Biokinetics: Macro- versus micro-approach

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Summary. The elucidation of methodology (i.e. the approach to be used for the adequate quantification of bioprocesses) is a key area for the understanding and commercialization of biotechnology. This paper intends to fill the gap which has long been observed between the purely empirical approach followed in industrial practice and the mechanistic view used in fundamental biological sciences. According to a more systematic strategy in bioprocess design and development the formal macro-approach represents the basis of all engineering considerations. This fact, however, is not to be taken to mean that the macroscopic is an alternative to the microscopic approach; the two are supplementary, as only mechanistic studies are able to give a detailed interpretation of macroscopic behavior and offer a chance to clarify the black-box by identifying, and thus avoiding, process bottlenecks. This will be demonstrated in the case of baker's yeast processing, where the group of Prof. Fiechter has contributed significantly, and also in the case of antibiotic production processing.

Key words. Mathematical modelling; bioprocess kinetics; macroscopic principle; microscopic/mechanistic approach; formal macro-approach; regime analysis; process bottlenecks; model bioreactors; systematic strategy.

Introduction

The quantification of bioprocess kinetics has had a long tradition since Monod carried out his fundamental work. In accordance with the mechanistic paradigm which still dominates the scientific world – even though some strong indications exist that we are at a turning point where we are beginning to switch to a holistic view – much knowledge has accumulated to clarify meachnisms, i.e. to 'know why'. On the other hand, industrial practice and process engineers handle bioprocesses, under the stress of commercialization, quite differently and on a purely empirical basis. Somehow – on a basis of their feelings – they use the mechanistic facts as a background for their 'know how', which is very often top secret. With this situation the exchange of knowledge between the pragmatic and the scientific levels is hindered, leading to an under-developed methodology in process design. It is the ambition of this paper to build a bridge by first demonstrating the validity of the (formal) macroapproach as the scientific justification of this engineering approach, and then to show the supplementary enrichment of the macroscopic view by the microscopic mechanistic approach.

Approaches in bioprocess kinetic modeling

It is well known from the literature[19] that three main kinds of models exist:

a) the (pure) numerical fit model,
b) the (mechanistic) microkinetics model,
c) the (macroscopic) formal kinetics model

However, their evaluation is rarely clearly explained.

a) Pure numerical fitting methods are without biological relevance, as they use 'blind' parameters in arbitrary mathematical functions e.g. polynomials. They are preferred by control engineers owing to their simplicity and usefulness, as they represent an overall picture of individual cases.

b) Microkinetics, based on mechanisms derived from fundamental work, are widespread in biochemistry as they represent a 'better' understanding. There, mathematical models include parameters which can clearly be interpreted in detail on the basis of mechanisms (e.g. enzyme kinetics, Michaelis–Menten coefficients, and all other descriptions of mechanisms, including modifications concerning inhibitors etc.). Microkinetics are thus quite complex; all parameters are not measurable and any satisfactory interpretation depends on the significance of mechanisms, which is a matter for speculation.

c) Formal kinetics, on the other hand, take into account that no mechanism can be regarded as true, that mechanisms cannot be clarified in 'economic' time for engineering tools, and that mechanisms are far too complex to be handled for process design purposes. Even in the century of computers, the main problem is the experimental verification of model parameters.

In this situation, the only way out is to use the concepts of formal kinetics[19], as they do not pretend to be the truth. However, they are based on carefully-designed experiments. (Physical transport phenomena must be clearly excluded, so that they do not influence the bioprocess; therefore special bioreactors, mainly on the bench-scale, are needed. These reactors are called 'perfect' bioreactors or 'high-tech' bioreactors. These laboratory-scale reactors achieve pseudohomogeneity, e.g. by applying the highest possible power input for the sake of transport processes for heat, impulse and mass). It should be mentioned here that Monod-type kinetics are formal kinetics. Even though increases in our knowledge since the days of Monod have led to a better understanding of metabolism and/or cell structure, formal kinetics are not necessarily unstructured types of model. Biomass (X), often represented by the rather unsatisfactory measurement

of cell dry weight, can easily be understood and handled by multi-compartment models like that of Williams[9,33].

Two compartments are often used for structuring the biomass; the active, structural compartment includes the protein synthesizing system and RNA, while the genetic compartment contains mainly DNA in the genome. Most important to the understanding of such structured biomass models is the fact that an exact experimental verification does not exist. Thus both compartments again represent a formal approach with some increase in plausibility resulting from the more detailed consideration.

Furthermore, structuring of models does not only involve structuring the biomass, but also includes structuring the metabolic pathway[26,28] as well as the cell function e.g. cell age, activity and synchronization.

The Monod equation has the same form as the Michaelis–Menten equation. It should be clear that the difference lies in the meaning of the parameters (K_s vs K_m and μ_{max} resp. $q_{s,max}$ vs v_{max}); this has been explained elsewhere[19,20]. When Monod speculatively applied enzyme kinetics the main reason was perhaps his belief that microbial growth had something to do with enzyme kinetics. Under ideal conditions, the enzyme-bottleneck with its K_m-value can be estimated and can be brought to a closer correlation with the K_s-value of the cell/substrate constellation in the case of very small cell diameters. Nevertheless, typically for this formal approach, the parameters stem from adapting them to significant experiments, therefore they are called adaptation parameters, with apparent character but without mechanistic interpretability (e.g. μ_{max}, K_s).

An additional comment needs to be made, as some misunderstanding exists concerning the possibilities of formal kinetics. The argumentation concerning formal kinetics is not yet finished. Criticism exists which states that formal kinetics are nothing other than a pure numerical fit, with some mechanistic probability due to the similar form of the equation, as researchers prefer equations when they are more plausible, and accept them more easily – on the basis of their feelings. This argument demands a clear answer, which is given here in two statements.

Firstly, what is called mechanistic work is also nothing else than curve-fitting; even interpretation is given on a logical basis with mechanisms. There can be no doubt that all mechanisms are speculative and will remain so, as all efforts bring us only to the point that a mechanism seems to be valid as long as no other describes the experiment 'better' or as well as the original one. Furthermore, in the case of real-life bioprocesses, mechanisms are still unclear and much too complex to be treated. Secondly, the most essential feature of formal kinetics, which is practically overlooked, is connected with the significance of the macroscopic principle. Thus, the term 'formal macro-approach' is to be seen as an entirety representing the scientific justification of adequacy according to the holistic view.

The formal macro-approach

The formal macro-approach is based on the quantification of the significant macroscopic process variables ('key variables') i.e. biomass (unstructured), substrates, O_2, CO_2, products, heat. According to the macroscopic principle, it is sufficient to verify process behavior at the 95% level of all balances[27]. All intermediary metabolites do not influence the macrobalances of a bioconversion system in a reactor. This is in agreement with Einstein's dictum that "everything should be made as simple as possible, but not simpler". Microscopic aspects of bioprocesses can also be handled on the same level, e.g. microscopic stoichiometry can be reduced by handling only C, H, O, N and sometimes S and P. Macroscopic stoichiometry, e.g. yield coefficients, can be explained by this approach. It must be stated here that stoichiometry (balancing methods) plays a powerful role in process design and must be regarded as an essential supplement to kinetics[19,27,29].

The formal macroapproach is graphically illustrated in the scheme of a bioprocess in figure 1. The macroscopic input/output variables X, S, P, O, C, H_v are indicated together with the areas of biological phenomena (kinetics, stoichiometry, thermodynamics), physical phenomena (mass, heat and impulse transfers) and interactions between them (mainly viscosity, shear, morphology). The message of the utmost importance in connection with the formal macro-approach, assuring its powerful status, is the fact that *all* (macroscopic) phenomena are to be handled together, and not as a sum but as an *integral entirety,* representing the bioprocess by means of a simplified but adequate network. A case study using this holistic approach was described by the author[16], where beyond process kinetics and stoichiometry (growth, consumption of S and O_2, production of P, CO_2 and heat) the missing link for a systematic process analysis and design was thought to be the interdependence of the oxygen transfer rate with viscosity and morphology.

This unconventional and even revolutionary concept can be better accepted by the scientific community if the philosophical background of this approach is clarified. Theoretical science was heavily influenced by the work of Varela, Maturana and Uribe[33], treating the 'organization of living systems' (see also Jantsch[13]). The following sentence summarizes the holistic view: "One can hope to find a much simpler description of systems, when looking for dynamic rules of the procedure of processes and for criteria of the overall behaviour in a higher semantic level. Thereby a new 'macroscopic simplicity' will be discovered, which is not on the microscopic-mechanistic level."

The formal macro-approach is in entire agreement with this view, using the following principle of thinking and

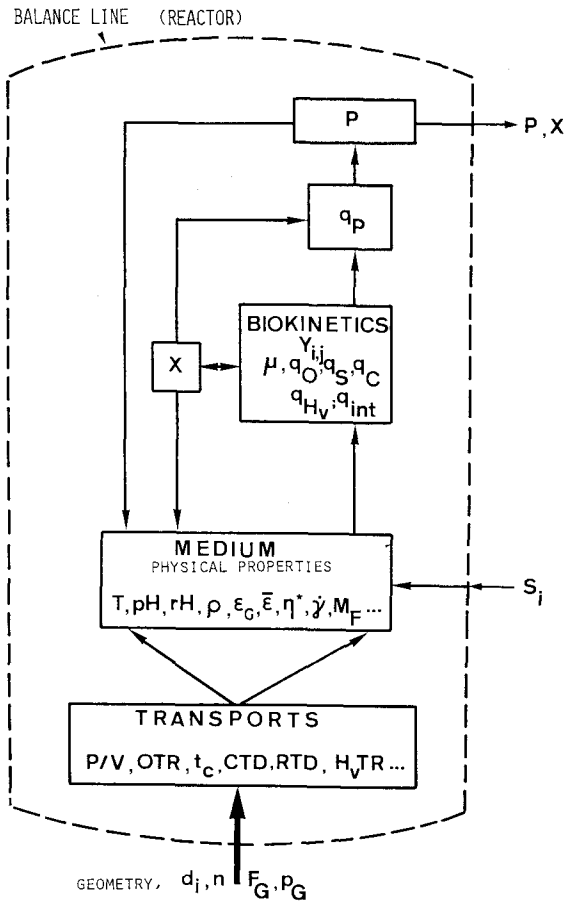

Figure 1. Schematic representation of the concept of interactions between physical transport phenomena (OTR, heat TR, P/V, t_c, CTD and RTD) and biokinetics (specific growth rate μ and specific consumption q_S resp. production q_P of significant process variables and cell internal compartments q_{int}). The physical properties of the medium represent the "missing link" for modelling: temperature, pH-value, redox potential rH, density ϱ, gas holdup ε_G, mean energy dissipation $\bar{\varepsilon}$, shear rate γ, 'Specific viscosity' $\eta^* = \eta_a \cdot x^{-1}$ as a quantitative estimation of "engineering viscosity" and M_F = morphology factor.

working: A living organism is an integral entirety and must be understood as a network of coordinating activities in the cells non-linearly interacting with the physical and chemical environment and not as a sum of separate parts in metabolism, genes, chromosomes, cell tissues and the environment in the reactors[17].

Systematic strategy in bioprocess analysis and design

The area of engineering sciences within the multi- and interdisciplinary field of biotechnologies, which is regarded as the 'trunk' of the tree of biotechnology, where the results of all fundamental sciences (the 'roots') are brought together and transferred to the whole variety of bioindustries (the 'fruits'), a systematic strategy has developed mainly as a result of the bioprocess technology schools in the Netherlands (T.U. Delft), Switzerland (ETH Zürich), and Austria (EJU Graz). This strategy is summarized in figure 2. Methodology includes the working principles of regime analysis and mathematical modeling and can be applied in two areas:

1. Industrial scale bioreactor systems in the case of a bioprocess which already exists and is running in industrial practice.
2. Lab-scale bioreactors in the case of newly-designed bioprocesses which are to be transfered to the technical scale later on.

Regime analysis means a four-step activity[19,31]:
to define/decide significant process variables;
to quantify them all by adequate measurements;
to derive rate constants i.e. characteristic values for rate or time constants;
to compare these values with the result that the slowest rate or the largest time governs the overall process rate (= 'process bottleneck', rate determining step, rds).

It has already been mentioned that carefully designed experiments need as a prerequisite the test of pseudohomogeneity. This, again, is a case of applied regime analysis. The consequence of regime analysis of industrial, pilot and bench-scale bioprocesses is that further information is needed for systematic analysis and design procedures. This information is obtained from laboratory-scale investigations:

A) – 'ideal' biokinetics in 'perfect bioreactors', in order to quantify the biological system[18,19]. The following systems can be regarded as lab-scale 'perfect bioreactors':
  - the multi-purpose 'horizontal stirred tank reactor' according to Moser;
  - the completely mixed microbial film reactor of Atkinson;
  - the 'high tech-compact completely filled loop reactor' according to Fiechter;
  - any other bioreactor system fulfilling the 'test of pseudohomogeneity'[19], with high power input by stirrer, aerator and foam separator and with high-quality control of process variables including temperature, pH, dilution rate and feed rates, sampling and on/off line analysis.

B) – to quantify the physical phenomena in different bioreactor systems so-called 'bioreactor models' need to be used, representing the production-scale bioreactor system on a smaller scale. At the same time this quantification can be advantageously carried out with 'biotest-systems' or 'reference fermentations' mainly developed by the Fiechter group[1,14,19].

Well-known and quickly-responding bioprocesses such as synchronous growth of *Saccharomyces cerevisiae* are used as examples of biotest-systems[30]. The following bioprocesses are able to serve as 'biotest systems', because they fulfil at least some of the necessary prerequisites. They have simple and/or well-known metabolic pathways; measurements can be made rapidly and reproducibly; they grow on a synthetic medium with well-

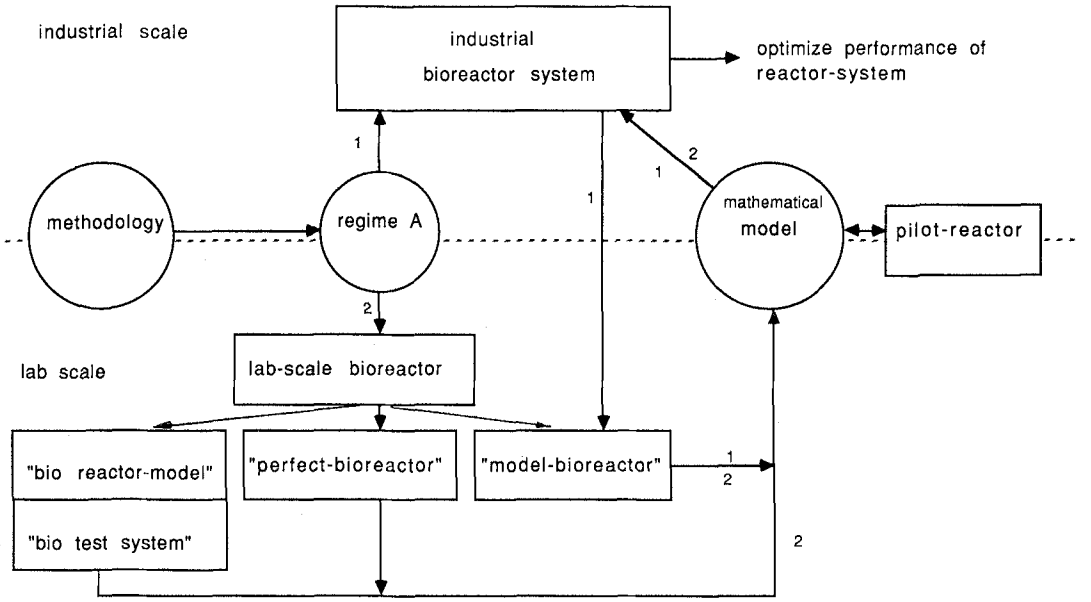

Figure 2. Strategy of bioprocess technology for the systematic design of bioprocesses and comparison of bioreactor system.

known composition and one limiting substrate (medium design!); they offer genetically stable strains without change in morphology:

- *Sacch. cerevisiae,* especially as a synchronously growing culture (glucose and oxygen effect);
- *Trichosporon cutaneum* (only O_2-effect);
- *Candida* strains (only glucose effect);
- *B. subtilis* and *E. coli* for O_2-depletion due to imperfect mixing;
- *Pullularia pullulans, Mucor javanicus, Rhizopus javanicus, Xanthomonas campestris* for testing shear sensitivity, and others.

C) – the transient behavior of bioprocesses under real process conditions in industrial scale bioreactor systems must be examined in so-called 'model bioreactors'[16,17,19]. Identified process bottlenecks e.g. mixing, shear, viscosity, gradients of concentrations, O_2, pressure, temperature must be experimentally simulated in specially shaped laboratory-scale bioreactors in order to derive an adequate dynamic model from these data.

The following bioreactor systems can be regarded as 'model bioreactors':

- for studies of gradients/transients in concentrations, pH, T:

CSTR: Continuous stirred tank, ("Chemostat", turbidostat, pH-auxistat etc.)

CPFR: Horizontal tubular reactor

- for OTR studies:
  - · horizontal tubular biofilm (thin-layer)
  - · falling film bioreactor and falling jet reactors
  - · bubble columns
- for biofilm studies
  - · sloping plane according to Atkinson
  - · vertical rotating drum
  - · horizontal rotating drum
  - · thin-layer bioreactor
- for mixing studies: two compartments combining CSTR[s] and/or CPFR[s]
- for shear and viscosity/morphology studies
  - · helical ribbon impeller tank
  - · liquid jet
  - · annular concentric rotating horizontal tubular reactor

Finally, all the pieces of information obtained are to be included in the hypothetical process model, which is to be experimentally verified or falsified in a fully equipped pilot-plant. The mathematical model derived from these efforts is then an adequate basis for process optimization etc.

Case studies – baker's yeast and antibiotic process kinetics

On this topic, only tendencies are referred to; for a more detailed discussion readers can go to the original references cited. However, from the trends shown in both case studies, several conclusions of general validity can be drawn.

Baker's yeast process kinetics

Over the years, a number of significant papers have appeared[3,4,7,20,24,28,31]. Comparison of these reports and of the different models used, which is rarely found in the literature, leads to several significant points[8]:

1) The complexity of mathematical models increases with time, as more complex situations are handled (non-

stationary processing, unbalanced growth, structured modelling). An exception is modelling for control purposes.

2) The aim of modelling has to be defined (physiology/control engineering/bioprocess design; batch or CSTR, quasi-or non-steady states).

3) The formal macro-approach has proved its equal validity, especially when using macroscopic mass and energy balancing[6]. In the case of descriptions of diauxic batch growth, the formal macro-approach comes to results identical with those of the mechanistic one[20]. However, it is not completely clear at present whether the macro-approach is still adequate in the case of modern process operational modes with sophisticated feed strategies and on-line measurements, where momentarily non-stationary conditions are dominant (repeated fed-batch with cyclic operation). Members of the Dutch school[32] take this view, and include e.g. acetic acid formation in their dynamic modeling, together with first order transfer terms as activity functions (cf. eq. 3b). On the other hand, the dynamics of ribosome synthesis have been proposed for this purpose, being also of first order transfer term on the macroscopic level[4].

4) Data from batch and chemostat experiments are still not transferable; there are still discrepancies.

5) A lack of data is evident, especially on effects of other compounds in the medium (e.g. amino acids) on product quality and kinetic data under true industrial process conditions (e.g. mixed substrate utilization, mixing behavior).

Here, on-line measurements of all significant process variables in a 'high-tech' bioreactor is to be recommended, as mentioned before.

The most essential facts for the setting up of a mathematical model (of any kind) are indicated in points 6–9:

6) Glucose metabolism has to be structured in oxidative and reductive pathways at the same time as corresponding terms are to be included in the equation for growth[28]:

$$\mu = \mu_{ox} + \mu_{red} + \mu_{eth} \qquad [1]$$

7) Beyond the diauxic growth pattern, baker's yeast cultures also exhibit non-diauxic behavior (where glucose and ethanol are consumed at the same time) as soon as glucose concentration declines below a critical value (s_{crit}) which is not a constant, but depends on known process conditions[35] as given by Moser[19,20]:

$$f_1 = \frac{1}{1 + s/s_{crit}} \qquad [2]$$

8) The repression (inhibition)-type consumption kinetics of ethanol in the second diauxic phase often show an apparent lag-phase[19,20]:

$$f_2 = \frac{1}{1 + p/K_R} \qquad [3a]$$

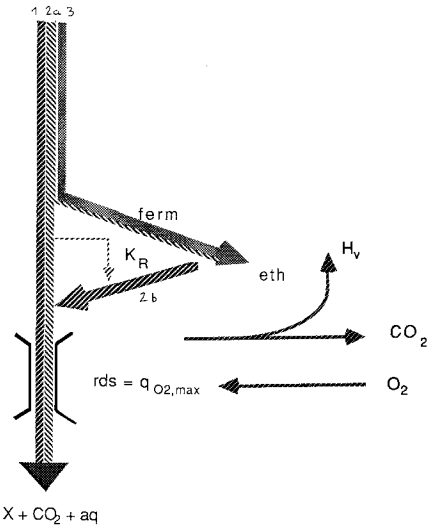

Figure 3. Flux-diagram of glucose with the process bottleneck of respiratory capacity ($q_{O_2,max,limit}$) in case of *Saccharomyces cerevisiae* type of yeast bioprocess with diauxie[28] exhibiting supracritical flux (fermentative/oxidoreductive process glucose → ethanol, case 3) and subcritical flux based on glucose (oxidative process glucose → biomass at $s \leq s_{crit}$, case 1 and case 2a with $s = s_{crit}$) and based on ethanol pool (oxidative process ethanol → biomass, case 2b). Generally the concept of $q_{O_2,max,limit}$ correspond to the concept of $q_{s,crit}$ resp. s_{crit}. Repression type kinetics in diauxic growth with konstant K_R[19].

resp. Bergter and Knorre[4]:

$$de/dt = \frac{1}{t_{L2}} \quad (1 - \mu_1/\mu_{1,max} - e) \qquad [3b]$$

where e = concentration of enzymes of oxidative pathway.

9) The process bottleneck is thought to be a limiting respiratory capacity[3,28]

$$q_{O_2,max,limit} = \frac{o}{K_O + o} (q_{s,max} Y_{O/S}) \qquad [4]$$

According to the holistic view, the most satisfactory way of considering living cells is to look for criteria for the dynamic rules of their overall behavior. Thus the diagram of figure 3, based on the dynamic flux of substrate, is comprehensive as a summary of process kinetics for baker's yeast. As a consequence of the respiration bottleneck, the key for understanding process kinetics is the corresponding 'critical S-flux' $q_{s,crit}$. Cases of supra- and subcritical fluxes have to be distinguished[28].

Antibiotic process kinetics

Not so many scientific papers appear in the literature in the case of antibiotic production processes as in the case of *Sacch. cerevisiae*. The most important articles concerning kinetics are given in the bibliography[2,3,5,10,11,21,22,23,25]. Several significant points will be summarized here, supplementing the general trends shown in the first case study (see points 1)–5) above):

10) Product formation clearly seems to be affected by a repression/inhibition type activity of the substrate. Even though the mechanism has not been finally elucidated [12], a formal kinetic equation was found to be adequate, being of the same form as equation 3a.

$$q_p(S) = q_{p,\max} \frac{S}{K_p + s(1 + s/K_R)} \quad [5]$$

with K_R = repression constant.

11) Growth is often affected by inhibition by substrate or product, as in the case of baker's yeast (K_{IPX}). Known equations can be taken, for example, for p-inhibition, including Contois kinetics and an apparent value for K_S (internal transport limitation):

$$\mu(s, p) = \mu_{max} \frac{S}{K_S, x(1 + p/K_{IPX}) + s} \quad [6]$$

As a consequence of this formal macro-approach, severe deviations from simple plots of production rate versus growth rate can be explained easily in the case of pleuromulin production [21].

12) This model approach leads to similar conclusions to those reached in the case of baker's yeast kinetics. Even though production rate is thought to be related to and controllable by growth rate [22], it becomes clear from mathematical modelling of the macroscopic kinetics (eqs 5 and 6), that in this case also the dynamic view, considering substrate-flux, becomes crucial. A plot of q_p vs q_s (not shown here; cf fig. 9 in Moser and Schneider [21]), demonstrates that a critical value $q_{S,crit}$ exists with extreme situations, as in case study one:

a) Supracritical: $q_S \geq q_{S,crit}$, then production decreases at increasing growth due to K_R

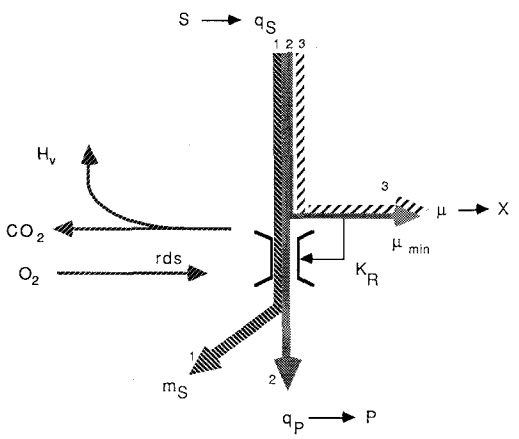

Figure 4. Concept of $q_{S,crit}$ in a dynamic flux diagram indicating a process bottleneck in case of antibiotic production as a result of a formal macrokinetic analysis [21]. As in figure 3 cases of sub- (case 1, S-flux for maintenance and 2 for derepressed production with a minimum growth rate corresponding to the critical S-flux given by the rate determining step, rds, equal to the bottleneck of respiration?) resp. supracritical flux (case 3) can be distinguished. Again repression (K_R) is active.

b) Subcritical: $q_{S,min} < q_S \leq q_{S,crit}$ where $q_{S,min}$ = value of S-flux for maintenance, then production becomes optimal at minimum growth rate (at high cell mass concentration).

The diagram of the dynamic substrate-flux shown in figure 4 demonstrates this concept of a critical S-flux $q_{S,crit}$. At the same time it indicates – in analogy to the baker's yeast process kinetics – that a process bottleneck obviously exists, which can also be the respiratory capacity of antibiotic-producing fungi.

Conclusions

The tendencies shown in the case studies of baker's yeast and antibiotic process kinetics can be summarized. Macroscopic and microscopic treatments of bioprocesses are supplementary and not contradictory, even though in the engineering working hierarchy the macroscopic level is more useful for design purposes. However, as has been demonstrated, the interpretation and better understanding of process bottlenecks can only be achieved by microscopic mechanistic work. The formal macro-approach as the center of process engineering considerations has on the one side the danger of anonymous curve fitting (numerical methods), and on the other side the danger of not seeing the wood because of all the trees representing 'mechanisms'. However, with the help of the philosophy mentioned, i.e. the holistic view, the criteria for the overall behavior and the dynamic rules of the integral entirety of all significant process variables (on-line quantification!) are the best guide-lines through the jungle of the unknown, in order to 'elucidate' the complexity of bioprocessing.

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Determination of oxygen gradients in single Ca-alginate beads by means of oxygen-microelectrodes

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Summary. Oxygen concentrations were measured in single Ca-alginate beads using polarographic microneedle electrodes. To obtain reliable results the effects of mechanical pressure on the electrode as well as the influence of free Ca^{2+}-ions had to be compensated. No oxygen gradients were detectable in cell-free alginate beads, whereas in beads with entrapped cells of *Enterobacter cloacae* steep oxygen gradients were observed. The steepness of these gradients depended on the bacterial growth in the gel. At the end of the logarithmic phase of growth the maximum depth of oxygen penetration into a bead of about 3 mm in diameter was in the range of 150 µm; i.e. nearly 70% of the volume of the alginate beads was free of oxygen.
Key words. Oxygen gradients; Ca-alginate; pO_2-microelectrodes; immobilized microorganisms.

Introduction

Entrapment in alginate gels is a technique often used for the immobilization of microbial cells. One disadvantage of the method is the restriction of substrate transport inside the particles to diffusion. This is especially important for oxygen, the maximum concentration of which in air-saturated media is only about 230 µmol/l (at 30 °C), so that in central parts of the immobilisates oxygen-limiting conditions may appear[12].

On the basis of experimental results concerning the oxygen uptake rate of immobilized cells[13], the maximum penetration depth of oxygen in alginate immobilisates was calculated to be 50–200 µm[6], whereas the critical particle diameter for a sufficient oxygen supply, depending on the cell concentration and respiration rate, could be calculated to be less than 1 mm[9].

According to these findings, the greater part of the alginate beads, the diameters of which are usually in the range of 2–5 mm, should be oxygen-free. Organisms in