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The Concept of Multiple-Nutrient-Limited Growth of Microorganisms and Some of Its Possible Applications in Biotechnology Processes

Thomas Egli*

Abstract. Justus von Liebig's 'Law of the minimum' states that usually one nutrient restricts the maximum quantity of biomass that can be produced within a system where all other nutrients are available in excess. This general rule has been applied also to the growth of microorganisms, *e.g.*, by adjusting the relative concentrations of the individual nutrients in growth media such that one of them, in the case of heterotrophic microbes usually the carbon source, determines the maximum cell density that can be obtained in a culture. However, recent experimental data from chemostats have demonstrated that growth of microbial cultures can be limited simultaneously by two or more nutrients. The experimental evidence supporting the limitation of growth by multiple nutrients is reviewed here, and a concept is presented that allows predicting the zones where multiple-nutrient-limited growth occurs. The information so far available indicates that multiple-nutrient-limited growth conditions allow to force cells into a physiological status that cannot be achieved under traditional single-nutrient-limited growth conditions. Two examples are given which demonstrate that cultivation of microbial cells under multiple-nutrient-limited growth conditions can be used to enhance the productivity of biotechnological processes.

1. Introduction

The environmental growth conditions strongly determine the behavior and metabolic abilities of microbial cells, and one of the most important parameters is certainly the availability of nutrients. Therefore, the restriction ('limitation') of specific nutrients is employed in many biotechnological processes to induce or enhance microbial product formation. Well-known examples are the use of phosphate-limited media to enhance the production of certain antibiotics or that of citric acid by cultivating the culture in a Fe-, Mn-, and/or Zn-limited environment [1].

*Correspondence: Dr. T. Egli
Department of Microbiology
Swiss Federal Institute
for Environmental Science and Technology
Überlandstrasse 133
CH-8600 Dübendorf
Tel.: +41 1 823 51 58
Fax: +41 1 823 55 47
E-Mail: egli@eawag.ch
http://www.eawag.ch/

Traditionally, in the media used for the controlled cultivation of microorganisms, the relative concentrations of individual nutrients are adjusted such that one of them restricts ('limits' [2]) the maximum quantity of biomass that can be produced, whereas all other nutrients are presented in excess. This design of growth media is based on the 'law of the minimum' formulated originally by *Liebig* in 1840 [3]. Based on this concept, microbiologists commonly assume that it is always one particular nutrient that determines the maximum amount of biomass that can be produced in a growth medium. However, a number of experimental studies exist in the literature which indicate that – in contrast to *Liebig*'s priniciple – microbial growth can be limited by two or even more nutrients at the same time. Here, the existing experimental evidence is reviewed, and it will be illustrated how such growth conditions can influence the composition and physiological performance of microbial cells. Furthermore, it will be shown that controlled limitation of microbial growth by multiple nutrients can be applied to biotechnological processes to optimize and enhance product formation.

2. The Phenomenon of Dual-Nutrient-Limited Growth

A number of experimental reports were published already in the seventies which indicated that growth of microbial cultures may be limited by more than one nutrient [4]. Clear experimental evidence was finally provided for the growth of bacterial and yeast cultures in the eighties [5]. An example for the observation made by all these researchers is given in *Fig. 1* for the growth of the bacterium *Hyphomicrobium ZV620* in a chemostat at a constant dilution rate, fed with media of different carbon/nitrogen ratios [5b]. Measurements of the steady-state concentrations of the formed biomass, the residual carbon (methanol) and nitrogen (ammonia) source in the culture, and the cellular content of the reserve material poly-(3-hydroxybutyrate) (PHB) indicate three distinct regimes of growth:

1) a clearly carbon-limited zone with nitrogen in excess at $C/N < 7.1$,
2) a distinctly nitrogen-limited growth regime where carbon is present in excess at $C/N > 12.6$, and
3) a transition zone when the feed ratio of

C/N was between 7.1 < *C/N* < 12.6, where neither residual carbon nor nitrogen was detected in the culture.

It is striking that, although growing apparently nitrogen-limited at *C/N* >7.1, the biomass concentration in the culture was still increasing linearly when additional carbon was added to the feed medium. It was shown that this increase in biomass was primarily a result of the intracellular accumulation of carbonaceous reserve material, in this case PHB [5b].

3. A Concept Allowing the Prediction of Multiple-Nutrient-Limited Growth Zones

Similar transition zones have been observed for the growth of various other microorganisms (most of the examples are given in [5]), and this prompts the question of whether the range and the position of the zones of single- and multiple-nutrient-limited growth can be predicted. An empirical approach was proposed by *Egli* and *Quayle* [5a] that allows to predict the boundaries of these zones for a microbial culture simply from the growth yields of the growth-limiting nutrients determined under single-nutrient-limited cultivation conditions. This is illustrated in the following example: the steady-state concentration of biomass (x) in *Fig. 1* can be calculated using *Eqn. 1* from the carbon utilized (*i.e.*, the difference between the feed concentration, C_0, and the actual concentration of carbon in the culture, *c*, and the corresponding growth yield measured under carbon-limited conditions ($Y_{X/C}$). Alternatively, x can be calculated from the nitrogen utilized (N_0–*n*) and the corresponding growth yield ($Y_{X/N}$) observed during carbon-limited growth. Right at the border between clear carbon limitation and the transition zone, the actual concentrations of carbon and nitrogen are very low in the culture compared to those in the inflowing medium. Therefore, *Eqn. 1* can be rearranged to obtain the carbon-to-nitrogen ratio (C_0/N_0) in the feed.

$$x = (C_0\text{-}c)Y_{X/C} = (N_0\text{-}n)Y_{X/N} \quad (1)$$

$$C_0/N_0 \approx Y_{X/N} / Y_{X/C} \quad (2)$$

From this, it follows that, for the prediction of the boundaries of the limitation regimes, only the growth yields measured under either carbon- (for the left-hand boundary) or nitrogen-limited conditions (for the right-hand boundary) have to be known. A comparison between the predicted and the experimentally observed boundaries confirms this for the culture of *Hyphomicrobium ZV620*. The growth yields for carbon and nitrogen determined experimentally under carbon-limited conditions were 0.99 and 7.19, respectively, whereas under nitrogen-limited conditions they were found to be 0.80 and 10.30. Hence, *Eqn. 2* predicts the boundaries of the transition growth zone between C_0/N_0 feed ratios of 5.4 and 12.5. These figures correspond well with the experimentally observed borders at 5.4 and 12.1 (*cf. Fig. 1*). This concept implies that, for a combination of two nutrients S_1 and S_2, a zone of dual-nutrient-limited growth should be observed only if $Y_{X/S1}$ and $Y_{X/S2}$ differ under S_1- and S_2-limited conditions, respectively, irrespective of the reason for the change in growth yield (*e.g.*, be it accumulation of reserve materials, increased dissimilation or maintenance energy requirement, product excretion, *etc.*). Furthermore, it implies that the wider the range within the growth yields can vary, the more extended the transition zone will become. From the fact that differences in cellular composition are usually more pronounced at low growth rates [6], one should therefore expect that slow growth would result in quite extended transition growth regimes.

The observation that the boundaries can be derived simply from the growth yields allowed the calculation of the extension of the zone of carbon/nitrogen-limited growth for *Klebsiella pneumoniae* from literature data [7]. The resulting figure illustrates that the extension of the carbon/nitrogen-limited zone should strongly depend on the growth rate of the culture (*Fig. 2*). Close to μ_{max}, the zone

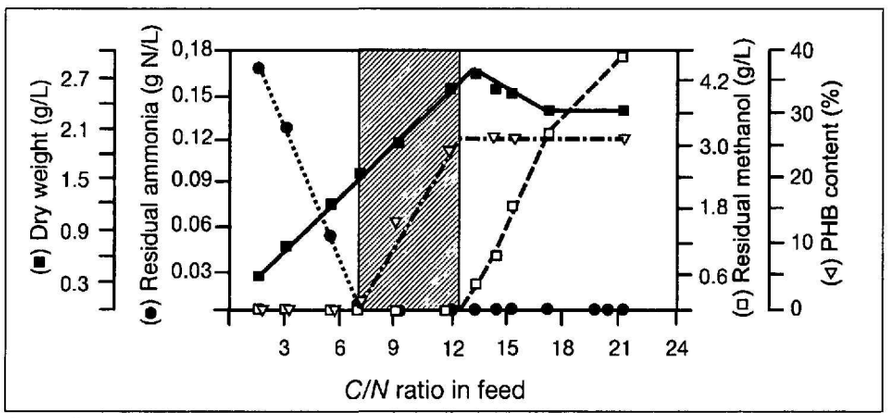

Fig. 1. *Concentrations of residual substrates and cellular PHB content in a culture of* Hyphomicrobium ZV620 *cultivated with methanol and ammonia in a chemostat at a constant dilution rate* (D = 0.54 h^{-1}), *as a function of the* C_0/N_0 *ratio in the feed medium.* Note that in this experiment, the concentration of nitrogen in the feed medium ($S_0(NH_4^+\text{–}N)$ = 223 mg l^{-1}) was kept constant, whereas S_0(methanol) was increased stepwise. Adapted from [5b].

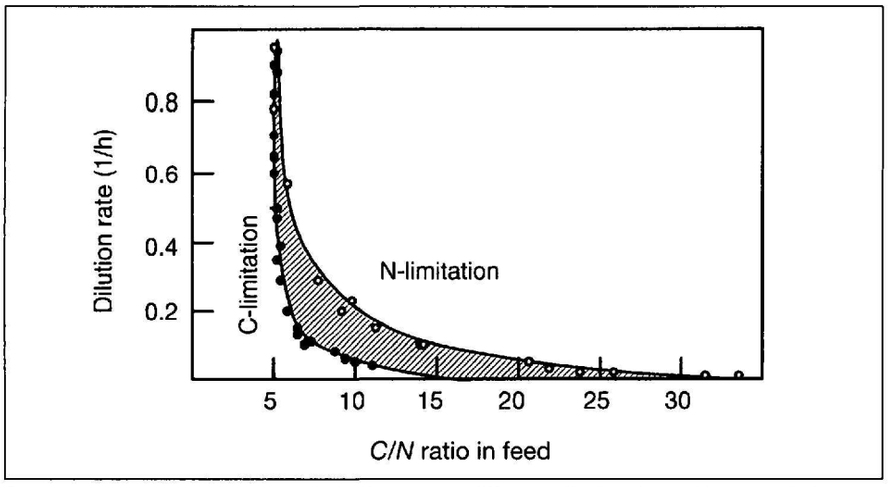

Fig. 2. *Predicted dual-nutrient-limited zone at different dilution* (growth) *rates during the cultivation of* Klebsiella pneumoniae *in continuous culture with glycerol and ammonia, as a function of the* C_0/N_0 *ratio in the inflowing medium.* Closed circles indicate data pairs below which growth should be carbon-limited, open circles those above which it should be nitrogen-limited. Adapted from [7].

should be very narrow, whereas during slow growth a rather extended zone of carbon/nitrogen-limited growth should become visible, and, at the same time, shift to higher C_0/N_0 ratios. An important consequence of this would be that, for example, cultivation of *K. pneumoniae* with a medium of a C_0/N_0 ratio of 8 would result in carbon-limited growth at low growth rates, while with the same medium, growth should become nitrogen-limited at high dilution rates.

The presence of a dual-nutrient-limited transition zone between clearly single-limited growth regimes has recently been reported for a number of different combinations of nutrients and microorganisms, including *Gram*-negative (*Acinetobacter* [8], *Pseudomonas* [9], and *Klebsiella* spp. [10]) and *Gram*-positive bacteria (*Bacillus* sp. [11]), and yeasts [5a][12]. However, in most reports, the information is limited to one particular growth rate, and I am aware of two cases where the extension and position of the transition zone has been investigated as a function of the growth rate [5c][13].

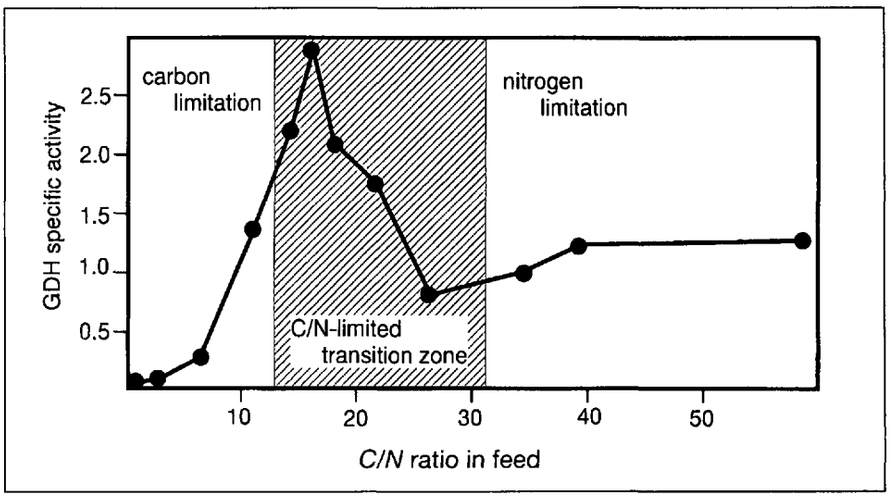

Fig. 3. *Specific activity of glutamate dehydrogenase* (GDH) *in cell-free extracts of the yeast* Hansenula polymorpha *during cultivation in continuous culture at a fixed dilution rate of 0.10 h^{-1}, as a function of the C_0/N_0-ratio in the inflowing medium.* The carbon source used was a mixture of glucose plus methanol (87.8% + 12.2%, *w/w*), and the nitrogen source was ammonia. The shaded area indicates the transition growth regime where growth was limited by carbon and nitrogen simultaneously. Adapted from [5a].

4. Application of Multiple-Nutrient-Limited Growth in Biotechnological Processes

The information presently available on the growth of microbial cultures under multiple-nutrient-limited conditions demonstrates that the composition and metabolic performance of microbial cells is rather flexible and that it can be controlled and manipulated to a great extent by modulating medium composition and cultivation conditions. Although to date, the use of multiple-nutrient-limited growth has hardly been considered for application in biotechnological processes, I would like to illustrate its potential in two examples.

The first example demonstrates the potential of these cultivation conditions for the optimization of enzyme levels. Typically, for the production of glutamate dehydrogenase (GDH), microbial strains (lacking the high-affinity ammonium-assimilating GS-GOGAT enzyme system) are grown under nitrogen-limited conditions, because the low concentrations of ammonia that cells experience in such an environment lead to overexpression of GDH [14]. Expression of this enzyme was investigated in the yeast *Hansenula polymorpha* during growth in continuous culture at a constant dilution rate with different *C/N* ratios in the inflowing medium [5a]. Surprisingly, the maximum activity of GDH was not found under nitrogen-limited conditions (as expected) but it peaked within the carbon-nitrogen-limited growth regime at a *C/N* ratio in the inflowing medium of roughly 15.9 (*Fig. 3*). At this *C/N* ratio, the specific activity of GDH was roughly double that found in purely nitrogen-limited cells.

The second example demonstrates the potential of dual-nutrient-limited growth conditions to tailor the composition of poly(3-hydroxyalkanoates) (PHA, an intracellular reserve material with plastic-like properties, which is biodegradable and therefore called 'bioplastic' [15]). It was shown that PHA-accumulating bacteria are able to introduce many different carbon compounds into the polymer, and that this leads to PHAs with different material properties [15b]. In a recent study done in our laboratory on the production of PHAs in *Pseudomonas oleovorans* from alkanoic acids, we found that it the tailoring of the composition of PHAs can be easily achieved under dual-(carbon/nitrogen)-limited growth conditions [9b]. The method makes use of the fact that, within the transition zone, the cells are still utilizing all the carbon fed (*cf. Fig. 1*). Hence, when *P. oleovorans* was cultivated within the carbon/nitrogen-limited zone and fed with different mixtures of octanoate/nonanoate the composition of the intracellularly accumulated PHA perfectly reflected the composition of the mixture of octanoate/nonanoate supplied in the feed medium. This example indicates the potential of multiple-nutrient-limited growth conditions for an easy and reproducible tailoring of PHAs of different composition.

To date, the influence of defined multiple-nutrient-limited cultivation conditions on microbial physiology and their potential application in biotechnological processes has hardly been investigated. As a result, properties, metabolic performance and potential of cells cultivated under multiple-nutrient-limited conditions are essentially unknown, unexplored, and thus unexploited. However, the two examples given above demonstrate some of its potential for innovative process design and optimization.

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[2] It should be pointed out that in microbiology, the term 'limitation of growth' is used today in two different ways. First, it is employed in a stoichiometric sense, *i.e.*, it indicates that a certain amount of biomass can be produced from a particular nutrient (or element). This is reflected in the growth-yield constants for the different elements, which are documented in the literature (see, *e.g.*, J.S.S. Pirt, 'The principles of microbe and cell cultivation', Blackwell, London, 1975). Second, this expression is also used to indicate that the microbial rate of growth, μ, is controlled by the (low) actual concentration (s) of a particular growth substrate, as described, for example, by the familiar equation proposed by *Monod* in 1942 [$\mu = \mu_{max} \cdot s/(K_s + s)$] (J. Monod, 'Recher-

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Preparation of Biotinylated and FITC-Labelled Phosphorylcholine Poly(acrylamide) Derivatives and Their Application for Protein Ligand-Binding Studies

Jiří Liberda[a]), Tomáš Trnka[b]), Jan Sejbal[b]), Věra Jonáková[c]), Marek Kraus[c]), and Marie Tichá[a])*

Abstract. Biotin- and FITC-labelled water-soluble poly(acrylamide) derivatives of phosphorylcholine were prepared by coupling either maleinylated (*a*) or periodate-oxidized (*b*) L-glyceryl phosphorylcholine to poly-(acrylamide-allylamine) copolymer. Biotinylated phosphorylcholine poly(acrylamide) derivatives of both types were tested with *Limulus polyphemus C*-reactive protein and were used for the study of the phosphorylcholine-binding properties of boar seminal plasma proteins. Binding sites for phosphorylcholine on the surface of bull sperms were visualized using a FITC-labelled derivative of the ligand.

1. Introduction

Suitable derivatives of phosphorylcholine that allow labelling are needed for the detection and the characterization of binding properties of proteins interacting with this ligand. A number of different types of phosphorylcholine-binding proteins exist that differ in their properties and functions. C-reactive protein (CRP) [1], frequently employed as a clinical index of acute inflammation, represents the most studied phosphorylcholine-binding protein. CRP is also found in the invertebrate *Limulus polyphemus* as a constitutive and major component of horseshoe crab hemolymph [2].

Another type of proteins possessing the ability to interact with phosphorylcholine was found among proteins isolated

*Correspondence: M.Tichá
[a]) Department of Biochemistry
Charles University, Albertov 2030
128 40 Praha 2, Czech Republic
Tel. 420 2 2195 2330, Fax. 420 2 2195 2331
E-Mail ticha@prfdec.natur.cuni.cz
[b]) Department of Organic Chemistry
Charles University, Albertov 2030
128 40 Praha 2, Czech Republic
[c]) Institute of Molecular Genetics
Academy of Sciences of the Czech Republic
Flemingovo nám. 2
166 37 Praha 6, Czech Republic