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Do Mutations Inevitably Affect Microorganism's Metabolites Production in Continuous Cultures?

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Abstract. *In fifty years, the production processes by microorganisms using continuous cultures have been very moderately imposed in industrial environments. Yet, these practices have significant advantages, both economically and from a pragmatic perspective. The main reason for this lack of interest lies in the fact that the chemostat (as the archetype of continuous culture) is widely used for the study of the biological evolution of microorganisms. Supposed to create a significant evolutionary pressure, these devices have been increasingly considered as generators of mutations able to defectively modify the "good" properties of the continuously grown producing cells. Over time, prejudices were developed to depreciate the value of these production processes. The viewpoint is not entirely unfounded, but we try to take stock of some excessive attitude, sometimes totally indefensible, and to rehabilitate a production process too often irrationally ruled out.*
Keywords: *chemostat, genetic drift, mutations, microorganisms' metabolites production, steady state*

I. Introduction

The industrial production of metabolites produced by microorganisms is an important part of the biotechnology market and of the "green chemistry". Despite a difficult start, since its discovery in the 1950s [1, 2, 3], it is generally accepted that the chemostat in particular, and continuous cultures in general, are widely recognized in the scientific community [4].The industrial production of many compounds is now more or less common and represents a significant part of the health, food, cosmetics (and the environment) market. A good representation (though sometimes a bit simplified) of these activities, already well known, can be found in [5]).
Among the advantages of industrial continuous cultures, Zeng and Sun (2010) [4] cite the high volumetric productivity, the savings in labor and energy, the uniform quality production, benefits in automation and process control, as well as an economy in culture media preparation and in downstream processing.

These authors endorse the benefits of basic research in genetics, biochemistry, and physiology, thanks to well-defined culture conditions characterizing continuous cultures. These "idyllic" qualities are however counterbalanced by some drawbacks, including the difficulty of maintaining sterile conditions over long periods and biofilm formation on the reactor walls, biosensors, etc. But the main object remains the genetic drift due to the selection pressure exerted in continuous cultures. These mutations result in instabilities in the continuity of the production. The phenomenon has been known since the early 80s, mainly with regard to GMOs (especially about plasmids - [6, 7 ,8].

However, this phenomenon is not general and only concerns some strains [4] Moreover, what is perceived by some operators as a drawback has developed to reverse metabolic engineering strategies [9] or evolutionary engineering [10]) which consist, simply put, to identify genotypes that will produce efficient production phenotypes. We want to emphasize that genetic drift exists, but is not common to all cultivated strains. In addition, the selection pressure it either, not always makes it possible to usefully change (or not) a strain. To quote Tyo (2008) [9] : « If growth selection cannot be used, as is often true when it is desired to increase the product, single cell measurement or microtiter plate screening can be employed for screening of desired mutants (emphasize added) ». This clearly shows that the selection pressure attributed to continuous cultures does not always influence a given production.

In 2006, [11] wrote: "Indeed, the historical introduction misses the aim of Novick & Szilard, (1950b) [12] who were more interested in the evolutionary applications of continuous culture than in the production of reproducible, steady-state bacteria (which was Monod's aim). ... The dichotomy between the desire for 'steady state' in functional genomics studies and the rapid selection for change needs to be appreciated. "Thus, Ferenci introduced a notion of cultural divergence between the assessment studies of stationary states for "French" basic research and "Anglo-Saxon" (Novick and Szilard) genetic drift and evolution of microorganisms. In the same article of 2006, Ferenci (2006) goes further and writes: "The third point missed by Hoskisson & Hobbs (2005) [13] is that there is actually no true steady-state in chemostats [...]." In our viewpoint, such a stance has seriously delayed the use of continuous cultures in industrial production, suggesting that such production process would invariably become unstable due to genetic drift. In addition, he questions the relevance of chemostat studies for the fundamental understanding of the physiology of microorganisms.

We believe that such dichotomous viewpoint is untenable at this time; we want to formulate some formal (theoretical) proofs to our debate, although it seems clear, now, that everyday industrial practice already partially overcomes this problem.

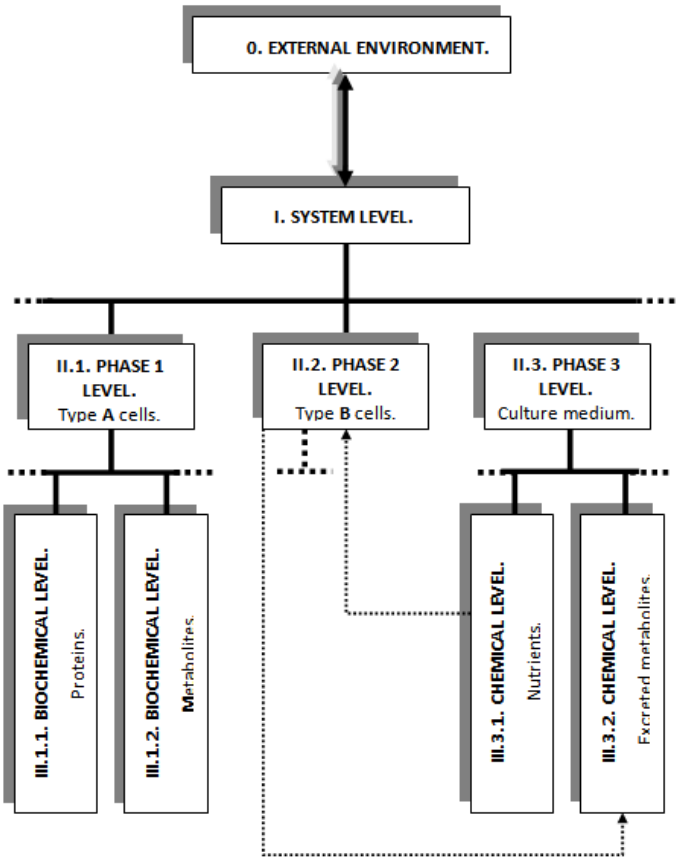

Figure 1. Representation of sub-levels of description in a biological system.

Four levels of description are represented (0 to III). The diagram shows a hierarchical structure moving from the most general level (the Universe) towards the most precise level (the biochemical level). Our analysis stands at Level I, the System Level (namely: the bioreactor.)

II. Material and Methods

The methods we use are those of modelling. Modelling methods of biological phenomena are numerous and it is not our intention to expose them here. For the sake of convenience, we will build upon a systems representation based on ordinary differential equations (ODEs), representing unstructured biological systems where representation at the cellular level is avoided. Based on Fig. 1, we principally place ourselves at the level of description of the bioreactor.

The methods are those of numerical simulations validated by an experimental validation.

In this work, we primarily turn towards the "non-experts" in mathematical modelling, using a voluntarily "naive" approach. Interested readers are invited to refer to more rigorous and precise literature, such as [14, 15, 16, 17].

2.1. Results.

2.1.1. General formalism.

Let an unstructured simple model (described at level I; see Fig. 1) of type:

$$\frac{dZ}{dt} = f\left(\{Y\},\{\lambda_i\},\{\theta_k\}\right) \tag{1}$$

$\{Y\}$ set of state variables (defining the system: biomass, substrate, ...);

$\{\lambda_i\}$ set of kinetic parameters (saturation constant, transmission coefficient ...);

$\{\theta_k\}$ set of physicochemical variables (P, T, dilution rate, pH,..., O_2, t).

One must note that in polyphasic dispersed systems we are considering here, the state variables are statistical values (average) ([14]). We define a polyphasic dispersed system (PDS) as a system consisting of several phases (solid, liquid, gas) intimately distributed into each other and, for the most, generally maintained in this dispersed state by external dispersion forces, such as mechanical agitation. The PDS are thus inherently unstable and, by definition, heterogeneous. To make them operational, we must introduce the concept of pseudo-homogeneity. This means that, despite the intrinsically heterogeneous nature of a system, there is some description level from which one can consider a volume element of this system as homogeneous (from a functional point of view). The ability to choose a level of description adequate to treat the system as homogeneous while in reality, he is not (pseudo-homogeneous) is the keystone of the definition of PDS.
The concept of pseudo-homogeneity can only be effective if a system can be divided into volume elements statistically equivalent from the viewpoint of their properties, that is to say by taking the average of the state variables at the level of a sufficiently small volume element comparing to all the system. As a result, the kinetic parameters are complex quantities (in the sense of "complicated") depending on these average values. (For a more extensive and rigorous development of these concepts, see [14]).

2.1.2. Steady state.
We define the steady state of (1) as

$$\frac{dZ}{dt} \doteq 0 \tag{2}$$

or

$$\lim_{t\to\infty} \frac{dZ}{dt} = 0 \tag{3}$$

Imagine now an open system (biological or not) comprising a producing step of compound *M*, *Π (M)*, and a disappearance step of *M, Δ (M)*. It writes:

$$\frac{dM}{dt} = \Pi(M) - \Delta(M) \tag{4}$$

Using (2), $\frac{dM}{dt} \doteq 0$, the steady state is

$$\Pi(M) = \Delta(M) \tag{5}$$

Imagine that this steady state is wrong.
Case 1: The production exceeds the disappearance: $\Pi(M) > \Delta(M)$. Using (3), it follows that

$\lim_{t\to\infty} \frac{dM}{dt} = \infty$ and $M = \infty$ which is obviously absurd (contrary to the conservation of matter).

Case 2: The disappearance exceeds the production: $\Pi(M) < \Delta(M)$. Using (3), it comes that $\lim_{t\to\infty} \frac{dM}{dt} = 0$ and after a sufficiently long time, we always get a trivial condition $M = 0$. (One "examines" an empty system!)

2.1.3. Conclusion:
The only way to have a realistic and non-trivial system is to satisfy the steady state (5). In other words, in a system like (4), the conservation of matter in a non-vacuum system requires that there exists a steady state (5).

III. Application to the representation of the biomass in the chemostat.

We can admit that the biomass in a chemostat is formalized as follows:

$$\frac{dX}{dt} = kX - DX \tag{6}$$

(Where *X* is the biomass, in g/L, for example)
The first term of the right side of (6) simply states that the production of *X* is autocatalytic (integration of this sole term in (6) shows an exponential growth). The second term is simply the expression of the hydraulic output of the biomass.
(6) can also be put in the form:

$$\frac{dX}{dt} = X(k - D) \tag{7}$$

and admits, according to (2) or (3), the following two steady states:

1. $X = 0$ (8.a)
2. $k = D$ (8.b)

State 1. is obviously trivial ("empty" chemostat, without biomass);
state 2. means that the biomass is equal to its output, as in (5). The biomass production is generally called "growth rate" and

$$k \equiv \mu(.) \tag{9}$$

where $\mu(.)$ is a complex function (not a constant).
Thus

$$\mu(.) = D \tag{10}$$

3.1. Note about the composition of the biomass.
The steady state (8.b) doesn't say anything about the quantitative value of biomass X. This is simply unknown at this stage. We can define biomass as the sum of all cellular components in the chemostat:

$$X = \sum_{i=1}^{N_i} p_i \tag{11}$$

where p_i is the i^{th} cellular component and N_i the number of cellular components.
For a given value of X, there are infinite ways to satisfy (11), and therefore:

$$X = \sum_{i=1}^{N_i} p_i \equiv \sum_{j=1}^{N_j} p_j \equiv \ldots \equiv \sum_{k=1}^{N_k} p_k \;;\; i \neq j \neq k \tag{12}$$

In other words, the steady state (10) neither depends on the total value of biomass nor its composition (number and/or nature of cellular compounds cf. (12)).

3.2. In conclusion.
We must admit that the law of mass conservation requires us to admit that the chemostat indeed has a steady state, or trivial ($X = 0$), or consistent with (10) and the growth rate is equal to hydraulic output (the "dilution rate" ratio of the input flow rate to the useful volume: $D = {Q}/{V}\left(t^{-1}\right)$). On the contrary, the total value of the biomass and its composition will be dependent on the growth rate modelling $\mu(.)$.
In other words, in a chemostat, there is a steady state ($\mu(.) = D; X \neq 0$) that does not imply anything about the composition of the biomass.

IV. Discussion

We demonstrated that the steady state of chemostat is a constraint imposed by the matter conservation law and, therefore, it does indeed indisputably exist. Equation (6) does not include the development of a biofilm often observed on the reactor walls, sensors, etc. in this case, the steady state can be changed ([18]) or largely delayed. Although extremely simple, the reasoning developed here to draw attention to a generally underestimated notion: the undefined composition of biomass at steady state without further assumption.
From our point of view, the composition of the biomass may depend either on its genome or its metabolism. It seems obvious that to different genotypes will correspond different phenotypes. As for the impact of metabolism, [19] demonstrate that the macromolecular composition of cells depends on the growth rate. An observation even more surprising relates to the elemental composition of the bacterial biomass: it is surprisingly little variation. [20] provided an average value for 16 species of bacteria and fungi, and obtained the following average formula: $CH_{1.79}O_{0.50}N_{0.20}$ with variations of the stoichiometry coefficient per carbon for hydrogen from 1.73 to 2.00, for oxygen from 0.43 to 0.56 and ranging for nitrogen, from 0.16 to 0.24. (These values are due to [21].) Concerning different species or different kingdoms, one can only be surprised by the extreme constancy of these values, despite the inevitable genotypic variability.
We ourselves were able to calculate elemental compositions for a single species (*Saccharomyces cerevisiae*) in different metabolic states. Table 1 shows the variation of the amount of biomass (on a constant average composition base); the overall reaction is the following (according to [22]):

$$C_6H_{12}O_6+c_1.O_2+c_2.0.15.NH_3 \rightarrow c_2.CH_{1.79}O_{0.57}N_{0.15}+c_3.C_2H_60+c_4.CO_2+c_5.H_2O \quad (13)$$

Table 1. Stoichiometry coefficients during *S. Cerevisiae* Crabtree effect.
D: dilution rate; S^0 substrate concentration at the chemostat inlet; stoichiometry coefficient: C_1, for oxygen; C_2 for biomass; C_3 for ethanol (EtOH). For $D<0.32\ h^{-1}$, metabolism is purely oxidative and EtOH is not produced; at $D>0.32\ h^{-1}$, metabolism is respirofermentative and EtOH is produced. (After [14], [23])

D (h^{-1})	S^0 (g/L)	c_1	c_2	c_3	METABOLISM
0.2	5	2.48	3.46	0.00	Oxidative respiration.
	10	2.37	3.41	0.00	
	30	2.35	3.41	0.00	
0.29	5	2.47	3.44	0.00	No EtOH production.
	10	2.36	3.40	0.00	
	30	2.34	3.40	0.00	
0.32	5	1.40	2.13	0.76	Respirofermentative mode. EtOH production.
	10	1.33	2.09	0.77	
	30	1.32	2.09	0.77	
0.36	5	0.85	1.46	1.13	
	10	0.80	1.42	1.14	
	30	0.80	1.42	1.14	
0.41	5	0.57	1.12	1.30	
	10	0.53	1.08	1.32	
	30	0.53	1.08	1.32	

The table clearly shows that the oxygen consumption only depends on the metabolic state, but not on the growth rate or on the substrate concentration at the inlet of the chemostat. Ethanol production is either zero or increases with the growth rate but doesn't depend on the substrate concentration at the entry. Regarding the amount of biomass, it is almost constant in the oxidative regime but decreases with *D* in the respirofermentative regime. The hypothesis of the elemental constancy of biomass ($CH_{1.79}O_{0.57}N_{0.15}$) is confirmed by the general mass balance, with a carbon recovery rate higher than 96% (see [14, 23]). Although we have no data on the subject, the genome of *S. cerevisiae* is likely invariable throughout the experiment, however, given the variability of the enzymes involved ([24, 25]), the expressed genes are necessarily different depending on the metabolic state ([26]). We conclude that the variability of expressed genes does not drastically interfere with the biomass composition. It is, therefore, justifiable to imagine that any modification of the genome not expressed, or not directly incriminated in a production process, has no impact on the production. The mutation of a microorganism is therefore only likely to interfere with a given industrial process but doesn't in any way imply an inevitable change in this process. We don't believe that this probability can be assessed a priori at the present time, and only an empirical approach will show whether continuous culture is stable and sustainable or not. Discard continuous cultures on the basis of prejudices or popular belief would be to deprive an often valuable and efficient production tool.

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