41 Modular control analysis: are membranes or cytosol more important for growth of the industrial yeast *Kluyveromyces marxianus*?

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Summary

An increase by almost 30% in maximum specific growth rate (μ_{max}) of the industrial yeast *Kluyveromyces marxianus* CBS 6556 has been observed between two successive steady states during more than 150 hours of pH-auxostat cultivation. The cell shape changed from spheroid/ovoid to elongated by which the surface to volume ratio increased. We here developed a quantitative description in terms of modular metabolic control analysis (modular-MCA) with the aim of understanding the implications of these findings. The experimental results can be readily understood if the control of membrane located processes on the maximum specific growth rate of *K. marxianus* is 0.9. It is hypothesized that the increase in surface area lead to an increased uptake capacity for all kinds of nutrients and that this enabled the cells to grow faster on mineral medium. These results may also have implications for commercial fermentations in which the production process depends on the μ_{max} of the *Kluyveromyces* strain. By pre-growing polymorphic production strains in a pH-auxostat one may select cells and develop strains with a considerably higher μ_{max}.

Introduction

Changes in morphology of microorganisms influence the description of their physiology because some cellular fluxes may depend primarily on cell volume and

others on the cell surface area [1]. The Dynamic Energy Budget (DEB) model [1] describes microbial growth on the basis of the uptake capacity and volume of the microorganism. Variation in morphology may change the cell's surface-to-volume ratio (s/v) which has been proposed to play a central role in controlling (in a proportional way) the specific growth rate of cells [1,2,3,4]. Therefore, we further investigated (theoretically as well as experimentally) the relation between the cell s/v-ratio and its maximal possible growth rate in a quantitative way.

Experimentally, Hennaut, Hilger and Grenson [10] have shown for the three substrates arginine, lysine and uridine, the transport of which is catalyzed by constitutive permeases [11,12,13], that the relative uptake rates decreased in proportion with the surface to volume ratio in a series of isogenic multiploid strains of the yeast *S. cerevisiae*. For other substrates such as methionine and leucine the transport of which is inducible [14], such a decrease was not observed. The interpretation of Hennaut, Hilger and Grenson [10] was that the cytoplasmic membrane is saturated (or nearly saturated) with the given constitutive permeases in a haploid strain, while the cell surface might become more and more limiting for permease insertion with increasing ploidy, since the increase in cell surface is smaller than the increase in cell mass or cell volume. If indeed control resides in membrane processes, and if the activity of these processes per cell increases with increasing membrane surface area per cell, selection for increased maximum growth rate should yield strains with increased surface to volume ratio's.

In the present study, an attempt was made to test and quantify this with modular MCA. By using the selective pressure on growth rate, which prevails in a pH-auxostat, we selected for *K. marxianus* cells with a higher μ_{max}. The observed increase in μ_{max} was accompanied by a transformation in cell morphology towards an increase in surface area relative to the cell volume. The concomitant changes in cellular growth rate and surface to volume ratio were in good agreement with the assumption that much of the control on the maximum growth rate resides in the membrane surface area.

Results and discussion

Theory: Calculation of control of membrane versus cytosol-located enzymes with modular MCA

In line with modular MCA [9] a suggestion of the location of potential control sites derives from considering the difference in surface to volume ratio (s/v-ratio) in relation to the μ_{max} of a microbial cell. Specifically, one may distinguish between the control contribution of transport processes relative to that of intracellular enzymes.

In the above perspective, one may regard the cellular metabolism as two modules of control sites: (i) module 1 exerts the summed control on μ_{max} of all the

membrane-located enzymes (C_m); (ii) module 2 exerts the summed control on μ_{max} of all the intracellular enzymes (C_c). The total control of both modules on μ_{max} must be 1 (total control):

$$C_m + C_c = 1 \tag{41.1}$$

A change in a flux such as growth rate may either be induced by a change in the group of membrane-located enzymes i (module 1) or by a change in the group of intracellular enzymes j (module 2):

$$C_m = \sum_{\substack{\text{module 1}\\ \text{membrane}\\ \text{located } i}} C_{e_i}^J = C_{e_1}^J + C_{e_2}^J + C_{e_3}^J + \ldots + C_{e_i}^J \longrightarrow d\ln J = \sum_{\substack{\text{module 1}\\ \text{membrane}\\ \text{located } i}} C_{e_i}^J \cdot d\ln e_i \tag{41.2}$$

$$C_c = \sum_{\substack{\text{module 2}\\ \text{intracellular}\\ \text{located } j}} C_{e_j}^J = C_{e_1}^J + C_{e_2}^J + C_{e_3}^J + \ldots + C_{e_j}^J \longrightarrow d\ln J = \sum_{\substack{\text{module 2}\\ \text{intracellular}\\ \text{located } j}} C_{e_j}^J \cdot d\ln e_j \tag{41.3}$$

An increase of an entire module, i.e., all of its enzymes to the same extent gives the following equation of the change in growth rate:

$$dlnJ = \sum_{\substack{\text{module 1}\\ \text{membrane}\\ \text{located } i}} C_{e_i}^J \cdot d\ln e_i + \sum_{\substack{\text{module 2}\\ \text{intracellular}\\ \text{located } j}} C_{e_j}^J \cdot d\ln e_j \tag{41.4}$$

A shorthand expression of eq. 41.4 gives:

$$d\ln J = C_m \cdot d\ln m + C_c \cdot d\ln c \tag{41.5}$$

When the specific growth rate increases one of two possible effects on the s/v-ratio may appear:

If the control of module 1 (C_m, control of all membrane located enzymes) on the specific growth rate is high, and there is space limitation for enzyme insertion, μ_{max} will increase with an increase in s/v-ratio. This increase in μ_{max} may only be proportional to the increase in surface area when the control of module 1 on μ_{max} is near 1 ($C_m \approx 1$, and therefore $C_c \approx 0$).

Space limitation for any intracellular enzyme is less likely than it is for membrane enzymes (see however [15]). Therefore, an increase in enzyme activity per cell is not likely to be induced by an increase in cellular volume. If module 2 rather than module 1 exerted most control on growth rate, then an increase in cell volume should not be expected to lead to an increased growth rate.

Thus, when an increase μ_{max} is accompanied by an increase in surface area relative to the volume, the initial control on μ_{max} may be postulated to reside mainly in

the cellular transport capacity of the membrane due to a space limitation for any constitutive transporter. Where growth is controlled by intracellular enzyme activities, or by an induced permease, such a concomitant change would not explain why μ_{max} increased. The Dynamic Energy Budget (DEB) model makes the former of these assumptions [1,3]. Measurements of changes in s/v ratio's between different maximum growth rates of one species could suggest the location of the control site and distinguish between transport processes (especially the group of constitutive permeases located in the cell membrane or in the space between membrane and cell wall) and the total of intracellular processes as controllers of growth rate; all other things being equal.

We now make eq. 41.5 suitable to implement our experimentally observed changes in s/v-ratio to our new developed modular MCA. Rewriting eq. 41.4 gives:

$$d\ln J = C_m \frac{dm}{m} + C_c \frac{dc}{c} \tag{41.6}$$

However, the flux J refers to a non-specific mass flow (unit mass per unit time) while the cellular growth rate (μ or j) is defined as specific flow to biomass formation (i.e., time^{-1}). Therefore, we rewrite eq. 41.6 using the following definition:

$$j = \frac{J}{m+c} \qquad \text{or} \qquad d\ln j = d\ln\left(\frac{J}{m+c}\right) = d\ln J - d\ln(m+c) \tag{41.7}$$

in which j is the specific flux to biomass formation, i.e., the specific growth rate, μ (h^{-1}). 'Specific' refers to 'per total cellular biomass'. This may, however, imply either of two definitions. Biomass may refer to volume related concentration of the intracellular components (enzymes) relative to either: (i) the total cellular volume, i.e., membrane plus intracellular space $(m+c)$, or (ii) only the intracellular mass (c). We follow the first definition. Therefore, using eq. 41.7 one can write for eq. 41.6 the following:

$$d\ln j = d\ln J - d\ln(m+c) = C_m \frac{dm}{m} + C_c \frac{dc}{c} - \frac{d(m+c)}{m+c} \tag{41.8}$$

When one rewrites eq. 41.8 with the summation theorem (eq. 41.1) one finds:

$$d\ln j = \left(C_m - \frac{m}{m+c}\right) d\ln m + \left(\frac{m}{m+c} - C_m\right) d\ln c \tag{41.9}$$

With eq. 41.9 we were able to quantitate the experimentally observed concomitant changes in maximum specific growth rate and surface to volume ratio obtained by selection of *K. marxianus* cells in a pH-auxostat.

Flux-control coefficients are related to enzyme activities, in the sense that they refer to reaction rates. Because the rate of many enzyme-catalysed reactions is proportional to the enzyme concentration (i.e., V_{max} to be proportional to [E]), we here assumed the latter, i.e., the concentration of enzymes in the membrane-surface area, to be proportional to the cell surface and that space-limitation for

enzyme insertion in membranes (saturated with transporters) exists. We measured the percentage increase in cell surface, and thus indirectly the increase in reaction rate based on the above two assumptions. No space-limitation in the cellular volume for cytosolic enzymes was assumed here, however (cf. [16]).

Experimental: Selection for increased μ_{max} by pH-auxostat cultivation and determination of changes in morphology by microscopic observation

Our aim was to select mutants of the yeast *Kluyveromyces marxianus* CBS 6556 with an increased maximum specific growth rate (μ_{max}) by use of a pH-auxostat [5,6,7]. An increase by almost 30% in μ_{max} of *K. marxianus* has been observed between two successive steady states during more than 150 hours of pH-auxostat cultivation [8]. Here, the term steady state refers to the situation of leaving the pH-auxostat undisturbed for at least 7 generations, which is normally defined for 'steady state' using continuous cultures. On the basis of the determined μ_{max}'s in the pH-auxostat, we estimated the ratio of the μ_{max} found in the first steady state to that found in the second steady state. For one experiment, out off at least three independent separate experiments, each resulted approximately the same outcome (any possible infection with faster growing microbes was excluded by checking the cells in both steady states by the CBS-organisation (Centraal Bureau voor Schimmel cultures) to be *K. marxianus* CBS 6556), this amounted to 1.39 ($\mu_{max2}/\mu_{max1} = 0.79/0.57$; see [8]). We also estimated the surface to volume ratio's on the basis of microscopic observations. We considered the cell morphology in the first steady state, as round spheroid and the shape in the second steady state, as elongated or long cylinder-like [8]. We estimated the length of the latter cylindrical cells to be about six times their width (or diameter, *d*). Moreover, both cell volumes did not change on the basis of geometric calculations ($V_{spheroid} = 0.113$ and $V_{cylinder} = 0.104 - 0.120$). Therefore, we made the assumption that the volume did not change significantly during the concomitant change in shape and μ_{max}. We estimated the volume and surface of the elongated cells, as found in the second steady state, in two ways: (i) Considering the elongated cells as cylinders including the surface area at both ends. (ii) Considering the elongated cells as filamentous cylinders with the ends as half spheroids. The ratio of the surface/volume ratio of the two cell types was approximated to 1.44–1.50. Consequently, when growth rate should be proportional to the surface to volume ratio, the prediction is that μ_{max} should increase by a factor in between 1.44 and 1.50. Consequently, the experimentally determined ratio of maximum growth rates (1.39) was not far from the ratio 1.44–1.50 predicted by the DEB-model [1].

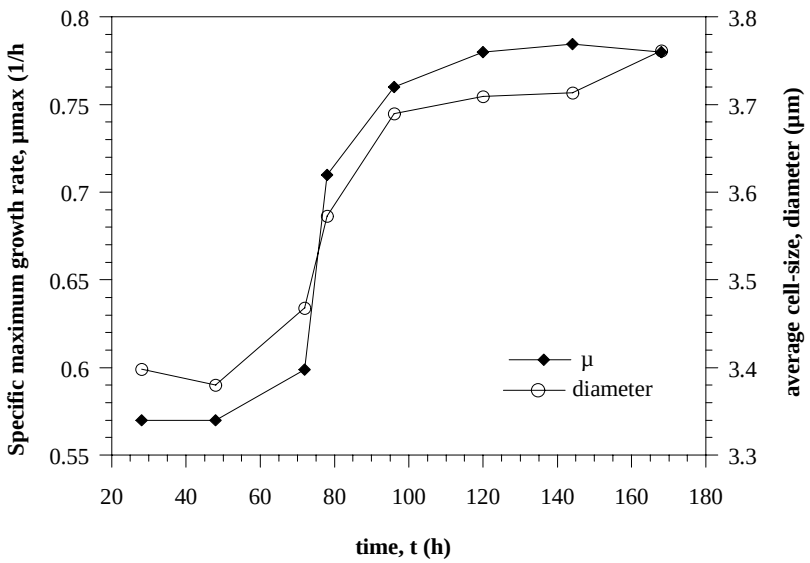

Fig. 41.1 Parallel change in maximum specific growth rate and average relative cell diameter of *K. marxianus* measured during 180 hours of pH-auxostat cultivation at 40°C. The average relative cell diameter was measured with a Coulter Counter.

Thus, experimentally we found that $d\ln j = 0.39$ (Fig. 41.1) and $d\ln m = 0.47$ (obtained from microscopic observation; see [8]); no significant change in volume was observed, thus specifying $d\ln c = 0$.

Therefore, eq. 41.9 gives:

$$C_m - \frac{m}{m+c} = \frac{d\ln j}{d\ln m} = \frac{0.39}{0.47} \tag{41.10}$$

Considering the plasma membrane located protein (m) to be about 10% of the total yeast protein content ($m + c$), thus: $m/(m + c)$ is about 0.10 gives the control of all membrane associated processes on the maximum specific growth rate of *K. marxianus* to be approximately:

$$C_m = \frac{0.39}{0.47} + \frac{m}{m+c} = 0.83 + 0.10 = 0.93 \approx 0.9 \tag{41.11}$$

This suggests that the control of the membrane surface on the maximum specific growth rate of *K. marxianus* is close to 0.9. To be more precise, these numbers correspond to the co-response coefficient relating specific growth rate to surface to volume ratio. Whether membrane surface area is involved mechanistically as proposed by DEB, requires further experimental testing.

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