield

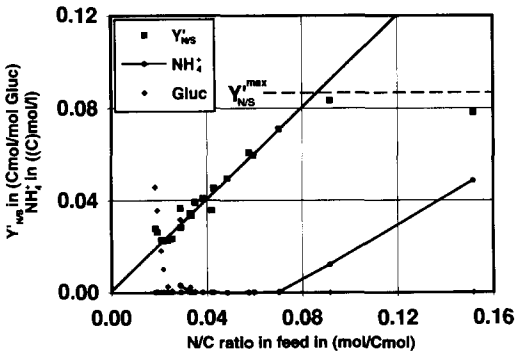

Fig. 5. Residual glucose concentration, residual ammonia concentration, and stoichiometric coefficient for nitrogen consumption ($Y'_{N/S}$) as a function of the N/C feed ratio for *S. cerevisiae*.

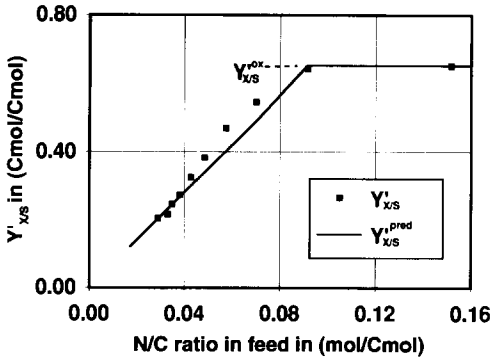

Fig. 6. Cmolar biomass yield ($Y'_{X/S}$) as a function of the N/C feed ratio for *S. cerevisiae*.

can be computed from the $Y'_{N/S}$ coefficient predicted on Fig. 4(b) by using a nitrogen balance

$$Y'_{X/S} = \frac{1}{x_3} Y'_{N/S} \approx \frac{1}{x_3} \frac{C_{N0}}{S_0} \tag{1}$$

where x_3 is the atomic coefficient for nitrogen in the Cmolar formula of biomass $CH_{x_1}O_{x_2}N_{x_3}$ and C_{N0}/S_0 is the ratio of nitrogen to carbon feed rate. As can be seen on Fig. 6, the measured points are well predicted by Eq. (1).

It could be hypothesized that *S. cerevisiae* reduces its biomass production and therefore its nitrogen uptake per unit of glucose consumed by degrading excess glucose through a reductive, or fermentative pathway, resulting in mixed oxido-reductive metabolism. Auberson and von Stockar [2] developed a simple mathematical model predicting growth stoichiometry of *S. cerevisiae* for the oxido-reductive states of its metabolism that resulted from O + C limitations. This so-called "aerobicity" model is used here to predict the ethanol yield and the heat yield directly from the behaviour of $Y'_{N/S}$ shown in Fig. 4(b).

Figure 7 shows that the metabolism did indeed shift to a more fermentative one at low N/C ratios in the feed, with concomitant ethanol production. However, the proposed model [2] (solid line) only predicts the product yields well at N/C ratios lower than 0.04, and seriously overestimates them at higher values. The question is how *S. cerevisiae* can reduce its biomass yield and hence its nitrogen consumption at an N/C feed ratio around 0.06, and simultaneously keep an almost fully respiratory metabolism as evidenced by an ethanol production of nearly zero.

The answer can be deduced from the calorimetric measurement shown in Fig. 8. The ordinate of this figure represents $Y'_{Q/X}$, the amount of heat dissipated per Cmol biomass grown. The aerobicity model predicts a smooth decrease from about 200 to 110 kJ $Cmol^{-1}$ with the onset of nitrogen limitation.

The measured heat yield is in agreement with the calculation for non-limiting, high N/C feed ratios. At lower ratios, however, $Y'_{Q/X}$ increases to values in spectacular discrepancy to the calculation. This shows clearly that uncoupled

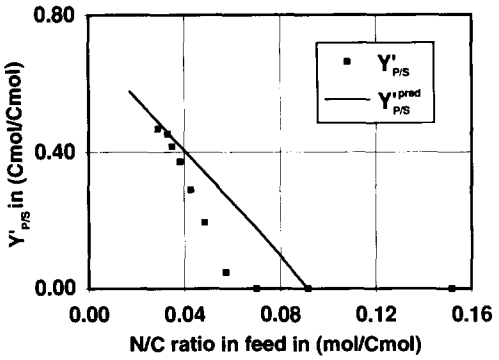

Fig. 7. Ethanol yield ($Y'_{P/S}$) as a function of the N/C feed ratio for *S. cerevisiae*.

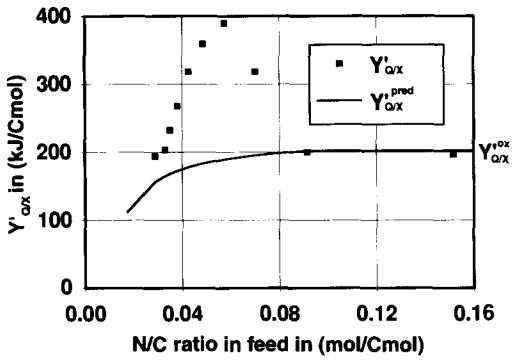

Fig. 8. Heat yield ($Y'_{Q/X}$) as a function of the N/C feed ratio for *S. cerevisiae*.

glucose oxidation must be used by the culture in order to degrade excess glucose while keeping growth and nitrogen uptake rates constant. A more detailed analysis shows that the shift to reductive metabolism only sets in at N/C feed rates which correspond to such a high dilution rate that the respiratory bottleneck is saturated ($q_0 \approx 4$ mmol H^{-1} g^{-1}).

5. Dual C + N limitation in *K. fragilis*

Figure 9 shows the existence of a dual limitation window at nitrogen to carbon feed rate ratios ranging from 0.08 to 0.03. The ratio of N/C uptake rates again behaves as predicted in Fig. 4(b). At the highest dilution rate, leading to N/C feed ratios below 0.03, nitrogen continues to limit the culture but the carbon limitation is lost, resulting in increasing amounts of unused glucose. In this region, $Y'_{N/S}$ again increases towards $Y'^{ox}_{N/C}$.

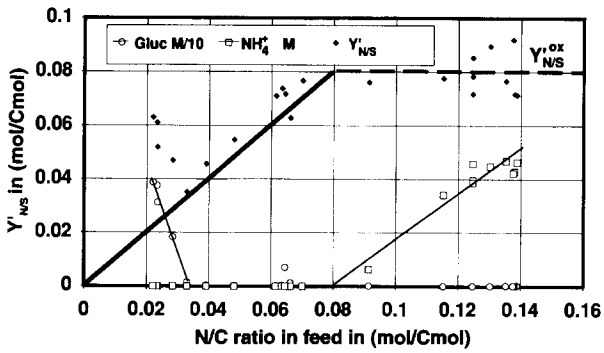

Fig. 9. Residual glucose and ammonia concentration, and stoichiometric coefficient for nitrogen consumption ($Y'_{N/S}$) as a function of the N/C feed ratio for *K. fragilis*.

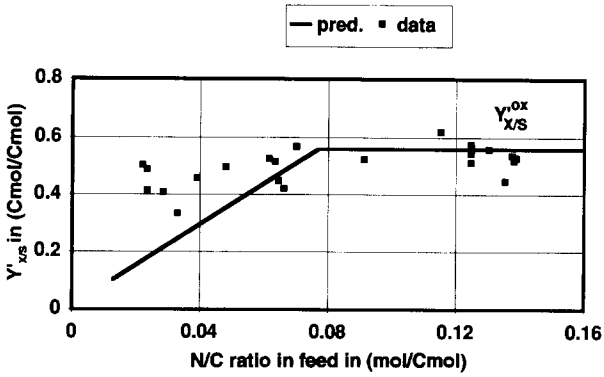

Fig. 10. Cmolar biomass yield ($Y'_{X/S}$) as a function of the N/C feed ratio for *K. fragilis*.

The biomass yield (Fig. 10) followed a similar pattern to that for *S. cerevisiae*, but variations in x_3 introduced a larger scatter and more deviation from the line predicted by Eq. (1).

As can be seen from Fig. 11, almost no ethanol is formed, which is in complete disaccord with the prediction based in the aerobicity model. The calorimetric heat yield measurements (Fig. 12) now show values that are about three times higher than the predictions in the dually limited range of N/C feed ratios. This confirms that the excess glucose has almost entirely been degraded by uncoupled oxidation rather than by fermentation. Hence, it is by uncoupling the oxidative metabolism that *K. fragilis* lowers the biomass yield and its need for nitrogen source when the latter becomes limiting.

6. Conclusions

Both *S. cerevisiae* and *K. fragilis* yeasts can undergo dual C + N limitations. The resulting shifts in metabolism can clearly be seen in calorimetric measurements. In

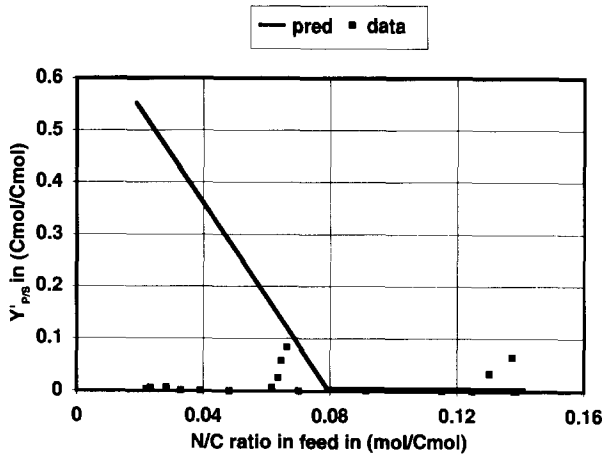

Fig. 11. Ethanol yield ($Y'_{P/S}$) as a function of the N/C feed ratio for *K. fragilis*.

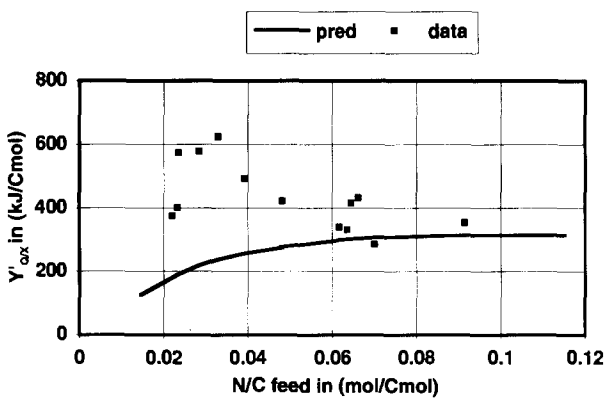

Fig. 12. Heat yield ($Y'_{Q/X}$) as a function of the N/C feed ratio for *K. fragilis*.

the present study, heat yield measurements have been used successfully to analyse the effect of dual limitations on metabolism.

Yeasts growing under dual C + N limitations lower the ratio of C/N carbon uptake rates (i.e. $Y'_{N/S}$) to the ratio of nitrogen to carbon source available, i.e. fed to the culture. This is effected by degrading some of the glucose by uncoupled respiration, which leads to a decrease of growth and nitrogen uptake per glucose consumed. In *S. cerevisiae*, some of the excess glucose is also catabolized by fermentation, although the main reason for this is not the dual limitation itself, but the saturation of the oxidative bottleneck that results from the high uncoupled respiration rates. The fact that no bottleneck is present in *K. fragilis* explains why it does not shift its metabolism towards fermentation.

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References

[1] U. von Stockar and I.W. Marison, Large-scale calorimetry and biotechnology, Thermochim. Acta, 193 (1991) 215–242.

[2] L.C.M. Auberson and U. von Stockar, A unified stoichiometric model for oxidative and oxido-reductive growth of yeasts, Biotechnol. Bioeng., 40 (1992) 1243–1255.

[3] U. von Stockar and I.W. Marison, Calorimetry of technical microbial reactions, Pure Appl. Chem., 65 (1993) 999–1002.

[4] L.C.M. Auberson and U. von Stockar, The role of the limited respiratory capacity in the *Saccharomyces cerevisiae* whi2 mutation, J. Biotechnol., 23 (1992) 35–54.

[5] M.A. Alexander and T.W. Jeffries, Respiratory efficiency and metabolic partitioning as regulatory phenomena in yeasts, Enz. Microb. Technol., 12 (1990) 2–19.

[6] M.J. Cooney, I.W. Marison, W. van Gulik and U. von Stockar, Calorimetric and stoichiometric analysis of growth of *K. fragilis* in continuous culture: nitrogen limitation imposed upon carbon limited growth, Appl. Microbiol. Biotechnol., submitted for publication.

[7] Ch. Larsson, U. von Stockar, I.W. Marison and L. Gustafsson, Growth and metabolism of *Saccharomyces cerevisiae* in chemostat cultures under carbon-, nitrogen-, or carbon- and nitrogen-limiting conditions, J. Bacteriol., 175 (1993) 4809–4819.

[8] I.W. Marison and U. von Stockar, A calorimetric investigation of the aerobic cultivation of *Kluyveromyces fragilis* on various substrates, Enz. Microb. Technol., 9 (1987) 33–43.